Brief report

Intracellular interaction of von Willebrand factor and factor VIII depends on cellular context: lessons from platelet-expressed factor VIII

Helen Yarovoi, Alan T. Nurden, Robert R. Montgomery, Paquita Nurden, and Mortimer Poncz

We have previously reported that ectopically expressed factor VIII (FVIII) is stored within platelets and is released upon platelet activation. Studies by others in various cell lines have suggested that having von Willebrand factor (VWF) coexpression is necessary for FVIII granular storage and for its secretion. We tested the importance of VWF coexpression for ectopic storage of FVIII in platelets and for its bioavailability. Transgenic mice expressing platelet-specific FVIII were crossed onto a VWF<sup>−/−</sup> background. Antigenic levels of platelet FVIII in these mice were nearly unchanged whether VWF was present or not. Whole-blood clotting times and FeCl<sub>3</sub> carotid artery injury correction demonstrated that platelet FVIII demonstrably improved the bleeding diathesis in FVII<sup>−/−</sup> mice independent of the platelets’ VWF status. Immunogold electron microscopy demonstrated that platelet FVIII is stored in platelet α-granules independent of the presence of VWF. It appears that FVIII’s interaction with VWF and its intracellular transportation, storage, and secretion differ greatly depending on the cell type. The molecular basis for these differences now needs to be elucidated. (Blood. 2005;105:4674-4676)

Introduction

We have been interested in the ectopic expression of proteins in platelets as a targeting strategy for modulating thrombosis. Recently, we reported on the ectopic expression of urokinase in platelets to enhance thrombolysis and of factor VIII (FVIII) to correct the bleeding diathesis of mouse FVII<sup>−/−</sup> animals. In both cases, the ectopic protein appears to be stored in the platelet and released only upon platelet activation. Also, in both cases, a potential carrier protein for the ectopic protein is expressed normally during megakaryopoiesis. For FVIII, the potential carrier protein is von Willebrand factor (VWF). Previous studies in cell lines have shown that VWF enhanced FVIII processing and secretion from Chinese hamster ovary (CHO) cells greater than 20-fold<sup>1</sup> and that in AtT-20, a neural-derived cell line that contains granules similar to Weibel-Palade bodies,<sup>2</sup> VWF coexpression was necessary for FVIII granular storage.<sup>3</sup>

We now addressed whether FVIII needs VWF for granular storage during megakaryopoiesis. Part of our motivation was that we had already observed that FVIII processing in megakaryocytes differed from that in CHO cells in that it was not secreted. In addition, such studies may provide a better understanding of the limitations of using platelets to deliver an ectopic protein to a site of interest.

We, therefore, crossed the transgenic mouse expressing platelet human B-domainless FVIII (hB-FVIII) with the previously described VWF gene–targeted disruption mouse<sup>6</sup> and examined the resulting mice for FVIII antigen and activity and for localization within the platelet. These studies showed that FVIII was efficiently stored in platelets independent of whether VWF was coexpressed. Upon platelet activation, the released FVIII was also equally biologically active independent of whether the platelets coexpressed VWF in a whole-blood clotting time (WBCT) and assay in an in vivo thrombosis model. Immunogold electron microscopy (EM) confirmed that most of the FVIII was found inside α-granules, independent of whether VWF was coexpressed.

Study design

Characterization of the studied mice

The mouse models allowing this study have been previously described. The VWF gene–targeted disruption mice involved a loss of exons 4 and 5 of the VWF gene with no VWF protein expression.<sup>5</sup> The FVIII<sup>−/−</sup> mice had a targeted disruption of exon 16 of the FVIII gene.<sup>7</sup> Platelet hB-FVIII transgenic mice were generated as previously described.<sup>2</sup> Appropriate littersmates served as controls. Studies were approved by the Animal Care and Use Committee of the Children’s Hospital of Philadelphia. The Student t test was used to assess statistical difference between groups.

Determination of antigenic and functional hB-FVIII levels

Levels of hB-FVIII antigen in the plasma and platelets of transgenic animals were determined using an anti–human-specific FVIII enzyme-linked immunosorbent assay (ELISA) kit (Affinity Biological, Hamilton, ON, Canada) in platelet releasate from platelet-rich plasma after activation by phorbol ester and collagen, comparing levels to hB-FVIII recombinant protein (ReFacto Laboratory Standard; Genetics Institute, Cambridge, MA).<sup>2</sup> FVIII activity level was determined by WBCT.<sup>2,8</sup> Results of both antigen and activity were determined per mL of blood sample, per platelet.

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count, and relative to another α-granule–specific protein, determined as previously described by us (Eslin et al10). FeCl3-induced arterial injury was also performed according to published procedures.10 In some experiments, the animals received an infusion of washed platelets obtained from the inferior vena cava of a single donor mouse to avoid plasma transfer prior to the application of the FeCl3 injury as previously described.1,2

Immunoegold electron microscopy

Washed platelets from individual mice were fixed and transported to Bordeaux at 4°C as in Wilcox et al.11 Preparations of ultrathin cryosections, antibody labeling, and EM were performed.11,12 Antibodies used were rabbit polyclonal anti–human VWF (Dako, Carpinteria, CA);11 a murine monoclonal (MBC 103.3) to human FVIII light chain;12 and a rabbit polyclonal to mouse fibrinogen (Molecular Innovations, Southfield, MI). Irrelevant mouse immunoglobulin G (IgG) was used as a control for MBC 103.3. Bound IgG was detected with a goat anti-rabbit secondary antibody adsorbed onto 10-nm gold particles (1:100 dilution of AuroProbe EM G10; Amersham, Les Ulis, France) and/or a goat anti-mouse secondary antibody adsorbed onto 5-nm gold particles (1:80 dilution of AuroProbe EM G10). A minimum of 50 sections was examined for each mouse. The electron microscope was a Jeol JEM-10 with camera and software provided by the manufacturer (Jeol, Tokyo, Japan). The Jeol objective lens used an EM-SHP10-DL polepiece. Images were handled in Canvas 8.0 for Macintosh (Deneba, Miami, FL).

Results and discussion

Determination of platelet FVIII antigenic and functional levels

The hB-FVIII mice expressed approximately 9 units of FVIII antigen in their platelets found in 1 mL of whole blood, which we have termed a plasma equivalency of approximately 9%.2 To define the importance of VWF for the presence of FVIII in platelets, ELISA studies of plasma and platelet release from hB-FVIII mice were done on hB-FVIII/FVIII-null mice that were either VWF+/−, VWF+/+, or VWF−/− (Figure 1A). The platelet level on a heterozygote background was nearly identical to that of the wild-type animal, but on the VWF−/− background it was 73% plus or minus 7% of the wild-type level (n = 12 per arm; P < .05). Since platelet counts and platelet factor 4 levels were identical among the 3 groups (data not shown), the results were also true when expressed on a per platelet basis. No FVIII activity was detected in any of the concurrently studied plasma samples (data not shown). WBCT studies are a particularly effective means of measuring platelet FVIII availability.2 WBCT measurements done on animals similar to those on whom the antigen assays were done were no different, regardless of whether VWF was present or not (n = 5 per arm; Figure 1B), with WBCT levels similar to those previously reported for the hB-FVIII/FVIII-null mice.2 To test in vivo functionality, platelets from the similar hB-FVIII/FVIII-null mice that were either VWF+/− or VWF−/− were infused into FVIII-null/VWF+/− recipients as were platelets from FVIII-null/VWF−/− animals. This study design should allow one to test the efficacy of platelet FVIII in the VWF−/− background. Transfused platelets increased platelet counts in the recipient animals by 24.7% plus or minus 2.7%. These studies showed that donor platelet FVIII was as effective in vivo in improving the bleeding diathesis in the FVIII-null recipients whether or not the donor platelets contained VWF. As in both settings, all studied animals developed complete occlusions lasting longer than 1 minute whereas none of the control animals did (n = 5 per arm; P < .05 for each arm to control). Three of the 5 mice receiving hB-FVIII/FVIII-null/VWF+/− developed stable occlusive thrombi lasting at least 5 minutes, whereas all 6 mice receiving hB-FVIII/ FVIII-null/VWF−/− platelets developed such thrombi.

Immunogold electron microscopy localization of platelet FVIII

There are several explanations for the approximately 25% decrease in platelet FVIII antigen on the VWF background (Figure 1A). These include the possibility that VWF affects platelet FVIII half-life by altering its storage site or its stability in that storage space or that without VWF some FVIII is secreted. Since this released FVIII would be in small amounts and since there would be no VWF to stabilize the circulating FVIII in these mice, the fact that there was no detectable plasma FVIII in the hB-FVIII/FVIII-null/VWF−/− mice does not eliminate the possibility that a portion of the platelet FVIII was secreted. To better understand the fate of the platelet FVIII, immunogold electron microscopy was carried out. In the hB-FVIII/FVIII-null/VWF−/− mice, the FVIII was mostly localized to α-granules (Figure 2A), often occurring in the same granules as the VWF, although labeling was not always coadjacent with the detected VWF. The platelet FVIII was found in similar granules and frequency in the hB-FVIII/FVIII-null/VWF−/− mice (Figure 2B). As expected, VWF was absent but these granules did contain fibrinogen, another α-granular protein2 (Figure 2C). No labeling was seen with an irrelevant mouse IgG (data not shown).
Thus, these studies support α-granular localization of ectopically expressed FVIII in platelets in a VWF-independent fashion. This provides further support for differences in intracellular processing of FVIII between cell types. In megakaryocytes, ectopically expressed FVIII is not secreted but stored in α-granules independent of coexpressed VWF. In contrast in fibroblast CHO cells, FVIII intracellular processing and secretion is markedly dependent on coexpressed VWF, and in neural AT-20 cells FVIII intragranular storage is dependent on coexpressed VWF. DDAVP (1-deamino-delta-D-arginine vasopressin) infusions in liver transplantation subjects and after gene therapy in dog models could be ectopically expressed in platelets: efficacy in hemostasis and thrombosis. Proc Natl Acad Sci U S A. 1998;95:9524-9529.

References


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