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Full Length Article

Alterations in markers of coagulation and fibrinolysis in patients with Paroxysmal Nocturnal Hemoglobinuria before and during treatment with eculizumab

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ABSTRACT

Background: Paroxysmal Nocturnal Hemoglobinuria is characterized by complement-mediated hemolysis and an increased thrombosis risk. Eculizumab, an antibody to complement factor C5, reduces thrombotic risk via unknown mechanisms. Clinical observations suggest that eculizumab has an immediate effect.
Objectives: A better understanding of the mechanism via which eculizumab reduces thrombotic risk by studying its pharmacodynamic effect on coagulation and fibrinolysis.
Methods: We measured microparticles (MP), tissue factor (TF) activity, prothrombin fragment 1 + 2 (F1 + 2), D-dimer and simultaneously thrombin and plasmin generation in 55 PNH patients. In 20 patients, parameters were compared before and during eculizumab treatment (at 1 and 2 hours, 1, 4 and ≥ 12 weeks after commencement).
Results: Patients with a history of thrombosis had elevated D-dimers ($p = 0.02$) but not MP. Among patients on anticoagulants, those with thrombosis had higher F1 + 2 concentrations ($p = 0.003$). TF activity was undetectable in plasma MP. Unexpectedly, thrombin peak height and thrombin potential were significantly lower in PNH patients than in healthy controls. Fibrinolysis parameters were normal. During eculizumab treatment D-dimer levels significantly decreased after 1 hour ($p = 0.008$) and remained decreased at ≥ 12 weeks ($p = 0.03$). F1 + 2 ($p = 0.03$) and thrombin peak height ($p = 0.02$) in patients not on anticoagulants significantly decreased at ≥ week 12. MP remained unchanged.
Conclusions: Eculizumab induces an immediate decrease of D-dimer levels but not of other markers. The decrease in thrombin peak height and F1 + 2 suggests that eculizumab reduces thrombin generation. Elevated D-dimer levels in untreated PNH patients with a history of thrombosis suggest possible value in predicting thrombotic risk.

1. Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare disease characterized by intravascular hemolysis and a highly increased risk of thrombosis [1]. The disease results from acquired mutations in the X-linked PIG-A gene in the hematopoietic stem cell. The PIG-A gene codes for an enzyme essential in the synthesis of glycosylphosphatidylinositol (GPI) anchors. The mutated hematopoietic stem cell produces a clone of blood cells with partial or complete deficiency of GPI-anchored proteins at the cell membrane. The percentage of granulocytes affected by this mutation reflects the PNH clone size. Deficiency of GPI-anchored complement inhibitors CD55 and CD59 on erythrocytes renders these vulnerable to complement-induced hemolysis.

One of the most serious features of PNH is a high risk of thrombosis, which has a major impact on survival and quality of life [2]. The cumulative 10-year incidence of thrombosis is estimated at 23-31% [2,3]. The risk of thrombosis is partly correlated with PNH clone size and is particularly elevated when the granulocyte clone size is > 50%. Nevertheless, thrombotic events occur also in patients with smaller clone sizes [3,4]. Thrombosis occurs frequently in vessels of the liver, mesenterium or central nervous system [1,2,5]. Primary prophylaxis with vitamin K antagonists (VKA) or low-molecular weight heparins (LMWH) (taken together as anticoagulant treatment (AC)) reduces the risk, but does not provide full protection [2,3]. No parameters are available to aid in estimating thrombotic risk in individual patients.

The mechanism of increased thrombophilia (reviewed in [6,7]) is incompletely understood. GPI-deficient platelets become activated upon complement activation [8] and may shed microparticles (MP) with

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procoagulant properties [9–13]. Additionally, free hemoglobin and arginase released from lysed erythrocytes activate endothelium and deplete nitric oxide (NO) from the circulation. NO normally inhibits platelet activation [14]. Furthermore, deficiency of GPI-anchored proteins involved in hemostasis such as urokinase plasminogen activator receptor (uPAR) and tissue factor pathway inhibitor (TFPI) may contribute [15–18].

Treatment with eculizumab, a monoclonal antibody to complement factor C5, has dramatically improved quality of life and survival in PNH patients [19]. It reduces hemolysis, improves anemia and hemolysis-associated symptoms, and prevents secondary organ damage such as renal failure [20–25]. Importantly, eculizumab is currently the only available treatment that significantly reduces thrombotic risk in PNH [26]. Helley and Weitz et al. have demonstrated reduced markers of coagulation activation, fibrinolysis and plasma markers of endothelial activation at week 5 of eculizumab treatment in PNH patients [27,28]. Case reports describe immediate abrogation of progressive thrombus formation upon administration of eculizumab [29,30]. This suggests that changes in coagulation and fibrinolysis markers occur very rapidly.

Here, we have studied coagulation and fibrinolysis in a large population of 55 untreated PNH patients. In addition, we have studied 20 patients before and during eculizumab treatment within a time window of one hour up to ≥ 12 weeks after the start of the first eculizumab infusion. We have assessed levels of procoagulant microparticles (MP), prothrombin fragment 1 + 2 (F1 + 2) and D-dimer levels as parameters of thrombin generation and fibrinolysis respectively. To study the interplay between coagulation and fibrinolysis, the Nijmegen Hemostasis Assay (NHA) was used, which allows simultaneous analysis of the plasma potential to generate both thrombin and plasmin [31]. All parameters were correlated with clinical parameters of PNH (LDH, PNH clone size and history of thrombosis).

2. Materials & Methods

2.1. Clinical Data and Samples

Clinical data and venous peripheral blood samples from 55 consecutive PNH patients were collected after obtaining written informed consent in the period 2006–2011 in the RadboudUMC. AC was prescribed when deemed appropriate according to the clinician's judgement. Generally, patients received AC if their PNH granulocyte clone size was > 50% or they had already suffered thrombosis. Eculizumab was given at standard doses of 600 mg in a 30 minute intravenous infusion once per week for 4 weeks and 900 mg per day once every two weeks as of week 5. Patients who commenced eculizumab treatment (n = 20) had an additional blood sample drawn immediately before the start of the first infusion. Follow-up samples were drawn at 1 and 2 hours after the start of the first eculizumab infusion, and before subsequent infusions at 1, 4 and ≥ 12 weeks. Control samples for the NHA were collected from 10 healthy volunteers. All samples were collected in 3.2% citrate, theophylline, adenosine and dipyridamole (CTAD) and were centrifuged within 1 hour at 4 °C for 15 min at 2000 g. Subsequently, plasma was aliquoted and stored at −80 °C until use. From a subset of patients, peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll Hypaque gradient separation. PBMCs were subsequently frozen in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Carlsbad, CA) containing 10% dimethylsulfoxide (DMSO) and stored in liquid nitrogen until use.

2.2. Microparticle ELISA

Microparticle procoagulant activity in PNH patients was determined using the Zymuphen MP-Activity kit (Hyphen BioMed, Neuville-sur-Oise, France). Briefly, this assay measures thrombin generation, catalyzed by the exposure of phosphatidylserine upon binding of microparticles to a microtiter plate coated with streptavidine and biotinylated annexin A5. Results were expressed as nanomolar phosphatidylserine equivalent by using a calibrator with a known microparticle concentration. MP were considered elevated if > 10 nM.

2.3. Tissue Factor (TF) Activity Assay

Functional TF activity was measured in plasma MP and lysed PBMCs of PNH patients, using a two stage amidolytic assay (detection limit 15 mU/ml) based on the ability of TF to accelerate the activation of FX by FVIIa as described previously [32].

2.4. D-dimer

D-dimer concentrations in PNH patients were determined using STA Liatest D-dimer (Stago Diagnostics, Asnières, France) according to the manufacturer's instructions. D-dimer values were considered elevated if > 500 ng/mL.

2.5. Prothrombin Fragment F1 + 2

Concentrations of prothrombin fragment F1 + 2 (F1 + 2) in PNH patients were determined using an enzyme-linked immunosorbent assay (ELISA) (Enzygnost F1 + 2 (monoclonal), Siemens Healthcare Diagnostics, Marburg, Germany) according to the manufacturer's instructions (reference values 69–229 pmol/l).

2.6. Nijmegen Hemostasis Assay (NHA)

The NHA was performed as described previously [31]. This assay simultaneously analyzes coagulation and fibrinolysis in plasma samples upon *in vitro* activation. In brief, the assay was mediated by ~0.3 pM human recombinant tissue factor (Innovin®, Siemens Healthcare Diagnostics, Marburg, Germany), and 1.7% (v/v) crude cephalin (Roche, Basel, Switzerland) as a source of phospholipids. Fibrinolysis was mediated by 190 IU/mL tPA (Alteplase®, Boehringer Ingelheim, Ingelheim am Rhein, Germany). The assay is subsequently started by the addition of 17 mM $CaCl_2$. Thrombin and plasmin generation are continuously measured using fluorogenic synthetic substrates (Chiralix, Nijmegen, The Netherlands) specific for thrombin (Bz-β-Ala-Gly-Arg-7-amino-4-methylcoumarin, final concentration 833 μM) and plasmin (bis-(CBZ-L-phenylalanyl-L-arginine amide)-rhodamine, final concentration 33 μM). Thrombin and plasmin-specific substrates are excitated at 355 nm and 485 nm and measured at emission wavelengths of 460 nm and 520 nm respectively. The procedure is performed in a 37 °C thermostated fluorometer (Fluostar Optima Fluorometer, BMG Labtechnologies, Offenburg, Germany) using Black polystyrene Fluotrac microtiter plates (Greiner Bio-One, Monroe, NC, USA). Seven parameters are derived from the NHA: a) lag-time, the time at which thrombin formation is initiated; b) thrombin peak time, i.e. the time when thrombin production reaches maximal velocity; c) thrombin peak height, the maximal velocity of thrombin generation; d) thrombin potential (AUC), i.e. the area under the curve which represents the total amount of thrombin formed; e) fibrin lysis time (FLT), the time between the initiation of thrombin generation and the time plasmin generation reaches maximal velocity; f) plasmin peak height, the maximal velocity of plasmin production and g) plasmin potential, area under the curve representing the total amount of plasmin generated.

2.7. Statistical Analysis

Data are expressed as medians unless otherwise indicated. Mann–Whitney-U test was used to compare differences between two groups. The Kruskall-Wallis test was used to compare differences between ≥ 2 groups. If significant differences were found, the data were further evaluated by the Dunn's test for multiple comparisons. The Spearman rank test was used to assess correlations with clinical characteristics. To