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Investigation of the phenotype heterogeneity in severe hemophilia A using thromboelastography, thrombin generation, and thrombodynamics

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ABSTRACT

Background: Hemophilia A (HA) patients with similar factor VIII levels can demonstrate varying bleeding tendencies. In particular, 10–15% of all severe HA patients (FVIII:C < 1 IU dL^{-1}) do not require regular replacement therapy. Modern global coagulation assays can help to detect and study this "mild" bleeding phenotype. Here, we investigated the coagulation status of different bleeding phenotypes using various types of global coagulation assays.

Materials and Methods: Ten HA patients with severe phenotype and eleven patients with mild phenotypes were included in the study. For each patient, thromboelastography (TE), thrombodynamics (TD), and kaolin- or tissue factor-induced thrombin generation (TG) were measured. TG in platelet-rich plasma (PRP) was investigated using our original modification when the thrombin generation curve showed two peaks, previously shown to depend on platelet activity. We also utilized TG and TD with the addition of thrombomodulin.

Results: The second peak amplitude and ETP of PRP TG were the only parameters that were significantly higher in mild bleeders (peak 41.6 ± 3.5 nM, ETP 1966 ± 169 nM*min) than in patients with severe bleeding (peak 28.3 ± 3.3 nM, ETP 1359 ± 130 nM*min).

Conclusions: Our results suggest that severe and mild HA phenotypes could be distiguished by TG assay in PRP suggesting that difference in platelet activity can be involved in the phenotype formation. According to our previous results we can suppose that the mechanism of the phenotypic heterogeneity is linked with TG mediated by PS-expressing platelets.

Introduction

Hemophilia A (HA) is an inherited X-linked recessive trait that is manifested as a bleeding disorder. People suffering from HA lack sufficient quantities of coagulation factor VIII (FVIII). The severity of HA is assessed using a FVIII activity assay (FVIII:C). However, patients that have identical FVIII activity can demonstrate quite different tendencies for bleeding. Indeed, 10–15% of severe hemophiliacs (FVIII: C < 0.01 IU dL^{-1}) have very rare bleeding episodes [1,2]. This phenomenon is often referred to as the "mild" clinical phenotype of hemophilia. The reasons and mechanisms behind the formation of the mild phenotype have remained unclear to date, but several hypotheses have been considered.

For example, clinical severity could possibly depend on the variability of the FVIII genetic mutations. Mutations in the FVIII gene that do not totally prevent protein synthesis (i.e., non-null mutations) could allow some residual FVIII activity that cannot be detected by the FVIII:C assay [3,4]. The second hypothesis is thrombophilic mutations [5], such as factor V Leiden [6–9] and prothrombin G20210A [7,8], or perhaps non-inherited prothrombotic markers that can be present in parallel with HA. A recent study reported that platelet suspensions isolated from the blood samples of severe hemophiliacs with the mild phenotype demonstrate higher level of phosphatidylserine(PS)-

Abbreviations: AMC, 7-amino-4-methyl-coumarin; APTT, activated partial thromboplastin time; CTI, Corn trypsin inhibitor; DMSO, Dimethylsulfoxide; ETP, Endogenous thrombin potential; FVIII, Coagulation factor VIII; FVIII:C, Clotting FVIII activity; HA, Hemophilia A; PC, Phosphatidylcholine; PFP, Platelet-free plasma; PRP, Platelet-rich plasma; PPP, Platelet-poor plasma; PS, Phosphatidylserine; RabTF, Tissue factor from rabbit brain; TG, Thrombin generation; TGA, Thrombin generation assay; TGC, Thrombin generation curve; TD, Thrombodynamics; TE, Thromboelastography; TF, Tissue factor; TM, Thrombomodulin.

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expressing platelets [10] as compared to that of severe bleeders [11]. Also, according to another study, platelets can significantly modify procoagulant activities in HA [12]. Note that in all studies that have investigated HA bleeding phenotypes, the patients that had the severe clinical phenotype also may have non-null FVIII mutations and thrombophilic markers or high levels of PS-expressing platelets. These facts indicate that all of the aforementioned mechanisms can interact to form a specific bleeding phenotype.

The ability to predict the HA patient's bleeding tendency is extremely crucial. Nowadays, various types of modern global coagulation tests, such as thromboelastography (TE) [13] and the thrombin generation assay (TGA) in platelet-poor plasma (PPP) or platelet-rich plasma (PRP) [14–16], can be used to offer bleeding predictions.

Some articles studied a correlation between the TGA parameters and the clinical severity [4,17,18]. It should be mentioned that in the studies [17,18] the ability of TGA to correspond with FVIII level was investigated as well. Also, study [19] has asserted that TE and TG can correlate with the FVIII level but the phenotypic heterogeneity not linked with FVIII:C was not discussed.

Recently, we have shown that such antiplatelet agents as dimethylsulfoxide (DMSO) and prostaglandin E1 can induce the appearance of a second peak in the thrombin generation curve (TGC) [20]. The second peak is mediated only by the PS-expressing platelets, while platelet α-granules and reactions on plasma phospholipids contribute to the first one. This effect can be used to analyze the impact of PS-expressing platelet-mediated thrombin generation (TG) to the total TG. According to the study [11] that points at the possible role of PS-expressing platelets in the formation of the clinical phenotype of HA the modified two-peaked TGA assay can be utilized to predict the tendency of bleeding.

Also, another coagulation assay called Thrombodynamics (TD, [21,22]) is based on the spatial aspects of coagulation. During this assay, the side light scattering from the growing fibrin clot is measured, and the velocity of clot growth is calculated. The initiation of coagulation is accomplished by means of a special activator with immobilized tissue factor (TF). TD parameters were shown to correlate with FVIII level [23,24].

In spite of the number of studies, the nature of phenotypic heterogeneity is poorly understood. Analogously, we now have the opportunity to use the great number of variations of global hemostatic assays, but we do not exactly know which of them is the best to predict the bleeding tendency. In this paper, we studied the possibility of TE, 5 variations of TGA and 3 variations of TD to correspond to the clinical phenotypes of severe HA.

Materials and Methods

The main conditions of the assays we used are presented in Supplementary Table 1.

Collection of HA Patient Blood and Plasma Preparation

The patients who participate in this research study gave their written informed consent. The study was approved by the Ethics Committees of the Center for Theoretical Problems of Physicochemical Pharmacology and National Research Center for Hematology. Blood was collected through venipuncture and immediately placed in a test tube with 3.8% sodium citrate (pH 5.5). The volume ratio between blood and citrate was 9:1. For all TD and most TG tests, the test tube also contained corn trypsin inhibitor (CTI) (Pushchino, Russia; 0.3 mg CTI per 10 ml of blood) to prevent contact activation. The patients claimed not to have received FVIII concentrate for 5 days prior to blood collection. PRP was obtained by centrifugation for 5 minutes at 100 g. To prepare PPP, blood was centrifuged for 15 minutes at 1600 g. PFP for TD was prepared from PPP by centrifugation for 5 minutes at 10 000 g.

FVIII:C, Detection of FVIII Inhibitors, and APTT

FVIII activity was assessed using a previously described one-stage method [25]. Since inhibitory antibodies against FVIII may influence the half-life of FVIII, the presence of these antibodies in the pre-infusion samples was assessed using the Nijmegen modification [26,27]. For APTT determination, the standard APTT kit (Renam, Moscow, Russia) was used.

Thromboelastography

TE was accomplished using a TEG® 5000 thromboelastograph® (Haemoscope Corp., MA, USA). A sample of 340 μl of citrated blood without CTI was placed in a TEG® flask and mixed with 20 μl of a solution containing 200 mM of $CaCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) and 72 pM of TF from rabbit brain (Renam, Moscow, Russia). The final TF concentration was 4 pM. To determine the TF activity, the Actichrome TF activity assay (American Diagnostica, Stamford, CT, USA) was used.

Preparation of Phospholipids

Phospholipids were prepared closely to ones described in [28]. Briefly, we mixed 24 μl of phosphatidylcholine (PC) solution and 6.5 μl of PS solution in a round-bottomed flask. Both the PS and PC were obtained from Avanti Chemicals (Ormeau, Australia). Then, the mixture was dried for 30 minutes under a nitrogen stream. After that, the phospholipids were resuspended with 1 ml of buffer A containing 20 mM HEPES and 145 mM NaCl (pH 7.5). All these compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA). The obtained mixture was shaken for 30 minutes at 57 °C. After that, the phospholipids were frozen and refrozen several times at −20 °C. Then the mixture was run through the extruder several times. The extruder pore size was 0.1 μm. Finally, the phospholipids were frozen at −20 °C to maintain lipid activity during storage. The final concentration of the phospholipids in the mixture was 1 mM.

Thrombodynamics

TD was made in PFP using a Thrombodynamics Analyzer (HemaCore LLC, Moscow, Russia). During the assay, the side scattering from the growing fibrin clot was measured every 15 seconds. The assay was utilized for three variations: in PFP only, in PFP with phospholipids, and in PFP with phospholipids and thrombomodulin (TM). A sample of 300 μl of PFP containing CTI was mixed with 12 μl of buffer B containing 750 mM HEPES, 145 mM NaCl (pH 7.4) for pH stabilization. Also, 3 μl of phospholipids and 6 μl of rabbit lung TM (Haematologic Technologies, Essex Junction, VT, USA) could also be added to this mixture. The final concentration of TM was 3 nM. After that, PFP was incubated for 10 minutes at 37 °C, and after recalcification with 6 μl of $CaCl_2$ (1 M), the sample was placed into prewarmed thin flat experimental chamber and put into the Thrombodynamics Analyzer. After that, a special activator with immobilized TF was put into the flask containing PFP, and the assay started. The clot size was calculated as described in [21,22], and finally, the time dependence of the clot size was obtained. In Supplementary Fig. 1, an example of such curves with and without additional phospholipids or TM is shown. The clot growth can be separated into two stages. These two stages can be characterized by the following two velocities of clot growth: the initial rate (i.e., the first 10 min) and the stationary rate (i.e., 10–40 min). If TM is present in the plasma, the stationary rate is very close to zero and can be substituted with the clot size after 40 minutes from the start of the assay.