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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.

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Thrombin generation assays

Five variations of the TGA were conducted. TG in PPP was investigated in the presence of 4 pM and 1 pM of TF. For the investigation of the role of the protein C system, we used TGA in 4 pM of TF-induced PPP with the presence of 3 nM TM. Additionally, we measured TG in kaolin-induced PPP. To investigate platelet-dependent two-peaked TG, we used 4 pM TF-induced PRP containing 1.6% DMSO. For all the TG assays excluding TG with kaolin activation, the citrated plasma samples contained CTI. In Supplementary Figs. 2 and 3, examples of the TGCs that we obtained are presented. The fluorescence of 7-amino-4-methyl-coumarin (AMC) was continuously measured at 37 °C using an Appliskan Multimode Microplate Reader (Thermo Scientific, Helsinki, Finland). The TGA mostly was utilized as described previously [20,29]. Plasma was mixed with the fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) solution in a volume ratio of 4:1. Initially, the substrate was stored at −20 °C in a 25 mM DMSO solution (Sigma-Aldrich, St. Louis, MO, USA). Before mixing with the plasma, the solution was diluted ten times with buffer A. Then 20 μl of buffer B was added to 500 μl of the plasma for pH stabilization. After that, 100 μl of this mixture was placed into the wells of 96-well flat bottom plate. Coagulation was triggered with 20 μl of a 100 mM $CaCl_2$ solution containing one of the coagulation activators per a well. The final DMSO concentration in all of the experiments was 1.6%. The coagulation activators in PPP were RabTF (i.e., 1 or 4 pM final plasma concentration) and the kaolin suspension from the standard APTT kit (200 times final plasma dilution, Renam, Moscow, Russia). The dilution degree of kaolin was chosen to obtain the ETP values close to the ones obtained using activation with 4 pM of RabTF. Also, for the PPP assay, 4 μM of phospholipids were present. For the PRP TG, we used recombinant human TF (4 pM final plasma concentration, Innovin, Dade-Behring, Marburg, Germany). To obtain a concentration of platelets in PRP equal to 150 000 platelets/μl, the PRP was diluted with autologous PPP. After adjustments in the TF and the substrate, the platelet concentration in the PRP became 100 000 platelets/μl. In our experiments [20], we have shown that the 1.6% DMSO present in the PRP sample decreases the PS exposure rate during platelet activation and induces the appearance of two peaks in the TGC but does not influence the endogenous thrombin potential (ETP) value. Therefore, all of our PRP TGCs had two peaks (Supplementary Fig. 3).

For calibration of fluorescence signal, two types of wells with non-clotting plasma were prepared. The first type contained 20 μl of buffer C instead of the activator to obtain the background fluorescence level. The second one (calibration wells) contained 2 μl of AMC (Sigma-Aldrich, St. Louis, MO, USA) solution in DMSO and 18 μl of buffer C instead of the activator. The final AMC concentration in these wells was 8 μM. Fluorescence was continuously measured at 37 °C using an Appliskan Multimode Microplate Reader (Thermo Scientific, Helsinki, Finland) (λ_{exc} = 355 nm, λ_{em} = 460 nm). The PRP was not stored for more than 1 hour before use. All results are reported as the averaged readings of duplicated wells.

Data analysis was carried out using Origin 6.0 or 8.0 software (Microcal Software, Northampton, MA, USA). The background fluorescence was subtracted from the signal of fluorescence in all of the wells. To calculate the AMC concentration, we used the appropriate calibration wells and considered the nonlinear fluorescence dependence on AMC concentration (inner filter effect [30]). The thrombin curve was obtained by differentiation of the time-dependent AMC curve (after its smoothing using 7 points averaging) and calculation of the thrombin concentrations using the previously measured kinetic constants for a given fluorescence substrate (K_M = 156 μM; k_{cat} = 46 min^{-1}). The substrate concentration corresponding to each time point was calculated by subtracting the concentration of produced AMC from the initial substrate concentration. So, we took into account the substrate consumption in our technique. The contribution of α_2-macroglobulin [14,30–32] was subtracted using a specially designed algorithm realized in Origin software as described previously [20,29].

Statistics

To compare the mean values of the coagulation assay parameters from the patients with mild bleeding phenotypes to those of patients with severe bleeding phenotypes, we used one-way ANOVA test.

Results

Patient characterization

21 HA patients (i.e., 11 with the mild phenotype and 10 with the severe phenotype) participated in this study. The basic FVIII:C level for each of the patients was not more than 1 IU dL^{-1} with exception to patient 7 (FVIII:C = 1.3 IU dL^{-1}) and patient 12 (FVIII:C = 1 IU dL^{-1}). In this study, we defined the mild bleeding phenotype according to the regular replacement therapy requirements and the frequency of bleeding. Patients with mild phenotype usually did not have bleeding episodes, but occasionally not more than two bleeding events could happen. Therefore, these patients did not require the regular FVIII replacement therapy. Some patients with this phenotype were on FVIII replacement prophylactics when facing surgical procedures. Table 1 details the age, the frequency of bleeding episodes, and the basic FVIII:C results for each patient. Most of the patients demonstrated non-detectable FVIII:C or close to 1 IU dL^{-1} (Supplementary Table 2). No one had detectable FVIII inhibitors. Two patients from the mild group and two from the severe group had definable FVIII:C results that were significantly more than 1 IU dL^{-1} even after the 5 day wash-out period (i.e., 5 days off their FVIII regimen). The APTT measurements did not differ between the patients of differing phenotypes (for the raw data, see Supplementary Table 2).

Thromboelastography

Supplementary Table 2 presents the TE parameter values for each patient. Fig. 1 shows that the TE parameters did not appear to be related to the phenotypic differences of the two groups.

Thrombodynamics

In Fig. 2, the values of TD parameters are shown. No parameters seemed to be related to either the mild or severe phenotype. Also, the addition of phospholipids did not affect the TD parameters.

Table 1
Participating patient demographics and HA characteristics.

Phenotype	ID		Age, year	Bleeding frequency, episodes per month (with prophylactics)	Basic FVIII:C, IU dL^{-1}	Number of arthroses
Mild	1	Bol	22	0-1	<1	2
	2	Shu	38	0-1	<1	0
	3	Der	31	0-2	<1	4
	4	Nuj	60	0-2	1	6
	5	Las	44	0-2	<1	6
	6	Bob	55	1-2	<1	4
	7	Kli	43	0-2(0)	1.3	3
	8	Sil	21	0-2(0)	<1	1
	9	Bov	33	0-1	<1	5
	10	Kho	78	0-2(0)	1	8
	11	Kom	21	0-1(0)	<1	2
Severe	12	Kop	44	2-4(0-1)	1	4
	13	Gor	32	2-5(0-1)	<1	1
	14	Fed	42	3-4(0-1)	<1	4
	15	Kir	57	4-6(0-1)	1	8
	16	Vol	19	3-5(0-1)	<1	3
	17	Var	26	2-3	<1	3
	18	Gul	35	6 and more (0-2)	<1	Not available
	19	Sho	29	1-4 (0-1)	<1	4
	20	Dya	46	2-3 (0-1)	<1	6
	21	Kas	41	2-4	<1	8

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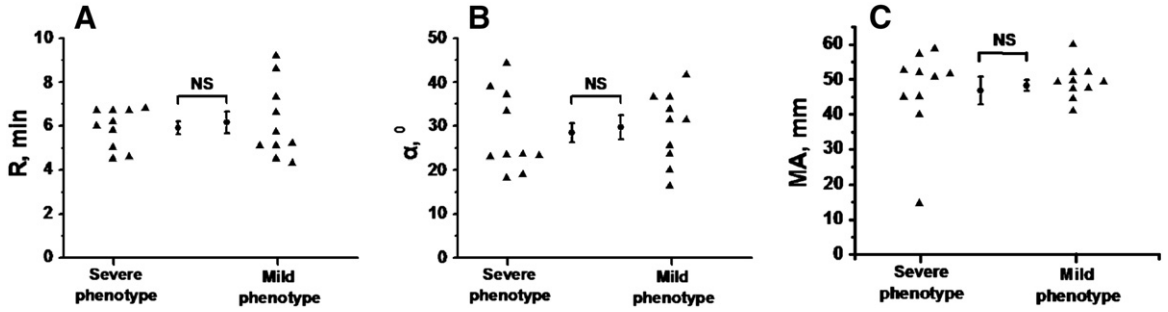

Fig. 1. Values of the TE parameters R (A), α (B), and MA (C) for patients with clinically severe and mild phenotypes of HA with their means and SE. A one-way ANOVA test was used to analyze the data. NS means not significant.

Fig. 2 and Supplementary Table 3 presents all of the TD parameter values. The TD parameters appear as not allowing to differentiate groups of hemophiliacs.

Thrombin generation in PPP

Fig. 3 shows ETP and thrombin peak values in PPP after activation with 1 pM or 4 pM TF and in the presence of TM. ETP and peak after the 4 pM TF activation were always higher than those after the 1 pM activation or in the case of TM addition. In three cases, the ETP and thrombin peak after 1 pM activation were not detectable and considered to be zero.

Interestingly, most of the kaolin activation samples demonstrated undetectable TG, which was in the sharp contrast with non-hemophilic plasmas that we assessed in our other studies (Supplementary Fig. 4). Healthy PPPs usually demonstrated detectable TG with ETP values close to ones obtained after 4 pM TF activation. However, four HA patients from the mild group and one from the severe group demonstrated detectable TG parameters. All of these TGCs seemed to have two peaks (Supplementary Fig. 5).

None of the parameters for patients in the mild group differed significantly from that of patients with severe phenotypes (Fig. 3). Supplementary Table 4 shows all the TG values for each patient.

Thrombin generation in PRP

Fig. 4 illustrates the parameters of the two-peaked TGCs in the PRP. The ETP and the second peak amplitude were the only parameters that differed significantly between the mild and severe groups. All of the PRP TG parameter values are presented in Supplementary Table 5.

Discussion

The main result of this study is that the second peak amplitude in our modified PRP TG test is significantly higher in patients with the mild HA phenotype as compared to that of patients with the severe

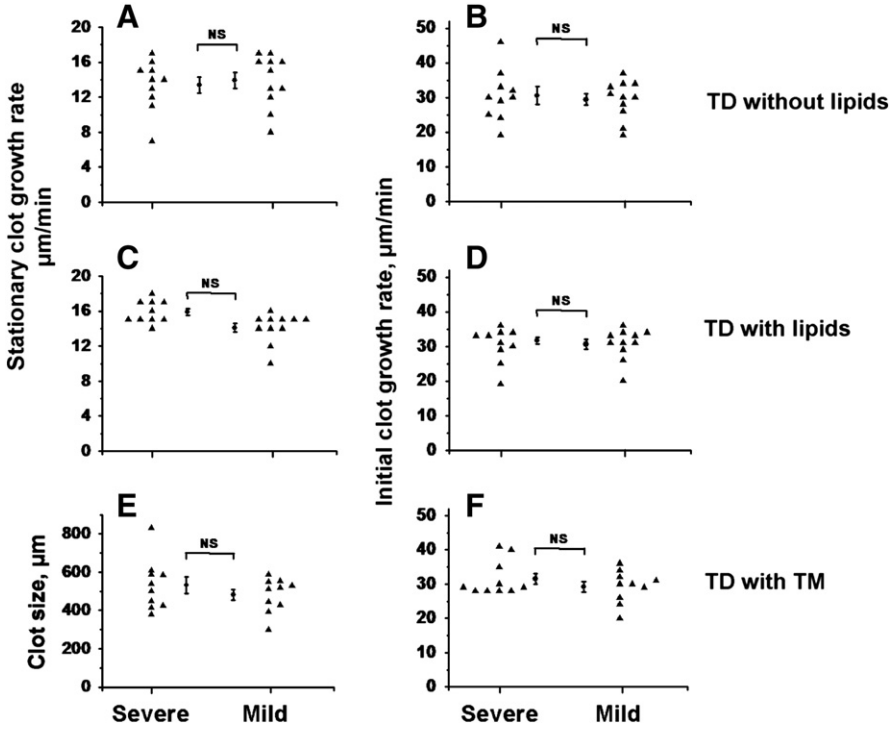

Fig. 2. Values of the TD parameters without lipids (i.e., stationary (A) and initial (B) clot growth rates), in the presence of lipids (i.e., stationary (C) and initial (D) clot growth rates) and with TM (i.e., clot size after 40 min of assay (E) and initial (F) clot growth rate) for patients with clinically severe and mild phenotypes of HA with their means and SE. A one-way ANOVA test was used to analyze the data.

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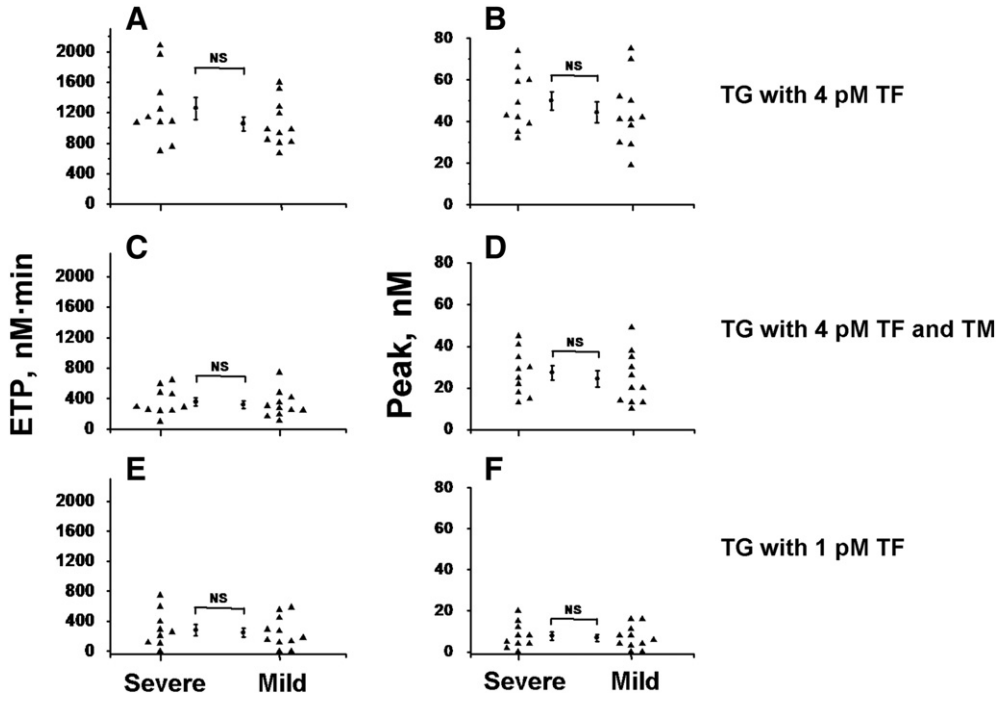

Fig. 3. Values of the PPP TG parameters (i.e., ETP (A, C, E) and peak (B, D, F)) for patients with clinically severe and mild phenotypes of HA with their means and SE. A one-way ANOVA test was used to analyze the data. Columns without P-values do not significantly differ from one another.

phenotype. None of the other tests revealed difference between the two phenotypes.

Our findings show that platelet-mediated TG is the best indicator of HA phenotype formation. Previously, we have shown that the second peak is mediated by the PS-expressing population of activated platelets [10,20]. The amplitude of this peak reflects the contribution of the platelet activation and the PS exposure on their surfaces. From this perspective our results are in agreement with those of Dale et al. [11], in which they showed that severe HA patients with the mild bleeding phenotype demonstrated significantly higher levels of PS-expressing platelets as compared to that of severe bleeders. Likewise, another study [4] showed that the ETP in PRP for those with the mild phenotype significantly differed from that of patients with the severe phenotype. Also, our results are in agreement with those of another study [18] in which a correlation between bleeding tendency and whole blood thrombin-antithrombin III complex generation was shown. In that study, the platelets in whole blood could significantly affect coagulation. On the other hand, another study [17] showed correlations between PPP TG and bleeding phenotype, but the patients included in this study had varying basic FVIII:C. In this case, clinical severity could be correlated with FVIII activity and TG parameters as well.

In addition to the general differences observed in the second peak amplitude between the two groups of patients in our study, great variations were observed in this parameter within the groups. Therefore, the patient with the severe clinical phenotype could have a rather high second peak amplitude and ETP on PRP. Similar results were also obtained in other studies [4,11]. According to the present and previous studies, we can conclude that despite the main role of the procoagulant platelet function in the formation of the mild HA phenotype, some additional mechanisms can also affect bleeding tendencies. In the literature, one hypothesis described the influence of other coagulation factors besides FVIII [33]. Such mechanisms cannot be detected by the PRP TG assay, but in some cases, other assays possibly can be used to assess these additional pathways. The fact that four of our patients showed detectable kaolin TG and only one severe patient demonstrated this phenomenon is very interesting. We can suspect that an additional approach to correct bleeding issues may lie in the contact activation system. Also, in one case (i.e., mild patient 8), when TM did not significantly affect TG, abnormalities in the protein C system seemed to contribute to additional hypercoagulability. Also the spatial aspects of coagulation can additionally influence on the bleeding tendency. In the case of severe patient 21 we observed rather low stationary clot growth rate in common while TGA on PRP demonstrated the similarity with mild

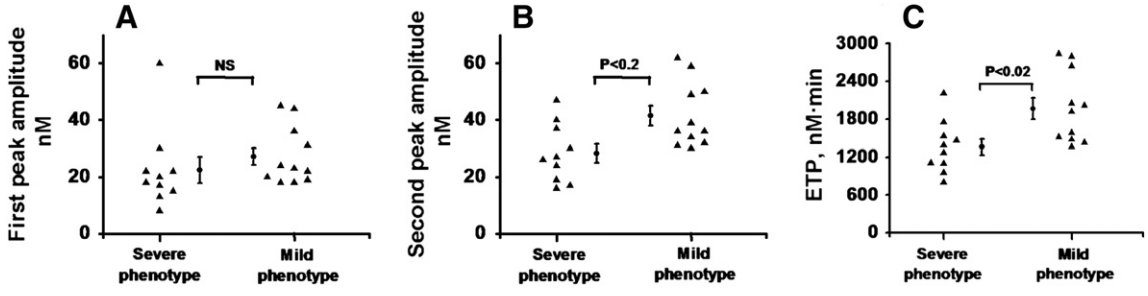

Fig. 4. Values of the PRP TG parameters (i.e., the first (A) and second (B) peak amplitudes and ETP (C)) for patients with clinically severe and mild phenotypes of HA with their means and SE. A one-way ANOVA test was used to analyze the data.

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phenotype. Analogous result but with TGA in PPP with 1 pM TF we obtained on the sample of patient 20. Thus, extrinsic pathway can also play a role in the formation of the clinical phenotype. Also, this patient demonstrated rather low amplitude of the first peak which allows us to suspect additional hypocoagulable impact linked with α-granules. The fact that most of our assays did not reflect the phenotypic heterogeneity in general can be also explained by existence of the strong mechanism located in the fibrinolytic system. Such a possibility has been previously reported [34].

It must be mentioned that while all TGAs demonstrated great variability, TD did not vary so much. Maybe further developing of this assay (for example, possibility to use PRP) can lead to appearance of a test which will be more sensitive to the phenotype but without great variations.

In our study we observed non-detectable kaolin TG for the most of patients while activation with TF showed the opposite result. This phenomenon can be explained by strong repression of the intrinsic pathway by lacking FVIII. In these conditions only extrinsic pathway can provide coagulation and in the kaolin-induced PPP this pathway is not activated. It can be supposed that increase of kaolin activation can make TG detectable.

In our research, we defined the mild bleeding phenotype mostly according to the frequency of bleeding. Unfortunately, in Russian Federation, the opportunity for regular and on-demand FVIII therapy has only been available since 2007. Because of this lack of access to proper treatment, even patients with the mild bleeding phenotype may now suffer from orthopedic disturbances. We did not use various specific bleeding scores [4,18] because we chose the patients for whom the phenotype was clear based solely on bleeding frequency.

Our study demonstrates the importance of conducting further investigations of platelet-mediated coagulation in HA. Following our results the new studies focused on the investigation of platelet functions like PS or α-granules expression and content (i.e. von Willebrand factor or factor V) upon various activation conditions should be done on hemophiliac platelets. These studies can more precisely elucidate the platelet signal pathways through which the bleeding phenotype correction can act. Also, a definition for the unknown mechanisms of phenotype formation is very urgent. These findings can provide information for the development of new drugs that can be used to prevent bleeding episodes.

Our modification of TGA in PRP also needs to be discussed. Previously, we showed that PGE_1 and DMSO both can also provide two-peaked TGC through this mechanism. So, we could use one of these compounds as the inhibitor of PS expression rate. Although DMSO is non-specific agent with poorly understood mechanism of action, we chose it because we cannot avoid it in TGA because DMSO is a solvent for fluorogenic substrate. If we used PGE_1 we would see the mixed effect of two compounds which can create some problems with interpretation of our results.

Predicting the bleeding tendency of HA patients reminds a very urgent problem. Existing standard assays still cannot be used to exactly define the phenotype for HA patients. Based on the results of our research, we have concluded that the main global assay that can be used in this field is TG in PRP. Perhaps our modification will be more useful than the more traditional method used in [4]. Note that developing the tests that could be used to better understand unknown mechanisms of bleeding phenotype modulation could help in bleeding prediction, therapy improvements, and improved quality of life for the people suffering from HA.

Disclosure of conflict of interests

M. Panteleev and F. Ataullakhanov are co-founders of HemaCore LLC, which holds several patents on the diagnostic use of spatial coagulation assays.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2013.04.004.

References

[1] Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. Lancet 2003;361:1801–9.
[2] van den Berg HM, De Groot PH, Fischer K. Phenotypic heterogeneity in severe hemophilia. J Thromb Haemost 2007;5(Suppl. 1):151–6.
[3] Oldenburg J, Pavlova A. Discrepancy between one-stage and chromogenic factor VIII activity assay results can lead to misdiagnosis of haemophilia A phenotype. Hamostaseologie 2010;30:207–11.
[4] Santagostino E, Mancuso ME, Tripodi A, Chantarangkul V, Clerici M, Garagiola I, et al. Severe hemophilia with mild bleeding phenotype: molecular characterization and global coagulation profile. J Thromb Haemost 2010;8:737–43.
[5] Franchini M, Montagnana M, Targher G, Veneri D, Zaffanello M, Salvagno GL, et al. Interpatient phenotypic inconsistency in severe congenital hemophilia: a systematic review of the role of inherited thrombophilia. Semin Thromb Hemost 2009;35:307–12.
[6] Franchini M, Lippi G. Factor V Leiden and hemophilia. Thromb Res 2010;125:119–23.
[7] Shetty S, Vora S, Kulkarni B, Mota L, Vijapurkar M, Quadros L, et al. Contribution of natural anticoagulant and fibrinolytic factors in modulating the clinical severity of haemophilia patients. Br J Haematol 2007;138:541–4.
[8] Shetty S, Ghosh K. Reduced clinical severity in a mutationally well-characterized cohort of severe hemophilia with associated thrombophilia.Am J Clin Pathol 2008;130:84–7 [quiz 146].
[9] Nowak-Gottl U, Escuriola C, Kurnik K, Schobess R, Horneff S, Kosch A, et al. Haemophilia and thrombophilia. What do we learn about combined inheritance of both genetic variations? Hamostaseologie 2003;23:36–40.
[10] Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. Blood 2000;95:1694–702.
[11] Saxena K, Pethe K, Dale GL. Coated-platelet levels may explain some variability in clinical phenotypes observed with severe hemophilia. J Thromb Haemost 2010;8:1140–2.
[12] Wartiovaara-Kautto U, Joutsi-Korhonen L, Ilveskero S, Armstrong E, Lassila R. Platelets significantly modify procoagulant activities in haemophilia A. Haemophilia 2011;17:743–51.
[13] Chitlur M, Sorensen B, Rivard GE, Young G, Ingerslev J, Othman M, et al. Standardization of thromboelastography: a report from the TEG-ROTEM working group. Haemophilia 2011;17:532–7.
[14] Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb 2003;33:4–15.
[15] Vanschoonbeek K, Feijge MA, Van Kampen RJ, Kenis H, Hemker HC, Giesen PL, et al. Initiating and potentiating role of platelets in tissue factor-induced thrombin generation in the presence of plasma: subject-dependent variation in thrombogram characteristics. J Thromb Haemost 2004;2:476–84.
[16] van der Meijden PE, Feijge MA, Giesen PL, Huijberts M, van Raak LP, Heemskerk JW. Platelet P2Y12 receptors enhance signalling towards procoagulant activity and thrombin generation. A study with healthy subjects and patients at thrombotic risk. Thromb Haemost 2005;93:1128–36.
[17] Beltran-Miranda CP, Khan A, Jaloma-Cruz AR, Laffan MA. Thrombin generation and phenotypic correlation in haemophilia A. Haemophilia 2005;11:326–34.
[18] Brummel-Ziedins KE, Whelihan MF, Gissel M, Mann KG, Rivard GE. Thrombin generation and bleeding in haemophilia A. Haemophilia 2009;15:1118–25.
[19] van Veen JJ, Gatt A, Bowyer AE, Cooper PC, Kitchen S, Makris M. Calibrated automated thrombin generation and modified thromboelastometry in haemophilia A. Thromb Res 2009;123:895–901.
[20] Tarandovskiy ID, Artemenko EO, Panteleev MA, Sinauridze EI, Ataullakhanov FI. Antiplatelet agents can promote two-peaked thrombin generation in platelet rich plasma: mechanism and possible applications. PLoS One 2013;8:e55688.
[21] Parunov LA, Fadeeva OA, Balandina AN, Soshitova NP, Kopylov KG, Kumskova MA, et al. Improvement of spatial fibrin formation by the anti-TFPI aptamer BAX499: changing clot size by targeting extrinsic pathway initiation. J Thromb Haemost 2011;9:1825–34.
[22] Soshitova NP, Karamzin SS, Balandina AN, Fadeeva OA, Kretchetova AV, Galstian GM, et al. Predicting prothrombotic tendencies in sepsis using spatial clot growth dynamics. Blood Coagul Fibrinolysis 2012;6:498–507.

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[23] Ovanesov MV, Krasotkina JV, Ul'yanova LI, Abushinova KV, Plyushch OP, Domogatskii SP, et al. Hemophilia A and B are associated with abnormal spatial dynamics of clot growth. Biochim Biophys Acta 2002;1572:45–57.
[24] Ovanesov MV, Lopatina EG, Saenko EL, Ananyeva NM, Ul'yanova LI, Plyushch OP, et al. Effect of factor VIII on tissue factor-initiated spatial clot growth. Thromb Haemost 2003;89:235–42.
[25] Morfini M, Lee M, Messori A. The design and analysis of half-life and recovery studies for factor VIII and factor IX. Factor VIII/Factor IX Scientific and Standardization Committee of the International Society for Thrombosis and Haemostasis. Thromb Haemost 1991;66:384–6.
[26] Verbruggen B, Novakova I, Wessels H, Boezeman J, van den Berg M, Mauser-Bunschoten E. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. Thromb Haemost 1995;73:247–51.
[27] Kasper CK, Aledort L, Aronson D, Counts R, Edson JR, van Eys J, et al. Proceedings: A more uniform measurement of factor VIII inhibitors. Thromb Diath Haemorrh 1975;34:612.
[28] Panteleev MA, Ananyeva NM, Greco NJ, Ataullakhanov FI, Saenko EL. Factor VIIIa regulates substrate delivery to the intrinsic factor X-activating complex. FEBS J 2006;273:374–87.
[29] Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV, et al. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. Thromb Haemost 2007;97:425–34.
[30] Hemker HC, Giesen P, AlDieri R, Regnault V, de Smed E, Wagenvoord R, et al. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. Pathophysiol Haemost Thromb 2002;32:249–53.
[31] Hemker HC, Giesen PL, Ramjee M, Wagenvoord R, Beguin S. The thrombogram: monitoring thrombin generation in platelet-rich plasma. Thromb Haemost 2000;83:589–91.
[32] Hemker HC, Wielders S, Kessels H, Beguin S. Continuous registration of thrombin generation in plasma, its use for the determination of the thrombin potential. Thromb Haemost 1993;70:617–24.
[33] Gissel M, Whelihan MF, Ferris LA, Mann KG, Rivard GE, Brummel-Ziedins KE. The influence of prophylactic factor VIII in severe haemophilia A. Haemophilia 2012;18:193–9.
[34] Grunewald M, Siegemund A, Grunewald A, Konegan A, Koksch M, Griesshammer M. Paradoxical hyperfibrinolysis is associated with a more intensely haemorrhagic phenotype in severe congenital haemophilia. Haemophilia 2002;8:768–75.

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