Evaluation of hemostasis and endothelial function in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab

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Supplementary Data

Methods

Endothelial microparticle quantification

Endothelial microparticles (EMPs) were quantified by flow cytometry, using anti-CD146 (clone TEA1/34) and anti-CD105 (clone 1G2)-phycoerythrin (PE), anti-CD45-phycoerythin-cyanin5 (PC5) (clone J33), and anti-CD54-fluoresceine isothiocyanate (FITC) (clone 84H10) (Beckman Coulter, Fullerton, CA, USA). Flow count beads (Beckman Coulter, Fullerton, CA, USA) were added to each sample to calculate the absolute number of microparticles. Samples were analyzed on an FC500 flow cytometer using CXP software (Beckman Coulter, Fullerton, CA, USA). EMPs present in plasma were analyzed according to their fluorescence and size. Briefly, EMPs were discriminated from background noise by their positive fluorescence intensity for CD146/CD105-PE, and a threshold was determined with isotype control PE conjugated antibodies. CD105 and CD146 antibodies used in combination rather than individually allow a higher mean fluorescence for EMPs detected to be obtained. To eliminate potential microparticles from T-lymphocyte subsets expressing CD146, only microparticles with negative CD45-PC5 fluorescence were considered as EMPs. Only events positive for CD146/105 and negative for CD45 were further analyzed, using a LogFS-LogSS dot plot (Figure S1). The EMP gate size was predefined by using fluorescent standard beads 0.3-1.1 µm in diameter (Sigma-Aldrich, St. Louis, MO, USA) on a Log forward-scatter (FS) cytogram. Results were expressed as the total number of EMPs per microliter of plasma. Expression of CD54 (ICAM-1) on EMPs was also determined. Each sample was analyzed for 120 seconds, in duplicate. The normal range was determined with samples from 30 healthy subjects.

Culture and characterization of circulating endothelial progenitor cells

Venous blood (40 mL) was drawn for endothelial cell progenitor culture from one patient (before eculizumab introduction) and one healthy control.

Mononuclear cells were isolated and cultured in endothelial growth medium (EGM-2) as described by Hur *et al.*[1] Endothelial colony-forming cells (ECFC)[2] appeared about two weeks after plating and were cultured to obtain enough cells for flow cytometry. FITC- or PE-conjugated monoclonal antibodies directed against CD14, CD31, CD55 and CD59, and isotype-matched controls, were purchased from Becton Dickinson (Franklin Lakes, NJ, USA); CD34, CD45, CD144, CD146 and isotype-matched controls were purchased from Beckman Coulter (Fullerton, CA, USA); CD87 (u-PAR), vascular endothelial growth factor receptor-2 (VEGFR-2) and isotype-matched controls were purchased from R&D Systems (Minneapolis, MN, USA). After staining with FITC- or PE-conjugated monoclonal antibodies, cells were analyzed on a FACScan device using Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA). Five thousand events were acquired for each sample.

Statistical analysis

Baseline patient characteristics and markers were compared according to anticoagulant therapy, using Fisher's exact test or the Wilcoxon rank sum test.

Marker kinetics between baseline and week 11 were analyzed with mixed model analysis of variance. Two fixed effects modeled the variation between baseline and week 5 and between week 5 and week 11, and random subject effects were included to account for correlations between measurements in a given subject. Model goodness-of-fit was assessed by examining residuals. When necessary, to obtain adequate models, data were log-transformed, or models with non-constant residual variance were used. The hypothesis that marker kinetics are affected by anticoagulant therapy at baseline was tested by adding such an effect and its interaction with time in the models. If no interaction effect was found, only the test for the whole cohort is presented. All tests were two-sided, and P values <0.05 were considered to indicate statistical significance. All analyses were performed using R 2.6.2 statistical software.

Results

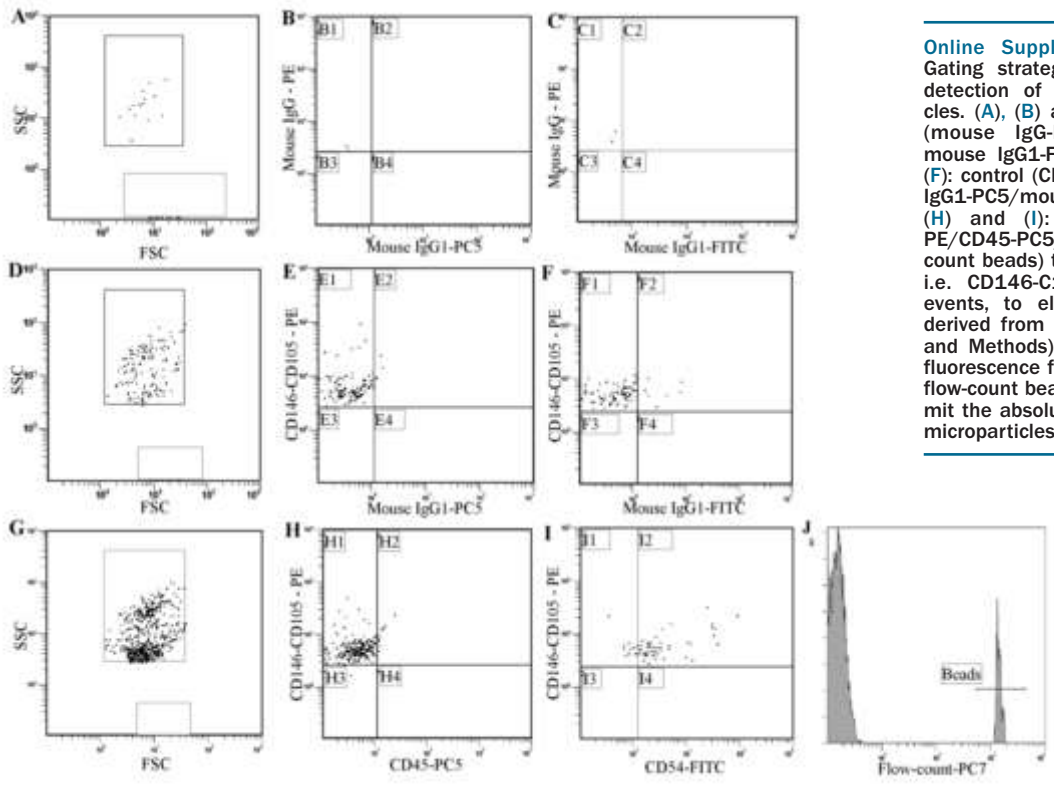
Surface marker expression on ECFCs

Because of the large amount of blood necessary, ECFCs were cultured from mononuclear cells of one patient and one control, and then

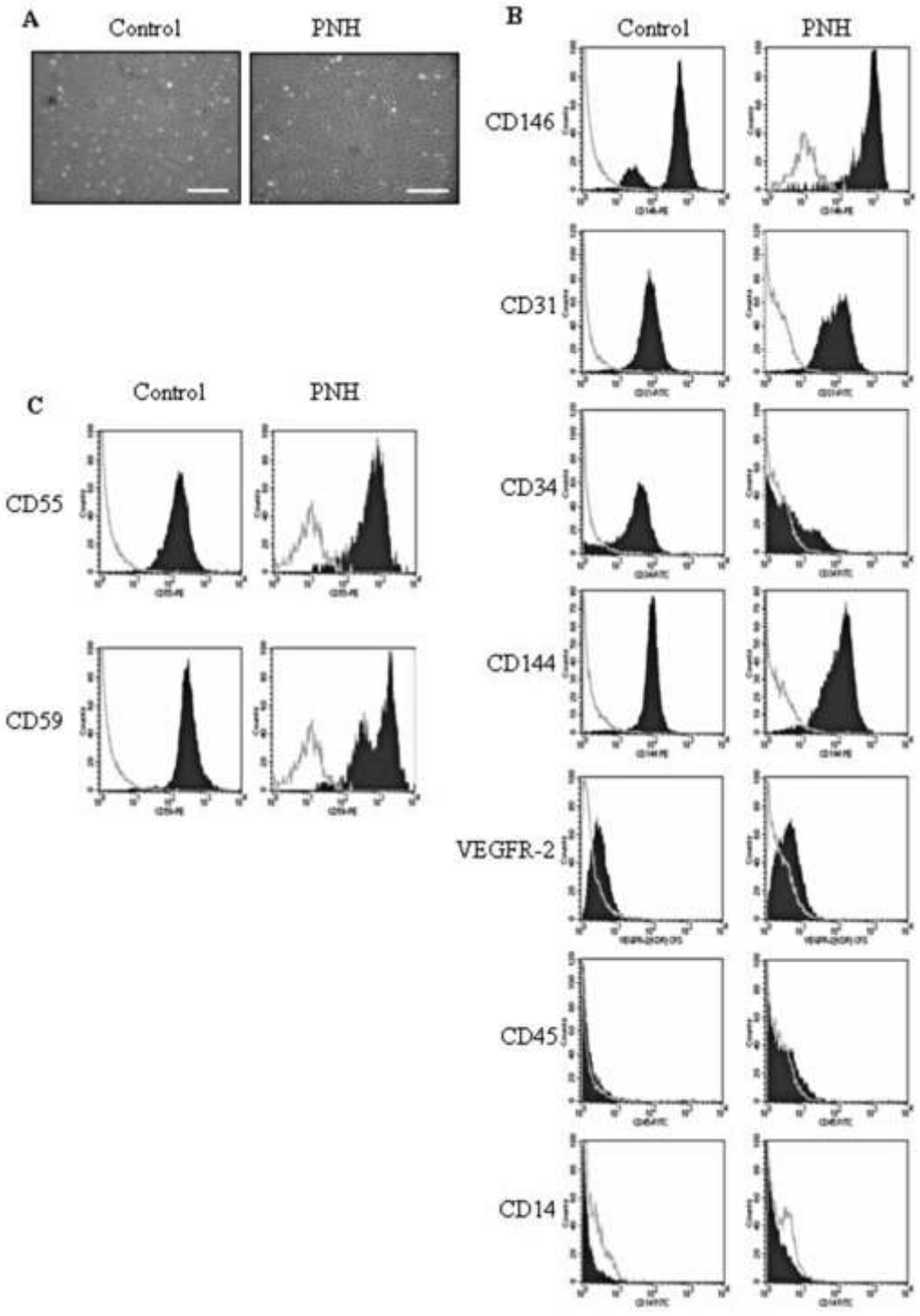
characterized by flow cytometry. ECFCs of both subjects appeared 15 days after plating in endothelial conditions, and were then cultured for 30 days (Figure S2A). They expressed CD31, CD34, CD144, CD146 and VEGFR-2 and not CD45 and CD14, confirming their ECFC profile (Figure S2B). ECFCs isolated from the PNH patient contained a small population that lacked CD55 expression (5% of cells). In addition to this small type III population, staining with anti-CD59 revealed an intermediate population (type II cells; 37%) (Figure S1C). This CD55- and CD59-defective expression on ECFCs suggests that endothelial cells may be affected in PNH. ECFCs from the healthy subject and the PNH patient did not express u-PAR (Figure S2C). Nevertheless, these results suggest a potential role of these GPI-deficient endothelial cells in the pathophysiology of thrombosis in PNH.

References

1. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004;24(2):288-93.
2. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 2007;109(5): 1801-9.



Online Supplementary Figure S1. Gating strategy for flow cytometric detection of endothelial microparticles. (A), (B) and (C): isotype control (mouse IgG-PE/mouse IgG1-PC5/mouse IgG1-FITC) tube. (D), (E) and (F): control (CD146-CD105-PE/mouse IgG1-PC5/mouse IgG1-FITC) tube. (G), (H) and (I): test (CD146-CD105-PE/CD45-PC5/CD54-FITC and flow-count beads) tube. (I) is gated on H1, i.e. CD146-C105-PE + and CD45- events, to eliminate microparticles derived from leukocytes (see Design and Methods). (J), histogram of PC7 fluorescence from all events; only the flow-count beads were positive to permit the absolute count of endothelial microparticles.



Online Supplementary Figure S2. Comparison of endothelial progenitor cells cultured from peripheral blood mononuclear cells of PNH patient and healthy control subject. (A) Phase contrast photomicrograph of ECFC (day 50). Scale bar = 100 μm. (B) Flow cytometry of proteins characterizing endothelial cells. (C) Flow cytometry of GPI-anchored proteins CD55, CD59 and u-PAR. Shaded peaks represent cells stained with antibodies against specific proteins; empty peaks represent corresponding isotype control antibodies staining.