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Fibrinogen E-fragment – A Cryptic Effector of Platelet Function.

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ABSTRACT

Accentuated platelet function is a significant factor in ischemic stroke. An understanding of a role of platelet function inducers might give insight into mechanisms of platelet contribution in thrombo- and atherogenesis. Assuming that fibrinogen degradation products may exert a direct effect on platelets, we have assessed the quantity and composition of soluble fibrin monomer complexes in blood plasma of ischemic stroke patients. It was shown that soluble fibrin monomer complex fraction contain significant quantities of fibrinogen E-fragment, which induced platelet hyper-reactivity *in vitro*.

Keywords: fibrinogen E-fragment, platelets, soluble fibrin monomer complex, ischemic stroke.

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INTRODUCTION

Degradation products of proteolytic activity and peptides that are released during protein processing often have bioactivities that are distinct to the parent protein [1, 2]. These peptides, or crypteins, appear to play a role in modulating such processes as angiogenesis, immune function and cell growth. Further discoveries in the field of peptidomics could reveal new opportunities for protein-based therapeutics [3-6]. Previous studies have identified a cryptic peptide derived from collagen IIIα that has chemotactic and osteogenic activity [7], Fc-peptides – fragments of the constant region of different classes of antibodies that exerted a fungicidal activity *in vitro* against pathogenic yeasts and could constitute the basis for devising new antifungal agents [8]. Several crypteins are undergoing advanced clinical trials.

In recent years, an increased attention is paid to the properties and characteristics of the individual fragments of fibrinogen and fibrin molecules [9]. It is proved that the fibrin/fibrinogen degradation products (FDP) are the direct cause of bleeding in some pathological states of the organism, can cause smooth muscle spasm, increase vascular permeability and enhance the effects of certain medications. Low molecular weight FDP inhibit platelet aggregation and high molecular weight FDP accelerate it [10]. It was shown that the FDP affect all stages of fibrinolysis – the interaction of plasminogen with fibrin, plasminogen activation, the rate of hydrolysis of fibrin clot and the interaction of plasmin with alpha 2-antiplasmin. Detection and quantification of FDP is one of the important methods for the diagnosis of thrombophilia. Of particular interest are reports of the FDP association with kidney disease and development of acute renal allograft rejection crisis [11, 12].

MATERIALS AND METHODS

Subjects. 66 Ukrainian patients with atherothrombotic ischemic stroke and 56 Ukrainian patients with cardioembolic ischemic stroke (mean age 73,62 ± 8,9 years, range 43 to 91) were enrolled in this study. Patients were admitted to the 1st and 2nd neurological units of Kyiv City Hospital № 4. The diagnosis of ischemic stroke was confirmed by neuroimaging (CT or MRI of the brain). All patients received a single orally administered dose of aspirin (325 mg) within 24 hours after admitting. The study did not include patients in coma, patients with severe respiratory failure and patients with suspected cancer.

Blood sampling. Venous blood was collected from the cubital vein from 8 to 9 am in a test tube with a solution of sodium citrate (38 g/l) in the final ratio of 9:1 within 24 hours after admitting.

Isolation of soluble fibrin monomer complex. 0.25 ml of blood plasma; 0.25 ml of 0.1 M phosphate-buffered saline containing 0.065 M NaCl, pH 7.5; 0.2% 6-aminohexanoic acid and 0.1% sodium citrate were thoroughly mixed, than 0.4 ml of 1 M phosphate-buffered saline, pH 7.5 was added, mixed and left for 30 minutes at 22ºC. The precipitate was centrifuged and washed thrice with 0.15 M NaCl and suspended in electrode buffer.

Obtaining of polyclonal anti-E-fragment antibodies. Rabbits were immunized subcutaneously with fibrinogen E-fragment in complete Freund's adjuvant with 1 week interval. Blood was taken two weeks after the final immunization. Ammonium sulfate solution at 45% saturation was used to produce IgG precipitation. The precipitate was than centrifuged at 1500 g for 30 minutes, dissolved in 0.05 M Tris-HCl buffer, pH 7.4 and subjected to gel-filtration on Sephadex G- 25 in order to remove ammonium sulfate. IgG fraction was applied to Protein A Sepharose HP and eluted with 100 mM glycine HCl, pH 2.0.

Western blotting was performed by probing the blocked membrane with primary antibodies against fibrinogen E-fragments. Secondary antibodies were detected using p-nitrophenyl phosphate.

Fibrinogen E-fragment obtaining. Enzymatic cleavage of fibrinogen was performed using plasmin (0.4 U/ml) at 20º C in 0.05 M Tris-HCl buffer containing 0.13 M NaCl, pH 7.4 for 5 hours. Inhibition of hydrolysis was performed with diisopropyl fluorophosphate (5×10^{-5} M) in 5 hours followed by plasmin removal by affinity chromatography on lysine-sepharose.

Preparation of platelet rich plasma (PRP). Blood was collected from the auricular artery of healthy rabbit into 3.8 % sodium citrate in a ratio of 9:1 and centrifuged at 150 g for 15 min. The supernatant was carefully removed by pipetting and used in subsequent procedures.

Flow cytometry. Forward light scatter (FSC), correlating with cell size and side light scatter (SSC), reflecting primarily cell internal fine structure and granularity, were examined. Fibrinogen E-fragment was used in final concentration of 75 mkg/ml.

Statistical analysis was performed using the SPSS and TotalLab Quant software.

RESULTS AND DISCUSSION

Soluble fibrin monomer complexes (SFMC) are the early marker of thrombophilia that represent the complexes of monomeric fibrin with fibrinogen or FDP. Detection of SFMC formed due to the activation of blood clotting by thrombin reveals a pathological process in the early, preclinical stages [13]. SFMC levels are not directly affected by therapy with heparin or thrombolytic agents and may contribute to an accurate diagnosis of thrombotic events. Increased soluble fibrin monomer is typical for the development of disseminated intravascular coagulation, autoimmune diseases and thrombophilia [14]. In the present study, we determined the plasma levels and composition of SFMC in atherothrombotic and cardioembolic ischemic stroke patients to identify and quantify the fibrinogen E-fragment (FgnE).

Quantitation of SFMC from blood plasma of atherothrombotic and cardioembolic ischemic stroke patients was performed using visual inspection technique with 6-aminohexanoic acid. We have found that SFMC in atherothrombotic ischemic stroke was 33,4±15,2 mkg/ml, while maximum of SFMC concentration (46,2±4,1 mkg/ml) was determined

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in 27% of patients. In patients with cardioembolic ischemic stroke SFMC was 37,0±11,8 mkg/ml and 25% of patients in this group had elevated level of SFMC (47,6±5,3 mkg/ml).

Isolated SFMC fractions were run on 4-12% gradient polyacrylamide gels under denaturing conditions. We have not revealed any significant differences in SFMC quantity and composition between atherothrombotic ischemic stroke patients and cardioembolic ischemic stroke patients. SFMC was 142,52±22,57 mkg/ml, which was significantly higher as compared with visual inspection with 6-aminohexanoic acid.

Following electrophoresis, proteins were transferred onto Amersham Hybond-C Membrane under standard conditions. Western blot detection was performed using polyclonal anti-E fragment antibody (Figure 1). The blotogram revealed the presence of FgnE free form (bands in the area of 50 kDa) and high molecular weight fibrinogen derivatives possessing peptide of interest or FgnE complexes (bands above 50 kDa). It was shown that total SFMC fraction comprises 25% to 50% of FgnE free form.

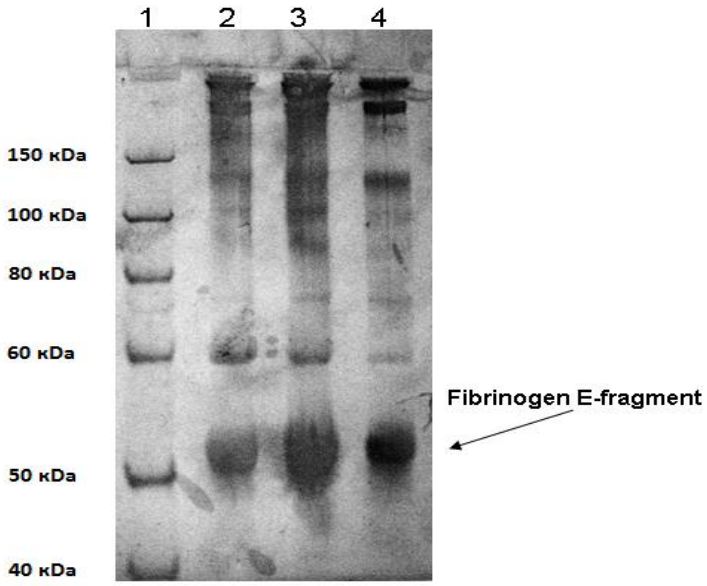

Figure 1: A typical resulting blotogram of FgnE in SFMC fractions obtained from blood plasma of ischemic stroke patients.

1 – molecular weight standards
2-4 – SFMC fractions

Investigation of FgnE properties has generated significant interest due to its biological activity and effect on hemostasis, in particular on the formation of atherosclerotic plaques [15-17]. Previous studies have shown that FgnE has the ability to activate prothrombin by non-enzymatic way and to induce catalytic activity of plasminogen molecule [18-20]. To elucidate what enhanced post-stroke platelet reactivity can be referred to we have examined FgnE-induced platelet behavior by flow cytometry. FSC/SSC based assay verified that the fragment

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was associated with accentuated platelet reactivity, which resulted in alterations of cell distribution (Figure 2).

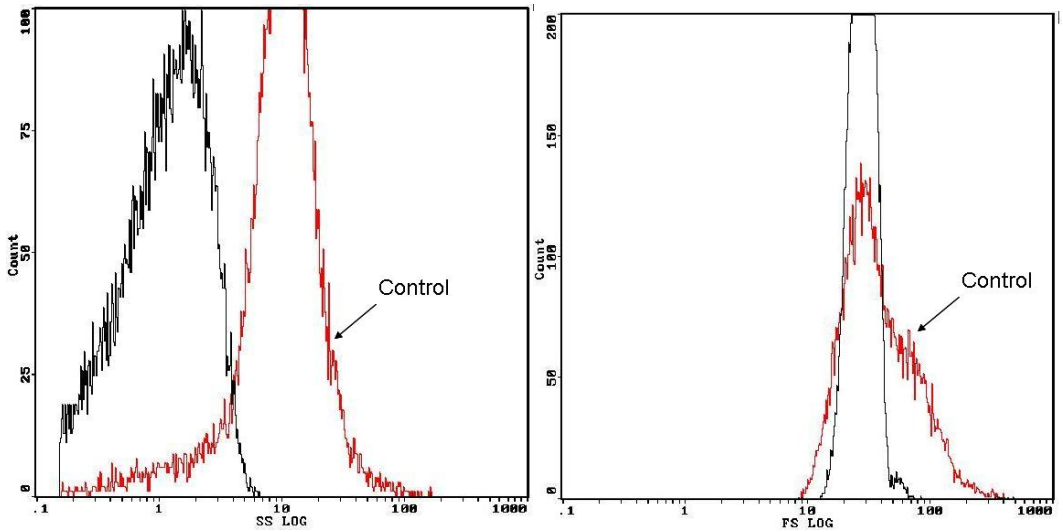

Figure 2: The effect of fibrinogen E-fragment on platelet granularity (SS LOG) and size (FS LOG).

Our findings suggest that SFMC fraction contains high quantities of fibrinogen E-fragment that could trigger a haemostatic response, causing development of thrombotic complications. Platelet activation by FgnE could be mediated by both clotting factors and platelet surface receptors. The receptors engaged in FgnE effect on platelets have yet to be identified. Previous studies have shown that whole fibrinogen binds to various integrins via RGD motifs in the D-domain of the fibrinogen molecule. However, FgnE lacks these domains, thereby FgnE binding may involve one or more novel, non-RGD sites [21].

CONCLUSIONS

Soluble fibrin monomer complex fractions obtained from blood plasma of ischemic stroke patients contain significant quantities of fibrinogen E-fragment, which induce platelet activation and formation of platelet aggregates *in vitro*, thus contributing to hemostatic process.

REFERENCES

[1] Menschaert G, Vandekerckhove TT, Baggerman G, Schoofs L, Luyten W, Van Criekinge W. J Proteome Res 2010; 9(5): 2051-2061.
[2] Diamandis EP. J Proteome Res 2006; 5(9): 2079-2082.
[3] Autelitano DJ, Rajic A, Smith AI, Berndt MC, Ilag LL, Vadas M. Drug Discov Today 2006; 11(7-8): 306-314.
[4] Ng JH, Ilag LL. IDrugs 2006; 9(5): 343-346.
[5] Bauça JM, Martínez-Morillo E, Diamandis EP. Clin Chem 2013 [Epub ahead of print].
[6] Schulz-Knappe P, Schrader M, Zucht HD. Comb Chem High Throughput Screen 2005; 8(8): 697-704.

[7] Agrawal V, Kelly J, Tottey S, Daly KA, Johnson SA, Siu BF, Reing J, Badylak SF. Tissue Eng Part A 2011; 17(23-24): 3033-3044.
[8] Polonelli L, Ciociola T, Magliani W, Zanello PP, D'Adda T, Galati S, De Bernardis F, Arancia S, Gabrielli E, Pericolini E, Vecchiarelli A, Arruda DC, Pinto MR, Travassos LR, Pertinhez TA, Spisni A, Conti S. PLoS One 2012; 7(3): e34105.
[9] Haas FJ, Schutgens RE, Biesma DH, Laterveer RH, Kluft C. Blood Coagul Fibrinolysis 2013; 24(3): 297-304.
[10] Kołodziejczyk J, Ponczek MB. Contemp Oncol (Pozn) 2013; 17(2): 113-119.
[11] Horan JT, Francis CW. Semin Thromb Hemost 2001; 27(6): 657-666.
[12] Shlipak MG, Fried LF, Crump C, Bleyer AJ, Manolio TA, Tracy RP, Furberg CD, Psaty BM. Circulation 2003; 107(1): 87-92.
[13] Hetland O, Knudsen A, Dickstein K, Nilsen DW. Blood Coagul Fibrinolysis 2002; 13(4): 301-308.
[14] Ieko M, Naito S, Yoshida M, Kanazawa K, Mizukami K, Sato H, Yui T, Nakabayashi T, Hirabayashi T, Oguma Y. Tohoku J Exp Med 2009; 219(1): 25-31.
[15] Thompson WD, Smith EB, Stirk CM, Wang J. Blood Coagul Fibrinolysis 1993; 4(1): 113-115.
[16] Stirk CM, Kocher A, Smith EB, Thompson WD. Atherosclerosis 1993; 103(2): 159-169.
[17] Bootle-Wilbraham CA, Tazzyman S. Marshall JM, Lewis CE. Cancer Res 2000; 60(17): 4719-4724.
[18] Makogonenko E, Yakovlev S, Slominsky A, Korolchuk V, Grynenko T, Sokolovsky L, Druzhina N, Kolesnikova M, Chernyshov V, Cederholm-Williams S-A. Ukr.Biochem.J. 2000; 72 (4-5): 99-109.
[19] Kudinov SA, Andrianov SI, Lezhen TI. Biokhimiia. 1988; 53(10):1684-90.
[20] Skomorovskaia EV, Grinenko TV, Kudinov SA, Zolotareva EN. Biokhimiia. 1991; 56(3): 458-66.
[21] Brown NJ, Staton CA, Rodgers GR, Corke KF, Underwood JC, Lewis CE. Br J Cancer 2002; 86(11): 1813-1816.