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Eculizumab decreases the procoagulant activity of extracellular vesicles in paroxysmal nocturnal hemoglobinuria: A pilot prospective longitudinal clinical study

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ABSTRACT

Introduction: Paroxysmal nocturnal hemoglobinuria (PNH) is a disease characterized by the susceptibility of blood cells to attack by the complement system, inducing extracellular vesicle (EV) production. Thromboembolism is the leading cause of death in this condition. Eculizumab, a humanized monoclonal antibody which inhibits the C5 protein of the complement, reduces the thrombotic risk in PNH.
Materials and method: We conducted a pilot, prospective, open-label, longitudinal clinical study with six PNH patients treated with eculizumab. The aim was to measure, by flow cytometry, the EVs' production in the patients' platelet-free plasma (PFP) before and during the treatment. We also assessed the procoagulant activity in PFP using STA®-Procoag-PPL and thrombin generation assays (TGA). A high-sensitive version of TGA was also used to study the procoagulant profile induced by the EVs using EVs pelleted from PFP.
Results: We observed a decrease in platelet EV count with eculizumab treatment ($p < 0.05$). STA®-Procoag-PPL assay showed a decrease of the procoagulant profile induced by procoagulant phospholipids (PL) during treatment. These results were not confirmed by TGA on PFP, due to a lack of sensitivity. Thus, we used a high-sensitive version of TGA that enabled us to observe variation in the procoagulant profile induced by the EVs with eculizumab ($p < 0.05$).
Conclusions: Eculizumab has an impact on the extent of EV production and on the procoagulant profile induced by the procoagulant PL and the EVs. One factor in the antithrombotic action of eculizumab is its ability to decrease EV production and the procoagulant profile induced by PL and EVs.

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1. Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal hematological disease characterized by a PIG-A gene mutation in hematopoietic stem cells. This mutation results in deficiency of glycosylphosphatidylinositol (GPI)-anchored proteins and by the lack of complement regulatory proteins (CD55 and CD59) on the blood cell surface. The lack of these proteins causes susceptibility of the hematopoietic cells to a complement attack leading to hemolysis [1,2]. The links between complement activation and coagulation are well described and cause a hypercoagulable state that can lead to thrombosis in PNH patients [3–5].

Thrombosis sensitivity can also be caused by the lack of other GPI-anchored proteins, by the multiple consequences of the complement-mediated hemolysis, by the activation of some cells, but also by the production of extracellular vesicles (EVs) due to the complement attack [3–8].

EVs are subcellular structures derived from a parent cell and ranging in size from around 100 to 1000 nm [9,10]. They have many physiological and pathological roles and can be implicated in coagulation [11–13]. Indeed, external exposure of phosphatidylserine (PS) in the phospholipid bilayer of the EVs contributes to the prothrombinase complex formation leading to thrombin formation [14]. According to their cellular origin, they can also present tissue factor (TF) at their surface which binds to FVIIa for the

Abbreviation: PNH, paroxysmal nocturnal hemoglobinuria; EV, extracellular vesicle; PFP, platelet-free plasma; TGA, thrombin generation assay; GPI, glycosylphosphatidylinositol; PS, phosphatidylserine; TF, tissue factor; CHU, Centre Hospitalier Universitaire; NPP, normal pooled plasma; CAT, calibrated automated thrombogram; LDH, lactate dehydrogenase; FITC, fluorescein isothiocyanate; PL, phospholipids; LT, lag time; TTPeak, time to peak; ETP, endogenous thrombin potential; TFPI, tissue factor pathway inhibitor.

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initiation of the extrinsic pathway [15–17]. With these characteristics, some of the EVs are known to be prothrombotic and to have an impact on the pathophysiology of thrombosis in PNH patients [18–20].

Apart from the susceptibility to hemolysis and thromboembolism, clinical manifestations of PNH include pulmonary hypertension and dyspnea, fatigue, anemia, muscle dystonia and renal failure. Until 2007, the treatment of PNH consisted of supportive care: blood transfusions, preventive anticoagulation and folate supplementation [21,22]. The development of eculizumab (Alexion Pharmaceuticals, Cheshire, USA) improved patients' quality of life, by reducing symptoms and also reduced the thrombotic risk by 85% [23–25]. Eculizumab is a recombinant humanized monoclonal IgG2/4 k antibody which binds to the complement protein C5, thereby inhibiting its cleavage and preventing the formation of the membrane attack complex. Eculizumab preserves the early components of complement activation that are essential for opsonization of microorganisms and clearance of immune complexes. However, the exact mechanism underlying the decreased thrombotic risk with eculizumab is still poorly understood. The impact of eculizumab on the production of prothrombotic EVs is poorly documented.

We conducted a pilot, prospective, open-label, longitudinal clinical study on eculizumab-treated PNH patients to measure the production of EVs in plasma before and during treatment with eculizumab and to assess the procoagulant activity by STA®-Procoag-PPL assay and thrombin generation assay (TGA) using platelet-free plasma or a pellet of EVs.

2. Materials and methods

2.1. Study design

PNH patients were prospectively recruited to this study from October 2012 to June 2016. Informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki and approved by the local Medical Ethics Committee of the Centre Hospitalier Universitaire (CHU) UCL Namur Yvoir, Belgium (B039201214365). To be included, patients had to: have a PNH clone detected by flow cytometry of at least 5%, be at least 18 years old, and be vaccinated against *Neisseria meningitidis*. Patients were excluded if they had received hematopoietic stem cell transplantation, had a previous meningococcal infection or were pregnant.

2.2. Sample collection

All blood samples were collected and analyzed in the hematology laboratory of the CHU UCL Namur. Blood was sampled before the start of treatment (baseline), after 4 weeks of 600 mg eculizumab (just before dose adjustment) and after 11 ± 2 weeks of eculizumab (900 mg). Samples were obtained by venipuncture with a 21-gauge needle in the antecubital vein in patients with an empty stomach who had rested for 30 min (following the International Society of Thrombosis and Haemostasis recommendations for EV analysis) [26].

2.2.1. Platelet-free plasma (PFP) and normal pooled plasma (NPP) preparation

The blood used for the EV analysis was collected into 0.109 M sodium citrate tubes (Vacuette® Tube, Greiner Bio-One GmbH, Kremsmünster, Austria). To harvest plasma, the blood underwent a double centrifugation (at 2,500*g* for 15 min at room temperature). This technique allowed us to obtain platelet-free plasma (PFP) for EV characterization.

We also collected blood from 58 healthy controls into 0.109 M sodium citrate tubes to obtain normal pooled plasma (NPP), used as an internal control for calibrated automated thrombogram assay (CAT). The samples were treated in the same way as those from the PNH patients. The PFP from the patients and controls was then aliquoted and frozen at −80 °C. The aliquots were thawed for 4 min at 37 °C before the experiment.

2.2.2. Isolation of the EVs

The isolation was performed from both patients' PFP and NPP. 880 μl of samples were placed in a microcentrifuge polyallomer tube (Beckman Coulter, Marseille, France) to undergo high-speed centrifugation (at 100,000G for 90 min at 4 °C) using an Optima MAX Ultracentrifuge with TLA-100.3 rotor (Beckman Coulter, Marseille, France). After centrifugation, the supernatant was removed by keeping a volume of 130 μl in the tube. After centrifugation, the supernatant was removed by keeping a volume of 130 μl in the tube. We use this volume to resuspend the EV pellets. That allows to have a suspension with the EVs 6.7 times more concentrated than in PFP [27]. These concentrated EVs' suspensions were used in the high-sensitive TGA.

2.3. Laboratory procedures

2.3.1. Lactate dehydrogenase (LDH) measurement

Lactate dehydrogenase (LDH) was measured with the LDH VITROS assay on a VITROS 5600 analyzer (Ortho Clinical Diagnostics, New Jersey, United States) according to the manufacturer's recommendations.

2.3.2. D-dimer measurement

The measurement was performed on STA-R Evolution (Stago Diagnostica, Aisnières, France) equipment with the STA®-Liatest®D-Di (Stago Diagnostica, Aisnières, France) reagent according to the manufacturer's recommendations.

2.3.3. Flow cytometry

Flow cytometry was performed as previously described [28]. Before use, each monoclonal antibody was centrifuged at 15,000*g* for 10 min at 4 °C to remove aggregates. Reagents were stored at 4 °C in the dark until use. Thawed samples were then incubated with CD41a-PerCP-Cy5.5 (clone HIP8, BD Biosciences, Erembodegem, Belgium) to determine platelet origin. Annexin V-FITC (BD Pharmingen, Erembodegem, Belgium) was also used to label procoagulant EVs by binding to PS on the EV surface. EVs tagged with annexin V were defined as annexin V positive (+), other EVs were defined as annexin V negative (−). The incubation with antibodies was performed for 30 min at room temperature in the dark. The samples were then analyzed on a FACS Aria flow cytometer with FACSDiva V6.1.3 software (BD Biosciences, San Jose, CA, USA). The instrument was optimized following the standardized procedures for measurement of EVs provided by the International Society on Thrombosis and Haemostasis [29]. This procedure consists of a calibration with Megamix-Plus SSC beads (BioCytex, Marseille, France). These beads are optimized for use on the FACS Aria cytometer [30]. The separation index was used to evaluate the resolution on SSC parameters [31]. The number of events during an acquisition period of 120 s was recorded. With an identical volume of sample and a constant flow during each analysis (5.4 μl/min), the absolute concentration of EVs could be calculated.

2.3.4. STA®-Procoag-PPL assay

The STA®-Procoag-PPL kit (Diagnostica Stago SAS, Asnières-sur-Seine, France) was used to detect procoagulant PL in patients' PFP using a chronometric method. The method has been described by Devalet et al. [28]. Briefly, this assay measures clotting time, in the presence of factor Xa and $CaCl_2$, and all the coagulation factors (supplied from plasma depleted in procoagulant PL). In this way, the only source of procoagulant PL is provided by the patient's sample [32]. An increase in the clotting time underlies a decrease of procoagulant PL in the sample. The measurement was performed on the STA-R Evolution analyzer (Stago Diagnostica, Asnières-sur-Seine, France).

2.3.5. Calibrated automated thrombogram (CAT)

This technique allows the determination of the coagulant profile of a plasma sample by the analysis of curves related to the thrombin generated over time. The following curve parameters are determined: the lag time (LT), corresponding to the induction time, the time to peak (TTPeak) corresponding to the time to reach the peak concentration, when the maximum amount of thrombin is generated, and finally the endogenous thrombin potential (ETP) corresponding to the area under the curve. Two protocols were used

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