Interplay between circulating von Willebrand factor and neutrophils: implications for inflammation, neutrophil function, and von Willebrand factor clearance

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Supplementary Data

Supplementary Methods

Isolation of human neutrophils from peripheral blood of healthy volunteers

Whole blood (5-10 ml) was collected from several healthy volunteers in EDTA tubes (Sarstedt, Germany) after obtaining informed consent, in compliance with the Declaration of Helsinki principles and the ethical guidelines of Germany. The volunteers had no history of impaired VWF or neutrophil function. They were healthy, infection-free, and had not taken any medications within 48 hours before blood collection. Neutrophils were isolated from peripheral blood immediately after collection using the EasySep™ Direct Human Neutrophil Isolation Kit (StemCell Technologies, Germany), according to the manufacturer's instructions. On average, 1.5-3 $X10^6$ cells /ml blood were isolated. The number and viability of isolated cells were determined using the NucleoCounter® NC-202™ (ChemoMetec, Denmark), a dual-color fluorescent automated cell counter that uses acridine orange (AO) and DAPI to identify live and dead cells. The purity of isolated cells was assessed by flow cytometry as described below.

We additionally determined cell viability and apoptosis after a incubation time of 180 minutes (min) using the Nucle-oCounter® NC-202™ and flow cytometry (by assessing CD16 receptor shedding), respectively.

Flow Cytometry

After isolation or incubation of neutrophils, cells were washed in PBS and resuspended in staining buffer (PBS containing 2% FBS and 2 mM EDTA). Cells were then immunostained with two panels of antibodies containing either fluorochrome-conjugated antibodies (anti-CD66b-FITC, anti-CD45-APC, and anti-CD16- PE) or isotype controls (IgG1-FITC, IgG1- APE, IgG1- PE). All antibodies were purchased from Miltenyi Biotec, Germany. After washing, cells were resuspended in a staining buffer and subjected to cytometric analysis using a Navios EX Flow Cytometer (Beckman Coulter, Germany). The attained data were further analyzed using FlowJo software (LLC, Becton Dickinson, USA).

Evaluation of VWF binding to neutrophils upon activation by inflammatory factors

After the isolation of neutrophils, cells suspended in autologous plasma (plasma obtained from the supernatant of the same isolated cells, containing plasma VWF) were transferred to multi-well plates. They were then either left untreated (resting cells) or stimulated with either 10 ng/ml PMA (Sigma, USA), 5 ng/ml TNFα (Invitrogen, Germany), or 0.3 ng/ml, 0.5 ng/ml, and 0.7 ng/ml IL -8 (R&D, USA) for 15 min at 37ºC, 5% CO_2.

To confirm if VWF interacts with neutrophils through the Mac-1 receptor on their surface, we conducted a series of experiments where isolated neutrophils were treated with Neutrophil Inhibitory Factor (NIF; R&D Systems, Germany) at a concentration of 400 ng/ml for 30 minutes at 37ºC with 5% CO2. As a control, another set of neutrophils was left untreated with NIF. Following this treatment, all neutrophils were stimulated with PMA (10 ng/ml) for 15 minutes at 37ºC with 5% CO2. Additionally, resting neutrophils (neither treated with NIF nor stimulated with PMA) were included in the experiments.

Cells were then washed with PBS and subjected to IF staining and microscopic analysis to quantify VWF signal intensity, as described below. At least three independent experiments were performed for each condition.

Evaluation of VWF binding to neutrophils upon exposure to shear forces

To assess the effect of shear flow on the binding of VWF to neutrophils, we performed experiments under static or flow conditions using the Bioflux 200 instrument (Fluxion Biosciences). We compared the VWF signal intensity between shear-exposed and resting cells through IF microscopy analysis. Initially, the isolated cells were centrifuged (300xg for 5 min) to remove the supernatant containing autologous VWF. They were then resuspended in RPMI medium (Roswell Park Memorial Institute medium 1640; Gibco, USA) containing glutamine and 10% FBS. A pdVWF/FVIII concentrate (Haemate from CSL Behring, Germany) either recombinant VWF (rVWF; VONVENDI from Baxalta, now part of Shire, Austria)was spiked to the cell suspension to achieve a final VWF antigen level of 100% (100 IU/dl). One fraction of the cells was incubated at 37ºC for 30 min under static conditions, while another fraction was exposed to a shear flow of 10 dyne/cm^2 for 30 min at 37ºC. To confirm the binding of the spiked pdVWF/FVIII or rVWF to neutrophils and exclude any potential VWF production induced by shear flow in neutrophils, we repeated the experiments by exposing the cells to shear flow in the absence or presence of pdVWF/FVIII or rVWF.

Subsequently, the cells were collected and fixed for subsequent IF staining analysis. At least three independent experiments were performed for each condition.

Investigation of VWF internalization by analysis of intracellular trafficking

To determine whether VWF is internalized by neutrophils, we visualized and examined VWF transport by studying the co-localization of VWF with early (EEA1) and late (Rab7) endosomal markers over time by IF microscopy analyses. In the first set of experiments, neutrophils were either fixed immediately after isolation or incubated (without any treatment) for 60 or 180 min and then fixed for subsequent immunostaining. In another series of experiments, we examined VWF trafficking upon interaction with neutrophils after exposure to shear stress for 30 min in the presence of pdVWF/FVIII, as described in the previous section. Afterwards, neutrophils were incubated for an additional 30 or 60 min at 37ºC and 5% CO_2 before fixing cells. The IF staining, microscopic image acquisition, and quantification of VWF/EEA1 and VWF/Rab7 co-localization were performed as described below.

The experiments were independently replicated at least twice, with each replication performed in triplicate (n=6).

Immunofluorescence microscopy analysis

The resting or treated cells were transferred to ibidi µ-Slide 4 wells (with glass bottoms and coated with poly-L-lysine; ibidi, Germany) at a concentration of 500,000 to 700,000 cells per chamber. Cells were then fixed with 10% formalin and permeabilized using a PBS-azide solution containing TritonX100. Primary antibodies used for immunostaining included rabbit polyclonal anti-human VWF (DAKO, Denmark), sheep polyclonal anti-human VWF (Abcam, UK), sheep anti-human FVIII (Haemato-logic Technologies, USA), mouse anti-human CD11b (Santa Cruz Biotechnology, USA), mouse anti-human EEA1 (BD Biosciences, USA), and mouse anti-human Rab7 (Santa Cruz Biotechnology, USA). The fluorescent secondary antibodies were anti-rabbit/ or anti-sheep Alexa Fluor-488, anti-mouse Alexa Fluor-555, and anti-mouse Alexa Fluor-633 nm (all available from Invitrogen, USA). A corresponding negative control was prepared for each experiment by excluding the primary antibodies. Imaging of cells was performed using an Apotome.2 microscope (Carl Zeiss, Germany). Three-dimensional (3D) images of stacked Z-series of images were created using the program ZEN 2.6 (blue edition; Carl Zeiss, Germany). VWF signals and VWF/EEA1

and VWF/Rab7 co-localization were quantified by measuring the mean VWF intensity value (MIV) and Pearson correlation coefficients, respectively, for at least n = 25 cells (for internalization experiments under static conditions) or n=50 (for internalization experiments under shear flow, as well as measuring MIV) of the stacked Z images using the program ZEN 2.6.

Measurement of VWF content in the supernatant of neutrophils

To further confirm the internalization of VWF by neutrophils, we measured VWF antigen (VWF:Ag) in supernatants from resting and stimulated neutrophils and compared the amount of VWF with the amount in supernatants without cells (as a negative control). After neutrophils were isolated from whole blood (n=6), cell suspensions were centrifuged (300xg for 5 min), and supernatants containing autologous VWF were collected in new tubes. Cell pellets were then resuspended in the same autologous supernatant at a cell concentration of 6 $X10^6$ cells/1 ml. Cells were left untreated or stimulated with IL -8 (0.7 ng/ml), PMA (10 ng/ml), or TNFα (5 ng/ml) for 15 min at 37ºC. Cell supernatants were then collected after centrifugation (350 g for 5 min), and VWF:Ag levels were measured, as previously described, in cell supernatants and supernatants deprived of neutrophils.[15]

Purification of pdVWF/FVIII concentrates

The pdVWF/FVIII concentrate (Haemate, CSL Behring, Germany) was purified to eliminate potential impurities present in the Haemate concentrate (e.g., albumin), for use in experiments assessing the effect of plasma on neutrophil characteristics. The purification was performed by size-exclusion chromatography on the ÄKTA pure 25 system (Cytiva/GE Healthcare Life Sciences, USA) using a Superdex 200 Increase 10/300 GL column (Cytiva, Germany).The protein purity and presence of VWF in the fractions corresponded to the VWF peak were confirmed using SDS-PAGE gel analysis.

Western blotting and gel electrophoresis of neutrophil lysates

Neutrophils were either left untreated or stimulated with IL -8 (0.7 ng/ml; for 15 min at 37ºC) in the presence of autologous VWF. Subsequently, washed cells were either directly proceeded to cell lysis or first suspended in RPMI buffer and incubated for 60 or 180 min at 37ºC and 5% CO_2 before cell lysis. Cell lysis was performed in M-PER

reagent® (Thermo Scientific, USA) supplemented with a protease inhibitor cocktail tablet (Roch, Germany) according to the manufacturer's instructions. Western blotting of neutrophil lysate together with the lysates of cells producing VWF as controls (including healthy individual-derived ECFCs producing endogenous VWF and transfected HEK293T cells producing recombinant VWF) were performed using 4-15% Mini-Protean® TGX and Mini Trans-Blot® Cell systems under reducing condition (Biorad, USA), according to the manufacturer's guidelines. After blotting, VWF was visualized with rabbit polyclonal antibodies against human VWF (Dako, Denmark) and antibodies against rabbit IgG conjugated to horseradish peroxidase (Biorad, USA), as described elsewhere.[16]

To determine whether the VWF detected in the neutrophil lysate from the WB experiment was taken up from outside the cell, the purified pdVWF/FVIII (Haemate-P®) was fluorescently labeled using the FluoReporter™ FITC protein labeling kit (Invitrogen, USA) according to the producer's instructions. Neutrophils were then stimulated with IL -8 (0.7 ng/ml; for 15 min at 37ºC) in the presence of the fluorescently labeled VWF (at a VWF antigen concentration of 100 IU/dL), incubated, and lysed, as described above. To assess the integrity of pdVWF/FVIII after exposure to the stimulated neutrophils, cell lysates were subjected to gel electrophoresis on 4-15% Mini-Protean® TGX (Biorad, USA) at 180 V for 60 min under reducing condition. The fluorescence signal was detected using the ChemiDocTM MP imaging system (Biorad, Germany).

Investigation of the effects of VWF on the expression of neutrophils chaqracteristics

To evaluate impact of VWF binding on expression of neutrophice surface proteins, isolated, washed neutrophils (n=5 healthy individuals) were resuspended in RPMI medium (containing glutamine and 10% FBS) and stimulated either with IL -8 (0.7 ng/ml; for 15 min at 37ºC) or by shear forces (10 dyne/cm^2 generated by the Bioflux 200). Experiments were performed without or with the purified pdVWF/FVIII concentrate (Haemate, CSL Behring, Germany). Cells were then subjected to flow cytometric analysis described above to determine the surface expression of CD16, CD45, and CD66b proteins.

Further, we evaluated the impact of VWF on the formation of Neutrophil Extracellular Traps (NETs). Isolated neutrophils, in the presence or absence of pdVWF (at a VWF

antigen concentration of 100 IU/dL), were stimulated with PMA (25 ng/ml) at 37°C with 5% CO2 for different time points: 60, 120, 180, and 240 minutes. The cells were then fixed and subjected to immunofluorescence (IF) staining. The staining of the chromatin was done by using DAPI and Cit-Histone H3 antibody (ThermoFisher, Germany). The experiments were independently replicated at least twice, with each replication performed in duplicate (n=4).

Statistical analysis

The statistical significance of differences in data was evaluated with the unpaired Student's test using GraphPad Prism version 8.0.1 (GraphPadSoftware). Figures are presented as mean ± SEM. A p-value ≤.05 was considered to be statistically significant.