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Eculizumab effect on the hemostatic state in patients with paroxysmal nocturnal hemoglobinuria

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ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by a hypercoagulable state associated with acute hemolysis. Eculizumab is used to reduce the intensity of intravascular hemolysis in PNH patients.
The hemostatic status of three patients with PNH was assessed during eculizumab treatment by D-dimer assay and the global assays: thromboelastography (TEG), thrombin generation test (TGT), and thrombodynamics (TD). In the state of hemolytic crisis before the therapy D-dimer concentration was increased in two patients accompanied by hypercoagulation changes in TEG parameter angle (α). TD parameter the clot growth velocity (V) revealed hypercoagulability while TGT parameter ETP was within the normal range in all patients.
The lactate dehydrogenase (LDH) activity decreased during the 8 months of eculizumab therapy. The physical health was improved, the frequency of hemolytic crisis decreased. Patients periodically exhibited hypercoagulable state: the mean values $\alpha = 38 \pm 11°$ (with normal range 20–40°), ETP = 1311 ± 442 nM·min (with normal range 800–1560 nM·min), V = 31 ± 4 μm/min (with normal range 20–29 μm/min). During the eculizumab therapy two patients had the repeated clinical manifestation of acute hemolytic crisis, the parameters of the global tests were increased compared to the previous measurement.
The global hemostasis tests TEG, TGT and TD revealed hypercoagulability in patients with PNH during eculizumab therapy.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH), also referred to as Marchiafava–Micheli syndrome, is a rare acquired blood disorder characterized by complement-induced intravascular hemolytic anemia, hemoglobinuria and thrombosis [1–4]. Episodic hemolytic crises are common for PNH patients. Crises usually occur spontaneously; sometimes they are triggered by hypothermia, infection or fatigue [3–7].

The hemostatic system of patients with PNH is characterized by a hypercoagulable state [7–10]; the risk of thrombosis in these patients is 62 times higher than the risk in general population. Thrombosis is the main cause of death in PNH [3–10]. The presumed mechanisms contributing to hypercoagulability in PNH are diverse and include circulating procoagulant microparticles, platelet activation, abnormal expression of adhesion molecules on vascular endothelial cells, chronic hypofibrinolysis, limited bioavailability of nitric oxide, and factor Va (activated by the circulating microparticles, which were derived from complement-lysed abnormal red blood cells) [10–19].

The thrombotic risk in PNH is directly associated with complement-induced intravascular hemolysis. The PNH patients who have increased LDH, abdominal pain, hemoglobinuria occurring during hemolytic crisis are 1.5 times more likely to have thrombosis compared with a standard risk of 30–40% during acute hemolysis [20–22]. However, the underlying mechanisms leading to thrombotic complications during hemolytic crisis in patients with PNH remain unclear [23–26].

Eculizumab is a monoclonal antibody that binds the complement component protein C5, thereby preventing complement-induced lysis of erythrocytes, reducing intravascular hemolysis, hemoglobinuria and transfusion dependence in patients with PNH. In clinical trials in patients with PNH, eculizumab was associated with 3-fold reductions in the frequency of the hemolytic crises, transfusion requirements and thromboembolic events. Additionally, eculizumab was associated with improvements in PNH symptoms, quality of life and survival [27–30].

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Thus, Hillmen et al. [28] showed that the number of thromboses was reduced from 39 to 3 episodes in 195 patients on eculizumab therapy. However, there are few research studies of hemostatic state in patients with PNH during eculizumab therapy and they are inconsistent. On the one hand, the markers of coagulation activation such as D-dimer, TAT-complex, prothrombin fragments F1 + 2 were reported to be significantly reduced during the treatment; on the other hand it was shown that the formation of procoagulant microparticles in patients continued, and moreover, about 20% of patients still marked the increased concentration of D-dimers and prothrombin fragments F1 + 2 during the therapy [29,30]. None of the studies involved the global hemostatic assays which are known to be highly sensitive to hypercoagulable changes. In the present study, in addition to the conventional tests (clotting tests, fibrinogen concentration, D-dimer assay) we used the global methods: thromboelastography (TEG), thrombin generation test (TGT) and thrombodynamics (TD) to monitor the state of the coagulation system in three patients with PNH. The results of three patients with PNH before and during the 8 months of eculizumab therapy showed that, despite the reduction of hemolysis and the frequency of hemolytic crises from 3–6 to 0–1 episodes in 8 months, the global hemostasis tests support the tendency for hypercoagulability in patients with PNH.

Materials and methods

Patients

Three patients with PNH from the Department of Rare Diseases, National Research Center for Hematology, were enrolled in the study. Clinical data of the studied PNH patients on admission are shown in Table 1. There was an increased LDH activity and reticulocytosis in all patients. The clinical protocol was approved by the National Research Center for Hematology Ethics Committee.

Therapy

The eculizumab treatment was administered as recommended by the manufacturer; all patients received a low-dose regimen of 600 mg weekly for 4 weeks followed by 900 mg every 2 weeks for the next 8 months. The half-life of eculizumab is 272 ± 82 h; it should be administered at the recommended dosage regimen time points, or within two days of these time points to maintain a constant drug concentration in blood. We assume that during the entire treatment, patients were under continuous action of the drug: it is known that the peak concentration of eculizumab is observed at week 26 (194 ± 76 pg/ml), and the minimal working concentration (35 mg/kg) is reached within an hour after the first administration [31–34]. Patient A was on the anticoagulant therapy with enoxaparin 1.2 mg/day during the first three weeks of eculizumab treatment. There was no any other therapy for other two patients during the eculizumab therapy.

Reagents

Thromboplastin was obtained from Renam, Moscow, Russia; the fluorogenic substrate Z-Gly-Gly-Arg-AMC from Bachem, Bubendorf, Switzerland; phosphatidylcholine (PC) and phosphatidylserine (PS) were obtained from Avanti Chemicals, Ormeau, Australia; Thromborel S, Test Thrombin Reagent, and D-dimer PLUS were obtained from Dade Behring, Germany; and Thrombodynamics kit was from LLC HemaCore, Russia.

Blood collection and plasma preparation

Blood samples were drawn into 9 ml vacuum tubes (Monovette, Sarstedt, Germany) with 0.106 M sodium citrate buffer. The blood samples were processed by centrifugation at 1500 ×*g* for 15 min to obtain platelet-poor plasma (ppp), part of the plasma was subsequently subjected to centrifugation at 10,000 ×*g* for 5 min to obtain platelet-free plasma (pfp) [35].

Clotting time tests, fibrinogen and D-dimer assays

The following tests were performed using fresh ppp samples and the aforementioned reagents: activated partial thromboplastin time (APTT), prothrombin index (PI), thrombin time (TT), fibrinogen and D-dimer concentrations. All tests were performed in the Coagulation Laboratory of the National Research Center for Hematology, using a Sysmex CA-1500 (Sysmex Corporation, Japan) automated analyzer, according to respective manufacturer's instructions.

Thromboelastography

Citrated Native Thromboelastography (TEG) was performed using a TEG 5000 Hemostasis Analyzer System and disposable cups (Haemonetics Corporation, MA, USA). The assays were performed 10 to 30 min after blood collection using citrated blood samples (340 μl) recalcified with 20 μl of 0.2 M $CaCl_2$. The angle alpha (α), the tangent to the thrombloelastographic clotting curve was used for analysis (Fig. 1a).

Thrombin generation test

Sample preparation and experiments were performed as described in [36]. Briefly, ppp was placed in the wells (80 μl/well) of a 96-well flak-bottom micro titer plate. Thereafter, 20 μl of the fluorogenic substrate (the final concentration was 400 μM) and 20 μl thromboplastin (the final concentration was 4 pM) with phospholipid vesicles (the final concentration was 4 μM) were added into each well. Phospholipid vesicles were prepared by extrusion from PC and PS at a percentage ratio of 80:20, as described previously [37]. The kinetics of accumulation of the fluorescing reaction product 7-amino-4-methylcoumarin was recorded for 60 min with a fluorimetric reader (Appliskan; Thermo Fisher Scientific, Finland) ($\lambda_{ex.} = 355$ nm; $\lambda_{em.} = 460$ nm). For all calculations, the program OriginPro 8.0 (OriginLab Corporation, USA) was used. To calculate the area under the thrombin-time curve for a sample, we determined the total amount of thrombin generated in that sample over the period of 50 min endogenous thrombin potential (ETP) (Fig. 1b) [38].

Thrombodynamics

The Thrombodynamics (TD) spatial clot growth assay was performed using Thrombodynamics Analyzer and Thrombodynamics kit

Table 1
Clinical and demographic data of the studied PNH patients.

Patient	Age, years	Sex	PNH, years	Anticoagulant therapy	Steroids	WBC (10^9), cells/l	Hb, g/l	Platelets (10^9), cells/l	Reticulocytes, ppm	LDH, U/l
Reference values						4.0–9.0	130–160	180–320	2–10	208–378
A[a]	29	M	6	Enoxaparin 1.2 mg/day	No	**2.8**	**54**	178	**101**	**6569**
B[a]	33	M	2	No	No	**3.6**	**53**	**85**	**52**	**1874**
C[a]	27	F	4	No	No	**2.9**	**67**	171	**32**	**3173**

The bold indicates the changes in parameters.
[a] Transfusion dependent patients.