Fibrinolysis parameters in the acute and post ischemic stroke patients

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ABSTRACT

Analysis of fibrinolysis of the haemostasis system of the patients with acute ischemic stroke as well as one year past acute phase of ischemic stroke were performed. The significant inhibition of fibrinolytic potential of the post stroke patients were showed. The development of both pathological conditions accompanied by lengthening of time of the euhlobulin fraction lysis and FXIIa-dependent fibrinolysis. Moreover cardioembolic ischemic stroke were inherent more threatening performance. Lower activity of plasminogen as well as α2-antiplasmin in blood plasma of the patients with atherothrombotic ischemic stroke was showed. Both tested pathological conditions were accompanied with abnormal concentrations of t-PA and PAI-1. Even one year past stroke attack the disorders in fibrinolysis still saved.

INTRODUCTION

A critical prevalence of stroke bring high mortality and significant disability population, a fairly high percentage of stroke recurrent are evidence of the relevance of the stroke problem in medical and social terms (Adamson et al., 2004; Jonathan et al., 2002). The pathogenesis of ischemic stroke consists of a number of mechanisms, including disturbances in platelets, coagulation, anticoagulation and fibrinolytic links of haemostasis play a key role (Wolfe, 2000). Fibrinolytic capacity of blood plasma is able to characterize the ability to organism to confront possible thrombotic threats in condition of hypercoagulation. Even in some pathological conditions which on its beginning are unrelated with haemostasis disorders, there are certain decreases in fibrinolytic potential, indicating in this way a possible threat of thrombotic complications (Lowe et al., 2002; Mehta et al., 2003; Swarowska et al., 2014). Especially important is evaluation of fibrinolytic potential of haemostasis after a certain time after the disease as a guarantee of absence of the risk of repetitions. As known firstly the term "fibrinolysis" was proposed in 1893 by A. Dastre, to describe the physiological process of fibrin lysis (Macfarlane et al., 1946; Vyremyeyenko et al., 1988).

Intensive study of fibrinolysis process started in the 50s of the XX century. Key proenzyme of the fibrinolysis system is plasminogen (Pg) - the single stranded plasma glycoprotein with a molecular weight 92 kDa, which after activation of endogenous or exogenous activators is converted to the active enzyme (Wallen and Wiman, 1980). Plasminogen is found in all body fluids, some tissues and blood components (Dobrovolsky et al., 2002; Zubayrov, 2000; Lijnen, 1996). Plasminogen is synthesized predominantly by the liver cells, although its synthesis was detected in the kidneys, eosinophils, in brain microglia cells and testis (Raum, 1980). Normally, the concentration of plasminogen in the blood varies within the limits 1.2-2 μkM, however, plasminogen level in the blood varies with different physiological processes (Lijnen, 1996).
Actions of fibrinolysis system are provided by a set of enzymes - activators, which are capable to convert plasminogen into its active form - plasmin. First of all, potential fibrinolytic activity is stipulated to (tissue plasminogen activator) tPA as well as inhibitors such as plasminogen activator inhibitor (PAI), or α2-antiplasmin which are inhibitors of serine proteases, insignificant impact also belong to FXIIa (Smith et al., 1985). Thus the most defining role belongs to TAP i PAI-1 in the regulation of enzymatic lysis of fibrin clots (Katrii et al., 2016).

Tissue plasminogen activator (tPA) is the only proteinase hemostasis which is constantly secreted by endothelial cells in the active form (Dobrovolsky and Titaeva, 2002). tPA - single stranded glycoprotein with a molecular weight 70 kDa, a double-stranded molecule is formed due to the hydrolysis of the peptide bond by plasmin, kallikrein or factor FXa. Under physiological conditions, tPA activates Pg preferably in the presence of macromolecules (fibrin or other component of extracellular matrix) or cellular receptors (Silverstein et al., 1985). Upon activation of plasminogen occurs reorganization of its in active double-stranded form - plasmin, due to the specific peptide bond cleavage. At the same time two strands are linked together by two disulfide bridges. In most cases the single-stranded form of serine proteases are inactive proenzymes, tPA is an exception to this rule is formed by the active center, whereby shows its catalytic activity and exhibits the ability to bind to the inhibitors, such as PAI Dobrovolsky and Titaeva, 2002; Vyeremyeyenko et al., 1988; Lijnen, 1996).

There is also the factor XIIa (Hageman factor) - dependent activation pathway of plasminogen is associated with activation of kallikrein-kinin system and the complement system. However, according to this pathway activation plays a non-significant role in fibrinolysis (Lijnen, 2001; Ichinose et al., 1986). It is known that patients who have a deficiency of the factors (factor XII, prekallikrein, high molecular kininogen) of the contact phase of blood coagulation may suffer from thrombosis due to insufficient activation of fibrinolysis (Barkagan, 1988).

PAI-1 is a single stranded glycoprotein with a molecular weight 52 kDa, whose half-life in the bloodstream is 5 to 15 minutes. Up to 40% tPA is in the complex form with PAI-1 (in ratio 1:1). Under physiological conditions, in the plasma PAI is a 3 molecular forms (Stefansson et al., 1998; Debrock and Declerck, 1997; Alessi et al., 1991). PAI-1 is synthesized by endothelial cells and megakaryocytes. Upon activation of platelets during thrombus formation PAI-1 secreted from the α-granules, which leads to a high local concentration of the inhibitor and stabilize the fibrin clot matrix (Dobrovolsky and Titaeva, 2002). The literature suggests the hypothesis that high levels of PAI-1 in the blood line can cause damage to the vessel walls. But the role of a general or local increasing of the PAI-1 level in the development of atherothrombotic phenomena still is contradictory. However, there is an assumption that the low level or absence of PAI-1 leads to bleeding or blood clotting delay (Henri et al., 1997).

α2-antiplasmin is synthesized in the liver and secreted as a single stranded glycoprotein with a molecular weight of 67 kDa. α2-antiplasmin provide inhibitory effect on plasmin in three ways: prevent the binding of plasmin on fibrin; inhibits the proteolytic activity of the enzyme; is covalently attached to fibrin (Dobrovolsky and Titaeva, 2002). In the presence of fast-acting plasmin inhibitor can exhibit its activity only in spot of formation, remains bound to fibrin. If the connection of plasmin to fibrin is broken, and it releases into the blood, thus there is a momentary inactivation of plasmin. Furthermore inhibition of plasmin in the blood stream α2-antiplasmin has an important role in the stabilization of fibrin. During fibrin polymerization plasminogen and α2-antiplasmin incorporated in approximately equal amounts. Moreover, α2-antiplasmin binds and then stitched with the factor XIIIa which that part of fibrin, which is primarily cleaved by plasmin (Kimura and Aoki, 1987; Weisel et al., 1999).

Determination of fibrinolytic potential is important criteria system status of fibrinolysis, its ability to prevent thrombotic complications. In this frame the purpose of this study is to investigate the fibrinolytic potential changes in the organism under the different subtypes of ischemic stroke in both the acute phase of the disease and one year after suffering disease. Potential fibrinolytic system was evaluated determining total time euhlobulins lysis, activity of plasminogen, plasminogen tissue activator (tPA) and its inhibitors such as α-2-antiplasmin, plasminogen activator inhibitor type one (PAI-1), FXII-dependent fibrinolysis.

MATERIALS AND METHODS

Blood plasma samples were taken from 35 healthy donors and 123 patients: 66 patients with atherothrombotic ischemic stroke (AIS) and 56 patients with cardioembolic ischemic stroke (CIS) during the first 6 hours of the acute phase of the disease onset and after one year past stroke attack from 86 patients: 66 patients with AIS and 56 patients with CIS. Patients were hospitalized in neurological department of the Hospital №4 (Kyiv, Ukraine). The diagnosis of ischemic stroke was confirmed through computed tomography or magnetic resonance imaging. At the first day of admission to hospital, all patients received aspirin 325 mg orally, then 100 mg aspirin daily. From the second day of hospital stay, patients received low molecular weight heparin in prophylactic dose. All donors and patients or their relatives had been warned about the conduct of clinical research and provided written agreement about participation. Current experiment was approved by the ethics committee from ESC “Institute of Biology and medicine”, Kyiv, Ukraine.

Fasting blood samples were collected from the cubital vein of all patients on the 1st day of hospitalization as well as from the absolutely same group of patients one year past acute phase of disease. Blood was collected into 3.8% sodium citrate (9:1). Blood plasma was separated by further centrifugation at 2,000g for 15 min at the room temperature and stored at −20° C until assays were performed.

The plasma levels of plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA) were measured by standard enzyme linked immunosorbent assay (ELISA) techniques.
(Hockfield et al., 1991; Kemeny, 1991). The ELISA plates were coated overnight at 4°C with blood plasma samples previously diluted 10-fold with 50 mM Tris-HCl containing 130 mM NaCl, pH 7.4. After being washed, plates were blocked with 5% nonfat dry milk in 50 mM Tris-HCl containing 130 mM NaCl, pH 7.4 for 1 h at 37 °C and washed again. Then plates were incubated for 1 h at 37 °C with specific primary antibodies against the t-PA, PAI and vWF (Millipore, Germany). Plates were washed and incubated for 1 h at 37 °C with corresponding secondary antibodies (Sigma, USA) conjugated to horseradish peroxidase. After washing, substrate (o-phenylenediamine and hydrogen peroxide) was added. Plates were read at 492 nm by a microplate spectrophotometer (QuantiTm, BioTek Instruments, Inc). The healthy donor's samples concentration was set as 100%, and changes in concentration are given as percentage of controls.

For the measuring of potential plasminogen activity the medium of incubation included 50 mM Tris-HCl containing 130 mM NaCl, pH 7.4; 25 ml of blood plasma; 3 mM chromogenic substrate S 2 2 5 1; and 5-10 µM of streptokinase was prepared (Meltzer, 2010). The incubation in the 37°C of medium was performed. Registration of absorption was carried out in two-wave mode at the primary 405 and references 492 nm wavelength by the microplate spectrophotometer. The intensity of the tested process was proportional to the color intensity followed released of p-nitroaniline from the chromogenic substrate. For the healthy donors blood plasma the plasminogen activity was equal 100 ± 5 % (Kozlov et al., 2013).

To determine the α2-antiplasmin activity the analogical procedure was performed (Zabczyk et al., 2011). But instead of streptokinase the 0.5 cu/ml of plasmin was add. For the healthy donors blood plasma the α2-antiplasmin activity was equal 80 -100 % (Kozlov et al., 2013).

For the measurement of Hegeman (FXIa) dependent fibrinolysis the mixture was prepared: 8 ml of H₂O, 0.2 ml of 1% acetic acid; 0.5 ml of blood plasma and 0.5% kaolin. The mixture is carefully stirred and incubated at 37 °C for 30 minutes, then centrifuged for 6 minutes at 1500 rpm. The precipitate was dissolved in 0.5 ml of 50 mM Tris-HCl containing 130 mM NaCl, pH 7.4. Clot formation was induced by addition of the equal volume of 0.025 M calcium chloride solution. After clot formation the time (in minutes) of complete clot lysis was determined (Kozlov et al., 2013).

At the next step the determination of the time for euglobulin's fraction lysis in blood plasma was performed. In a conical glass tube 0.2 ml of blood plasma; 1.8 ml of H₂O; 150 ml of 0.25% acetic acid were added. The mixture was carefully stirred and incubated for 30 min at 4 °C. Fraction of euglobulins was precipitated by centrifugation at 700 g for 15 min per 4 °C. The precipitate was dissolved in 250 ml 50 mM Tris-HCl containing 130 mM NaCl, pH 7.4 (Bratchik, 1993). The total fibrinolytic activity was determined by the time (in hours) of complete lysis of fibrin clot formed in blood plasma from the euglobulins fraction which was induced by addition of the equal volume of 0.025 M calcium chloride solution (Hrytsiuk, 1994).

**RESULTS AND DISCUSSION**

The potential of fibrinolysis depend upon the regulation of the plasminogen conversion to plasmin. The activity of plasmin is tightly regulated by three main components of the plasminogen activator system: plasminogen; tissue-type plasminogen activators; urokinase type plasminogen activators; and plasminogen activator inhibitor type 1. Important that cascade is modulated by feedback loops that include Pg and activator inhibitor 1.

To characterize the fibrinolytic level of hemostasis its key parameters were analyzed (Table 1).

The proteolytic activity of plasmin is the principal determinant of the degree of proteolysis and fibrinolysis at the sites of vascular injury. The activity of plasmin is tightly regulated by three main components of the plasminogen activator (PA) system: plasminogen (Pg), plasminogen activators urokinase type (uPA), and tissue-type (tPA); and plasminogen activator inhibitor-1 (PAI-1). Results suggest the lower activity of Pg as well as its inhibitor in blood plasma of the patients with ischemic stroke both in acute phase and one year past. In acute phase the Pg activity of patients with AIS was on the 15% lower in comparison with healthy donors. Besides one year past acute phase the level of Pg activity become closer to the norm and was on the 9% lower for the patients with AIS. Even one year past stroke attack the disorders in fibrinolysis still saved. Maybe for the full rehabilitation longer period is required.

The level of Pg inhibitors in the patients with stroke showed the controversial results. Pathological conditions observed during the acute phase of both subtype of ischemic stroke were accompanied with abnormal level of t-PA and PAI-1 (Figure 1, 2). The changes in the level are given as percentage of controls. Some amount of patients with the level of inhibitors close to the norm observed in patients with both subtypes of ischemic stroke. 25% of the patients one year past CIS showed the higher level of PAI-1 on the 30-35%. And just 15% of the patients with acute CIS had a higher level of PAI-1 on the 20%. The 20% of the patients with one year past AIS showed on the 30-35% higher level of the PAI-1. And according the acute AIS 40% of the patients had on the 20% higher level of PAI-1. For the tPA level the analogical situation was observed for both subtypes of stroke. Moreover one year past stroke attack the disorders in fibrinolysis still saved. Thus 10% of the acute CIS patients had on the 20 % higher tPA level. One year past CIS 35% of the patients showed on the 20 % higher level but 15% of the patients had the 25-30% lower level of tPA. 50% of the acute AIS patients showed on the 20-25 % higher tPA level but one year past 55% of the patients had on the 20-40 % higher level and 20% of the patients on the 25-30% lower tPA level. Analysis of PAI-1 level in stroke conditions, showed a close relationship with concentration of tPA in blood plasma. No doubt the fact that 90% of PAI-1 aimed to inhibit the physiological action of tPA in the bloodstream. Any violation of such connection may be due to adaptive or compensatory phenomena in the hemostatic system during this pathological state of the organism.
Fig. 1: Level of the PAI-1 in the blood plasma of patients with ischemic stroke.

Fig. 2: Level of the tPA in the blood plasma of patients with ischemic stroke.

Fig. 3: The level of FXIIa-dependent fibrinolysis in patients with ischemic stroke.

*Statistically significant changes in comparison with healthy donors, # statistically significant changes in comparison with acute phase.
The next investigation showed significant extension of level of factor XIIa-dependent fibrinolysis especially for the acute phase of the both stroke subtype (Figure 3). Thus for the acute AIS the tested parameters was 2.8 times and for the CIS 3.5 times longer. One year past stroke the index become more close to the norm but still showed significant disorders in fibrinolysis. For one year past acute phase of AIS the XIIa-dependent fibrinolysis time was 1.9 times and for CIS 2.3 times longer comparing with the healthy donors. Thus, the detected changes are the evidence of the low fibrinolytic capacity of blood plasma of the stroke patients even one year past stroke attack.

Another one index of the state of fibrinolysis is the time of euglobulin fraction lysis. As known the main factors which determine current index are the PAI-1, Pg, FII and α2-antiplasmin. Thus euglobulin lysis time was significant extended for all tested group of patients (Figure 4). In average it was 5 times longer for both subtypes of acute ischemic stroke and 3 times longer one year past acute phase stroke.

**CONCLUSION**

Analysis of fibrinolytic level of the haemostasis system of the patients with acute ischemic stroke as well as one year past acute phase of ischemic stroke showed significant inhibition of fibrinolytic potential of the organism. Thus the development of both pathological conditions accompanied by lengthening of time of the euglobulin fraction lysis and FXIIa-dependent fibrinolysis. While for cardioembolic stroke were inherent more threatening performance. Results suggest the lower activity of plasminogen as well as α2-antiplasmin in blood plasma of the patients with AIS. Tested pathological conditions observed during the acute phase of both subtype of ischemic stroke were accompanied with abnormal level of t-PA and PAI-1. Even one year past stroke attack the disorders in fibrinolysis still saved. Maybe for the full rehabilitation longer period is required.

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