Investigating the pathomolecular mechanisms an in-frame heterozygous von Willebrand factor large deletion in a von Willebrand disease patient with significant bleeding history

H. Yadegari1, N. Marquardt1, V. Ivaskevicius1, O. Rawley2, L. Swystun2, D. Lillicrap2, J. Oldenburg1 (1Bonn, Germany, 2Kingston, Canada)

Bleeding disorders - Basic science
17.02.2017, 08:00 - 09:15

Objective: This study aimed to elucidate disease pathogenesis and genetics etiology in an index patient (IP) diagnosed to have type 3 von Willebrand disease (VWD). The IP suffers frequently from bleeding episodes in spite of prophylaxis treatment. The genetic investigations revealed only a de novo heterozygous large deletion of exons 4-34 of von Willebrand (VWF) gene. However, this single defect could not explain low VWF antigen (VWF:Ag) levels and severe clinical demonstration of the IP. The current ex vivo study investigated the molecular pathological mechanisms by which the detected large deletion impairs VWF biosynthesis.

Methods: Blood outgrowth endothelial cells (BOECs) were isolated from peripheral blood of IP and healthy individuals. RNA was extracted from cultured BOECs, and coding region of VWF gene was amplified by RT-PCR. Seventy-two hours after seeding the BOECs, VWF:Ag levels in the medium and lysate of the BOECs were measured. Multimer analysis of the BOECs supernatant was performed. The subcellular location of VWF in BOECs was visualized by immunofluorescence antibody staining and subsequent confocal microscopy scanning.

Results: The BOECs RNA analysis assured biosynthesis of normal VWF transcription originating from wild type allele as well as an aberrant transcript with exons 4-34 deletion. Surprisingly, the mean of secreted VWF:Ag levels from the patient-derived BOECs was only slightly reduced compared with those of the healthy donors (63% vs. 72% respectively). Multimer analysis of the secreted VWF showed loss of large and intermediate multimers along with shift in mobility of low molecular weight multimers. Additionally, confocal immunofluorescent analysis showed a relatively strong VWF staining in IP-derived BOECs. However, it seems that the Weibel-Palade-bodies in IP-BOECs are smaller compared to those within normal BOECs.

Conclusion: In conclusion, our results suggested that production of the VWF from second allele might not be disrupted. Rather it is speculated that in-frame deleted VWF (p.Asp75_Cys1948del) which lacks multimerization domains has a dominant-negative impact on multimerization process. Moreover, we presumed that the secreted chimeric VWF is cleared from the plasma circulate more rapidly than normal VWF. This assumption is remained to be confirmed by mouse model tools.
Possible role of protein disulfide isomerase PDIA1 in mechanisms of von Willebrand disease type 3

M. A. Brehm, S. Vollmers, T. Obser, R. Schneppenheim (Hamburg, Germany)

Bleeding disorders - Basic science
17.02.2017, 08:00 - 09:15

Objective: The hemostatic activity of von Willebrand factor (VWF) is highly dependent on its presence as a multimeric protein. The biosynthesis of VWF multimers involves formation of multiple disulfide bonds with different functions. First, in the endoplasmic reticulum (ER), intra-monomeric bonds are formed which are responsible for correct folding. Second, formation of bonds between the C-termini of two VWF monomers is catalyzed by protein disulfide isomerase isoform A1 (PDIA1) to produce VWF dimers. Third, multimerization is realized in the Golgi apparatus by formation of N-terminal inter-dimer disulfide bonds catalyzed by the VWF propeptide.

Mutations in the VWF gene, which result in lack of VWF in the patient’s plasma, lead to von Willebrand disease (VWD) type 3. Nevertheless, it has been shown that residual VWF may still be produced in the patient’s endothelial cells, which often is retained in the ER.

The aim of this study is to determine whether PDIA1 might be involved in this cellular retention of VWF mutants associated with VWD type 3.

Methods: We have investigated eleven VWD type 3 mutants by immunofluorescence after transient expression in HEK293 cells. Parallel staining of endogenous PDIA1 was used to visualize VWF-PDIA1-association and measurement of fluorescence intensities revealed influence of the VWF mutants on PDIA1.


Conclusion: While most of the propeptide mutants exhibited ER retention without an effect on PDIA1, three of them induced an increase in PDIA1 expression. The loss of cysteine residues caused formation of VWF-PDIA1 clusters that resulted in a VWF secretion defect and an altered PDIA1 localization and expression. The latter finding indicates that PDIA1 might be involved in mechanisms underlying VWD type 3 by mediating cellular retention of VWF cysteine mutants.
Synergistic effects of a procoagulant bispecific antibody and rescue therapy on thrombin generation - a potential safety risk?

R. Hartmann1, S. Knappe1, B. Goldstein2, B. Ewenstein2, L. Valentino3, F. Scheiflinger1 (1Vienna, Austria, 2Cambridge, United States, 3Bannockburn, United States)

Bleeding disorders - Basic science
17.02.2017, 08:00 - 09:15

Objective: Investigational non-factor products, such as ACE 910 (emicizumab), offer potentially new treatment for hemophilia patients with inhibitors. However, their uncertain and unregulated mechanisms of action raise multiple concerns regarding safety and efficacy in specific clinical contexts. As an antibody to FIX (a) and FX(a), ACE 910 lacks the inherent regulatory characteristics for hemostasis present in replacement factor and bypassing agents.

FEIBA is a plasma-derived, activated prothrombin complex concentrate. It has been used over 40 years to treat and prevent bleeding episodes in hemophilia A and B patients with inhibitors. Extensive prospective clinical study and post-approval pharmacovigilance data demonstrated that the product is safe and highly effective. A phase III study of emicizumab (NCT02622321) is currently being conducted in hemophilia A patients with inhibitors. To evaluate the treatment options for those experiencing breakthrough bleeding, we studied the in vitro thrombin generation profile of various combinations of FEIBA and a biosimilar version of ACE 910.

Methods: A biosimilar antibody to emicizumab (BS-Em) expressed in mammalian cells, purified, and biochemically characterized was analyzed in standard thrombin generation (TG) experiments using plasma from individual inhibitor patients. Therapeutic doses of BS-Em (20-600nM) in combination with various concentrations of FEIBA (0.05-1IU/ml) were tested. A normal range of TG was established using plasma from healthy individuals.

Results: The combination of FEIBA and BS-Em induced peak thrombin values of >500nM (600nM BS-Em/1IU/ml FEIBA), while the reference range for peak levels was ~50-120nM. FEIBA concentrations higher than 0.25IU/ml combined with 600 nM BS-Em induced peak thrombin values up to 5-fold of normal.

Conclusion: These in vitro experiments demonstrate the potential for excessive thrombin generation after co-administration of FEIBA and BS-Em at antibody concentrations expected to occur in patients participating in this study. Therefore, caution and clinical judgement will be required when treating potential breakthrough bleeds in patients receiving emicizumab, as AEs may occur.
Calcium binding sites of coagulation factor XIII (FXIII) A subunit and their functional relevance to the activation of FXIII A subunit

S. Singh 1, C. Krettler 2, C. Reinhart 2, J. Dodt 3, V. Ivaskevicius 1, J. Oldenburg 1, A. Biswas 1 (1Bonn, Germany, 2Frankfurt, Germany, 3Langen, Germany)

Bleeding disorders - Basic science

Objective: To check the functional relevance of three calcium binding sites on FXIIIA subunit by a combination of increasing concentrations of calcium saturated simulations and site directed mutagenesis performed on the recombinant FXIIIA (rFXIIIA).

Methods: rFXIIIA subunit was cloned, expressed and purified in-house, using the yeast based Pichia expression system. Twelve substitutions of two types were generated on the calcium binding site residues: A) substitution with a Lys residue to prevent calcium binding and B) substitution with an Asp residue leading to tighter binding using Geneart site-directed mutagenesis kit (Thermo Fischer Scientific, USA). Positive clones were expressed in yeast based Pichia expression system, and purified using two-step purification strategy (IMAC using His-TRAP, followed by Gel filtration). The purified proteins were tested for identification (by peptide mass fingerprinting) antigenic stability (by western blot), FXIIIA activity (by photometric assay), and FXIIIAa generation (by FXIII generation assay). The complete FXIIIA zymogenic crystal structure was subjected to classical MD simulation with different supra-physiological concentrations of calcium. The same process was repeated also exclusively for only the FXIIIA core domain

Results: In the simulations higher concentrations of calcium led to faster co-ordination of the calcium binding sites. We also observed cross-talk between the three calcium binding sites in terms of correlated motion. So far three clones with implemented mutations in the calcium binding sites were successfully purified. While these three clones were antigenically detectable easily, they had a tendency to degrade faster as compared to the wild type rFXIIIA under same buffer conditions. Amongst the three mutants, only one showed any activity which was very low (almost 1-10% of the wild type).

Conclusion: Calcium binding sites in FXIIIA subunit appear to be highly sensitive areas of the protein, which is why any kind of substitution here appears to compromise the activity or even antigen levels of the protein by causing misfolding of the protein. The three calcium binding sites in agreement to our previous reports also show interdependence and chronology during the activation process.
Hemophilia macrophages exhibit specific defects relating to wound healing

J. Pilch, D. Lessig, M. Bernard, E. C. Schwarz, H. Eichler, L. M. Knowles (Homburg, Germany)

Bleeding disorders - Basic science
17.02.2017, 08:00 - 09:15

Objective: Bleeding events in hemophilia can lead to synovial inflammation, cartilage damage and ultimately, joint destruction. It has also been shown that wound healing is delayed in hemophilia patients. Macrophages are specialized in promoting wound healing and tissue regeneration as they infiltrate in blood clot and phagocytose wound debris. We hypothesize that these macrophage functions are relevant to joint health. The objective is to define macrophage function in hemophilia with respect to polarization, phagocytosis and clot invasion.

Methods: Monocytes isolated from hemophilia patients and healthy individuals were treated with M-CSF or GM-CSF to induce macrophage differentiation. Macrophage differentiation was probed by phenotypic analysis as well as immunocytochemistry for TNFα, CD163 and CD206. Expression of Tie2 was assessed by flow cytometry. Phagocytosis was assessed using fluorescent labeled latex beads as well as red blood cells. To determine cell invasion, monocytes were embedded in a 3-dimensional matrix of clotted plasma, differentiated with M-CSF or GM-CSF and probed for podosome formation.

Results: M-CSF, which supports macrophage traits associated with wound healing and tissue repair, induced a spread and elongated phenotype in donor monocytes. Hemophilia monocytes, on the other hand, did not undergo this typical shape change and failed to express differentiation markers such TNFα or CD163 in response to M-CSF. Inflammatory differentiation in response to GM-CSF, however, was at least partially intact. Functionally, deregulation of hemophilia monocytes was reflected in deficits of M-CSF-dependent actions such as clot invasion and phagocytosis. These deficits could be explained by impaired filopodia formation, which may result from reduced expression of the receptor tyrosine kinase Tie2 on hemophilia monocytes.

Conclusion: Hemophilia macrophages are unable to promote adhesive interactions in response to M-CSF. As a result, M-CSF mediated regenerative macrophage functions such as phagocytosis and clot invasion are diminished while inflammatory macrophage functions in response to GM-CSF appear to be preserved. Together, our results suggest that hemophilia macrophages exhibit specific impairments of functions involved in wound healing, which may have important implications for resolving joint inflammation after bleeding.
Plasma derived FVIII concentrates induce neutrophil activation and neutrophil extracellular trap formation.

B. E. Kehrel, A. Bertling, M. F. Brodde (Muenster, Germany)

Bleeding disorders - Basic science
17.02.2017, 08:00 - 09:15

Objective: In this project, we studied whether pdFVIII may boost inflammation by the activation of neutrophils.

Methods: Three pdFVIII products (Octanate®, HaemateP® and Haemocytin®) and two rFVIII products (Kogenate®FS and Advate®) were studied. Neutrophils were gently isolated from fresh human blood by counter-flow elutriation. Low activation status after isolation was controlled by fibrinogen binding to CD11b. Whether FVIII concentrates induced generation of the reactive oxygen species (ROS) superoxide anion (O2.-) from nonactivated neutrophils was determined as the linear rate of superoxide dismutase-inhibitable reduction of cytochrome C. Activation of CD11b was analyzed by flow cytometry using an activation specific monoclonal antibody against CD11b labeled with PE. Associate formation in whole blood between platelets and neutrophils was measured by flow cytometry. Effect of factor VIII products on DNA release from isolated human neutrophils was measured by two methods, a fluorogenic assay using the fluorescent dye Syto13 binding to nucleic acids, and a sandwich ELISA against histone-DNA complexes detecting neutrophil extracellular traps (NETs) from FVIII treated human neutrophils. Net formation was in addition analysed by light microscopy.

Results: Significant activation of CD11b (MAC-1) was only observed on neutrophils treated with the used (0.1 -1 U/ml) pdFVIII, but not on neutrophils treated with rFVIII. pdFVIII induced neutrophil ROS production as well as the release of extracellular DNA (neutrophil extracellular traps, NETs), which was not observed with rFVIII. Fluorescence microscopy showed that pdFVIII induced neutrophil nets present alpha defensins (HNP1-3) and myeloperoxidase. In addition to experiments with isolated neutrophils, we studied the effect of FVIII on neutrophils in melagatran anticoagulated whole blood. Two of three tested pdFVIII clearly and significantly induced the formation of associates between neutrophils and platelets. In contrast, rFVIII had no effect. The activation effects of pFVIII on neutrophils were more pronounced in the presence of platelets.

Conclusion: Based on these in vitro results, pdFVIII products, in contrast to rFVIII products, may be proinflammatory by activating neutrophils. Further research aims to investigate the effect of factor VIII products ex vivo.

This work was supported by a grant of Bayer Vital GmbH, 51368 Leverkusen, Germany.