Cellular Stress Induced by Plasma-Derived Factor VIII Products

Martin Brodde^{a,b} Anja Müller^{a,b} Beate Kehrel^a

^a Experimental and Clinical Hemostasis, Department of Anesthesiology, Intensive Care and Pain Therapy, University Hospital Münster,

^b OxProtect GmbH, Münster, Germany

Keywords

Hemophilia · Cell stress · Factor VIII · Plasma derived · Recombinant

Summary

Background: We previously identified protein impurities in plasma-derived factor VIII (pdFVIII) products. The goal of the current experiments was to determine whether these impurities might have clinical relevance, by comparing the effects of pdFVIII and recombinant FVIII (rFVIII) products on cellular stress induction. **Methods:** The in vitro outcomes on cell stress sensors of 2 pdFVIII products and 1 rFVIII product were evaluated. Microparticle formation was assessed in cells treated with the 3 products. Effects on the mitochondrial membrane potential were measured in cells treated with clinically relevant concentrations of each product. **Results:** Microparticle formation was induced in platelets by 1 pdFVIII product and in monocytes and granulocytes by both pdFVIII products; the rFVIII product did not affect microparticle formation. Both pdFVIII products, but not the rFVIII product, significantly depolarized the mitochondrial membrane potential. **Conclusion:** The 2 pdFVIII products tested induced cellular stress in in vitro experiments. No such results were seen with the rFVIII product. Chronic activation of the cell stress defense system and chronic cell irritation may have clinical consequences for patients with hemophilia A.

Introduction

Hemophilia A is an inherited bleeding disorder resulting from a deficiency in clotting factor VIII (FVIII). Patients with hemophilia A receive infusions of FVIII products either on demand to treat bleeding episodes or prophylactically to prevent bleeding episodes and subsequent development of joint arthropathy. Currently available FVIII products are either plasma derived (pdFVIII; manufactured using human plasma) or recombinant (rFVIII; manufactured from cell lines using recombinant DNA technology). Patients with hemophilia A require lifelong treatment, and the quantities of FVIII products used are considerable, particularly when patients are treated prophylactically. In a retrospective analysis of a large US pharmacy database, the median annual consumption of rFVIII products among patients with severe hemophilia A was 1,442 IU/kg when used on demand and 4,060 IU/kg when used for prophylaxis [1]. Given the amount of FVIII product infused, especially prophylactically, patients with hemophilia A have considerable potential for exposure to impurities associated with the FVIII product they use.

The presence of blood-borne viruses, such as HIV, HBV and HCV, in pdFVIII products has been virtually eliminated through improved screening of blood donations and the use of viral inactivation techniques during manufacture [2]. The risk of viral transmission with rFVIII products is almost nonexistent because these products are not derived from human plasma [3]. However, other impurities that could affect patient health may be present in pdFVIII products. In an earlier study [4], we identified hematocyte-derived proteins and proteins of the complement activation pathway in several pdFVIII products of varying purities. Proteins isolated in some of the pdFVIII products tested included anaphylatoxin C3a, the platelet activation markers thrombospondin-1 and platelet factor 4, and leukocyte-secreted myeloperoxidase and α -

defensin [4]. Although the concentrations of these impurities were low, the presence of these proteins clearly indicates that some of the blood cells in the blood donations used as starting material for the purification process had been activated [4].

Whether lifelong exposure to such protein impurities in pdFVIII products adversely affects patient health is currently unknown. It is possible that chronic activation of the cell stress defense system and chronic cell irritation may have deleterious effects on patient health and could thus be of considerable importance in hemophilia treatment. To investigate whether pdFVIII impurities might be clinically relevant, the effects of pdFVIII and rFVIII products on the induction of cellular stress were compared.

Material and Methods

Venous Blood

Venous blood from healthy volunteers who had given their informed consent and not taken any medication during the last 2 weeks was anticoagulated with 0.32% sodium citrate or 20 U/ml fragmin. Platelet-rich plasma (PRP) was obtained by centrifugation at $180 \times g$ for 10 min at room temperature.

HMEC-1 Culture

A human microvascular endothelial cell line (HMEC-1) [5] was cultured in endothelial cell medium (PAA, Cöbe, Germany) and supplemented with 2 mM L-glutamine, 100 IU/ml benzylpenicillin, and 100 mg/ml streptomycin. The human microvascular endothelial cells (HMECs) were grown to approximately 90% confluence; the monolayer was washed twice with phosphate-buffered saline (PBS) and detached with Accutase® (Millipore, Billerica, MA, USA). The cell concentration was adjusted to 3,000 cells/ μ l.

FVIII Products

Three FVIII products were evaluated for their in vitro effects on cell stress sensors: a pdFVIII product with a moderate level of impurities (product 1, Haemotin®SDH; Biotest Pharma GmbH, Dreieich, Germany), a pdFVIII product with a relatively high level of impurities (product 2, Octanate®; Octapharma GmbH, Langenfeld, Germany) [4, 6], and an rFVIII product formulated with sucrose (rFVIII-FS, KOGENATE®; Bayer Pharma AG, Berlin, Germany).

Mitochondrial Stress

Whether clinically relevant concentrations of FVIII products generate mitochondrial stress in platelets and HMECs was examined as previously described by Leytin et al. [7]. Specifically, depolarization of the mitochondrial membrane potential ($\Delta\Psi$ m) was measured using the cell-penetrating lipophilic cationic fluorochrome JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide), which accumulates in the mitochondrial matrix, driven by the $\Delta\Psi$ m [8]. JC-1 exists as a green fluorescent monomer in the cytoplasm and forms red fluorescent aggregates in the mitochondria; depolarization of the inner mitochondrial membranes is associated with a decrease in the JC-1 aggregates. The JC-1 stock solution (3.8 mmol/l in dimethyl sulfoxide) was diluted with HEPES-buffered Tyrode's solution (PBS supplemented with 1 mmol/l MgCl_2 , 5.6 mmol/l glucose, 0.1% bovine serum albumin (BSA), and 10 mmol/l HEPES; pH 7.4) to a final concentration of 0.5 μ mol/l. Platelets or HMECs in solution were incubated for 60 min at room temperature with 0.5, 1, or 5 IU/ml FVIII product. Thereafter, JC-1 was added and the cells were incubated for 30 min at 37 °C. After addition of buffer, the cells were directly ana-

lyzed by flow cytometry, and the red fluorescence (emission maxima, 585/590 nm) indicative of JC-1 aggregates and the green fluorescence (emission maxima, 510/527 nm) indicative of JC-1 monomers were quantified. Depolarization of $\Delta\Psi$ m was quantified as a decrease of the JC-1 aggregates (fluorescence 2). Thapsigargin (10 μ mol/l) was used as positive control to induce cellular stress and apoptosis in these studies.

Microparticle Formation

The effects of FVIII products on cellular microparticle formation in platelets, monocytes, and granulocytes were evaluated. Positive controls were collagen 1 μ g/ml + thrombin 0.5 U/ml for the platelet experiments and thrombospondin-1 peptide (RFYVVMWK, 100 μ mol/l) for the monocyte and granulocyte experiments.

Gel-filtered platelets were stained with the fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) against glycoprotein IX (GPIX; mAb clone Beb-1; BD Biosciences, San Jose, CA, USA), after which they were incubated for 60 min at room temperature with different amounts of FVIII products and then analyzed by flow cytometry. To study platelet microparticle formation and resolve platelet-derived microparticles from background light scatter, acquisition gates were set to include only FITC-positive (GPIX-positive) events. Using such fluorescence thresholds, only platelets and platelet-derived microparticles were included in the analyses. To distinguish the platelet-derived microparticles from intact platelets, the forward scatter profile of untreated platelets was used to set the lower threshold, such that all events below this threshold corresponded to platelet-derived microparticles. Cell acquisition was performed on a FACSCalibur flow cytometer (BD Biosciences), and 15,000 FITC-positive events per sample were analyzed using Cellquest software (BD Biosciences).

Microparticle formation from monocytes and granulocytes from whole blood was similarly measured by flow cytometry. The blood samples were stained with fluorescent antibodies to identify monocytes/monocyte-derived particles (phycoerythrin (PE)-labeled mAb against CD14, clone M5E2; BD Biosciences) and granulocytes/granulocyte-derived microparticles (FITC-labeled mAb against CD16b, clone CLB-gran11.5; BD Biosciences). As described earlier, the lower limit of the forward scatter profile of microparticles derived from the monocytes and granulocytes was used to distinguish them from their respective intact cells. The forward scatter thresholds were set using untreated granulocytes and monocytes as controls, and 10,000 fluorescence-positive events per sample were analyzed using Cellquest software (BD Biosciences).

Statistical Analysis

Student's t-test was used for statistical analysis. The data shown represent the mean $\pm$ SEM (standard error of the mean).

Results

Mitochondrial Stress

In the mitochondrial stress experiments, both pdFVIII products, at concentrations down to 0.5 IU/ml, had significant dose-dependent negative effects on $\Delta\Psi$ m in platelets compared with rFVIII-FS and the negative control (p = non-significant for product 2 at 0.5 IU/ml vs. the negative control); no negative effects on $\Delta\Psi$ m were seen with rFVIII-FS (fig. 1A). The significant pdFVIII-induced decrease in $\Delta\Psi$ m was also seen in HMECs (fig. 1B). No significant differences were seen between the 2 pdFVIII products in either the platelet or HMEC experiments (fig. 1).

Fig. 1. Effect of factor VIII products on the mitochondrial membrane potential in **A** platelets and **B** HMECs. The mean fluorescence indicative of JC-1 aggregates is shown. The data shown represent the mean $\pm$ SEM. P values ≥ 0.05 were considered non-significant (NS). pdFVIII = Plasma-derived factor VIII; rFVIII = recombinant factor VIII.

Microparticle Formation

The studies in platelets to examine the effect of the different FVIII products on microparticle formation, which is indicative of mitochondrial stress and apoptosis, showed that 1 of the pdFVIII products, Octanate (product 2), induced significant microparticle formation in platelets at 4 different concentrations tested (1, 2, 5, and 10 IU/ml); the other pdFVIII product, Haemoctin (product 1), and rFVIII-FS did not induce significant microparticle formation in platelets compared with the negative controls (fig. 2A). In the studies examining microparticle formation in monocytes and granulocytes, both pdFVIII products induced significant microparticle formation in monocytes (at concentrations of 0.2, 0.5, 1, 2, and 5 IU/ml) and granulocytes (at a concentration of 0.5 IU/ml) compared with the negative controls, whereas rFVIII-FS did not induce the formation of microparticles in either of the cell types (fig. 2B, C). There was a significant difference between rFVIII-FS and the 2 pdFVIII products in the induction of microparticles in granulocytes (fig. 2C).

Discussion

Our study is the first to examine the effects of FVIII products on cell stress sensors. Cells respond to stressful stimuli initially through protective mechanisms; if these mechanisms fail to counteract the stress, the damaged cells are removed through programmed cell death [9]. Cell stress induced by FVIII products potentially could adversely affect patient health. We performed a series of experiments to gauge the effects of 2 pdFVIII products and rFVIII-FS on cell stress.

The mitochondrial membrane potential ($\Delta\Psi_m$) is an important measure of mitochondrial function and is used as an indicator of cell health [10]. Most apoptosis-inducing conditions involve disruption of the $\Delta\Psi_m$, resulting in a sudden increase in membrane permeability to solutes with a molecular mass of less than ~ 1.5 kDa [11]. Both of the pdFVIII products tested in this study had a clear effect on mitochondrial stress, significantly depolarizing $\Delta\Psi_m$ in a dose-dependent manner in platelets and HMECs. rFVIII-FS had no negative effects on $\Delta\Psi_m$ in either platelets or HMECs and did not differ significantly from the negative control.

Fig. 2. Effect of factor VIII products on micro-particle formation in **A** platelets, **B** monocytes, and **C** granulocytes. The data shown represent the mean $\pm$ SEM. P values ≥ 0.05 were considered non-significant (NS). pdFVIII = Plasma-derived factor VIII; rFVIII = recombinant factor VIII.

Depolarization of the inner mitochondrial transmembrane potential is an early marker of apoptosis [11]. Because apoptotic cells release microparticles [12], we also studied the effects of FVIII products on platelet microparticle formation. Microparticles are small vesicles released from the plasma membrane of most activated or apoptotic cells [12, 13]. Microparticles bear antigens and receptors [14–16] that can be transferred to cell types different from their cell of origin [17]. On platelet-derived microparticles, tissue factor and phosphatidylserine are exposed on the cell surface [18], a state that promotes a high level of procoagulant activity [19]. Microparticles can induce inflammatory responses [16] through the modulation of nitric oxide and prostacyclin production in endothelial cells, monocyte chemotaxis, and adherence to the endothelium. Additionally, microparticles are important markers for cardiovascular disease [20]. Elevated levels of circulating microparticles have been reported in patients with various cardiovascular conditions, including acute myocardial infarction, congestive heart failure, hypertension, and peripheral artery disease [12, 21]. In our experiments, compared with the controls, 1 pdFVIII product induced strong, significant formation of microparticles from platelets, and both pdFVIII products induced strong, significant microparticle formation in monocytes and granulocytes. No microparticle formation was seen with rFVIII-FS in platelets, monocytes, or granulocytes.

Our results indicate that the 2 pdFVIII products tested, but not rFVIII-FS, induce cellular stress in vitro. The negative effects of the pdFVIII products were seen in platelets, HMECs, and blood cells. Considering the frequency of FVIII infusions needed to treat severe hemophilia A, patients using pdFVIII products may be at risk of chronic activation of the cell stress defense system and chronic cell irritation.

Future experiments should examine the effects of pdFVIII products on cell stressors in human synovial cells, chondrocytes, osteoblasts, and osteoclasts. This is particularly important in hemophilia because chronic cell stress may contribute to inflammation and chronic hemarthrosis.

Acknowledgements

This work was supported by a grant from Bayer Vital GmbH, Leverkusen, Germany. Medical writing assistance was provided by Karen L. Zimmermann of Complete Healthcare Communications, Inc. (Chadds Ford, PA, USA) and was funded by Bayer HealthCare.

Disclosure Statement

The authors declared no conflict of interest.

References

- Epstein J, Xiong Y, Woo P, Li-McLeod J, Spotts G: Retrospective analysis of differences in annual factor VIII utilization among haemophilia A patients. *Haemophilia* 2012;18:187–192.
- Franchini M: Plasma-derived versus recombinant factor VIII concentrates for the treatment of haemophilia A: recombinant is better. *Blood Transfus* 2010;8:292–296.
- Musso R: Efficacy and safety of recombinant factor VIII products in patients with hemophilia A. *Drugs Today (Barc)* 2008;44:735–750.
- Brodde MF, Kehrel BE: Markers of blood cell activation and complement activation in factor VIII and von Willebrand factor concentrates. *Transfus Med Hemother* 2010;37:175–184.
- Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ: HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol* 1992;99:683–690.
- Clifton JG, Huang F, Kovac S, Yang X, Hixson DC, Josic D: Proteomic characterization of plasma-derived clotting factor VIII-von Willebrand factor concentrates. *Electrophoresis* 2009;30:3636–3646.
- Leytin V, Allen DJ, Mutlu A, Gylkhandanyan AV, Mykhaylov S, Freedman J: Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Lab Invest* 2009;89:374–384.
- Kroemer G, Galluzzi L, Brenner C: Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 2007;87:99–163.
- Fulda S, Gorman AM, Hori O, Samali A: Cellular stress responses: cell survival and cell death. *Int J Cell Biol* 2010;2010:214074.
- Duchen MR: Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. *J Physiol* 1999;516 (pt 1):1–17.
- Ly JD, Grubb DR, Lawen A: The mitochondrial membrane potential ($\Delta\psi(m)$) in apoptosis: an update. *Apoptosis* 2003;8:115–128.
- Boulanger CM, Amabile N, Tedgui A: Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease. *Hypertension* 2006;48:180–186.
- Wolf P: The nature and significance of platelet products in human plasma. *Br J Haematol* 1967;13:269–288.
- Gilbert GE, Sims PJ, Wiedmer T, Furie B, Furie BC, Shattil SJ: Platelet-derived microparticles express high affinity receptors for factor VIII. *J Biol Chem* 1991;266:17261–17268.
- Combes V, Simon AC, Grau GE, Arnoux D, Camoin L, Sabatier F, Mutin M, Sanmarco M, Sampol J, Dignat-George F: In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *J Clin Invest* 1999;104:93–102.
- Cloutier N, Tan S, Boudreau LH, Cramb C, Subbaiah R, Lahey L, Albert A, Shnyder R, Gobeze R, Nigrovic PA, Farndale RW, Robinson WH, Brisson A, Lee DM, Boilard E: The exposure of autoantigens by microparticles underlies the formation of potent inflammatory components: the microparticle-associated immune complexes. *EMBO Mol Med* 2013;5:235–249.
- Puddu P, Puddu GM, Cravero E, Muscari S, Muscari A: The involvement of circulating microparticles in inflammation, coagulation and cardiovascular diseases. *Can J Cardiol* 2010;26:140–145.
- Trappenburg MC, van Schilfgarde M, Marchetti M, Spronk HM, ten Cate H, Leyte A, Terpstra WE, Falanga A: Elevated procoagulant microparticles expressing endothelial and platelet markers in essential thrombocythemia. *Haematologica* 2009;94:911–918.
- Pereira J, Alfaro G, Goycoolea M, Quiroga T, Ocqueteau M, Massardo L, Perez C, Saez C, Panes O, Matus V, Mezzano D: Circulating platelet-derived microparticles in systemic lupus erythematosus. Association with increased thrombin generation and procoagulant state. *Thromb Haemost* 2006;95:94–99.
- Werner N, Wassmann S, Ahlers P, Kosiol S, Nickenig G: Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 2006;26:112–116.
- Lynch SF, Ludlam CA: Plasma microparticles and vascular disorders. *Br J Haematol* 2007;137:36–48.