Data Supplement

Materials

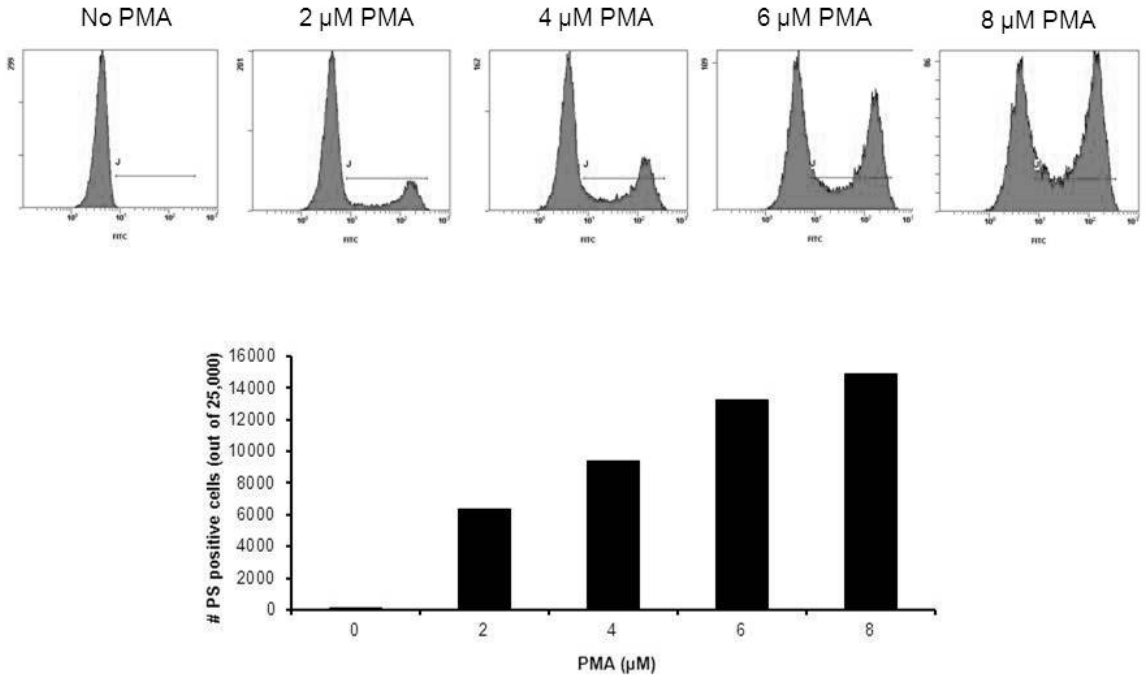
Fluorescein isothiocyanate (FITC)-labeled bovine lactadherin, FITC-EGR-chloromethyl ketone (FITC-EGRck) and RVV fX Activator were from Haematologic Technologies Inc. (Essex Junction, VT). Recombinant Tf_{1-243} was provided by Roger Lundblad and Shu-Len Liu (Baxter Healthcare, Duarte, CA). FITC-labeled annexin V was from Invitrogen (Grand Island, NY). PE-mouse anti-human glycophorin A and FITC-mouse anti-human CD71 were from Millipore (Billerica, MA). FITC-mouse anti human CD42b and CD45 were from BD Pharmagen (Sparks, MD). Ecarin was from Pentapharm (Basel, Switzerland). Recombinant hirudin and SpectozymeTH were from American Diagnostica (Stamford, CT). Recombinant human prothrombin (R155A, R271A, and R284A) (designated "rMZ") was a gift from Dr. Michael Nesheim (Queens University, Kingston, Ontario).[1] Bovine serum albumin, phorbol 12-myristate 13-acetate (PMA) and N-ethylmaleamide were from Sigma-Aldrich (St. Louis, MO). EZ-Link™ NHS-LC-LC-Biotin and ImmunoPure Streptavidin HRP Conjugate were from Thermo Scientific (Waltham, MA). TMB Microwell Peroxidase Substrate System was from KPL (Gaithersburg, MD). Serocluster™ PVC 96 well, non-treated plates were from Costar (Washington, DC). Human plasma fV, prothrombin, fX, and antithrombin were purified in-house as described.[2-4] Relipidated human Tf_{1-243}, immuno-depleted fV deficient plasma, corn trypsin inhibitor (CTI), D-Phe-Pro-ArgCH_2Cl (FPRck), αIIa, fVa and fXa were prepared in-house as described.[5-11] Affinity purified burro anti-human antithrombin and the monoclonal antibodies, anti-human thrombin and anti-human prothrombin fragment 2 were from the Antibody Core Facility (Colchester, Vermont). Synthetic phospholipid vesicles (75% phosphatidylcholine (PC) and 25% phosphatidylserine (PS)) from Avanti Polar Lipids (Alabaster, AL) were prepared as described.[12]

αTAT and mTAT ELISAs

The αTAT and mTAT assays are sandwich-style ELISAs. 96-well plates were coated with 5 µg/mL capture antibody (anti-human αIIa or anti-human prothrombin fragment 2 for αTAT and mTAT, respectively) in PBS pH 7.4, 16 hours before use and stored at 4°C. For the mTAT assay, the anti-prothrombin fragment 2 antibody was used due to the instability of the R155 bond. Plates were washed with HBS, 1% BSA for the αTAT assay or HBS, 1% BSA or 0.05% Tween-20 for the mTAT assay. Whole blood serum samples were diluted with HBS, 0.1% BSA for the αTAT assay (routinely 1:100-500) or HBS, 0.1% BSA, 0.05% Tween for the mTAT assay (routinely 1:50) and incubated for 2 hours at room temperature on an orbital rocker.

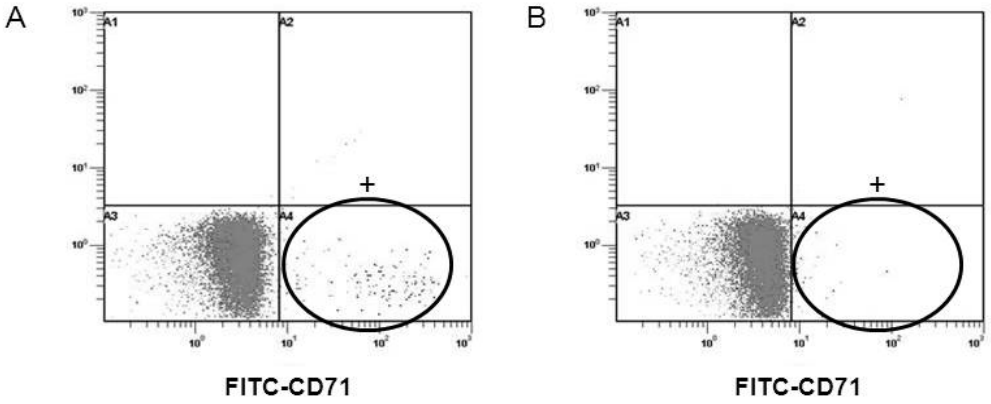
Plates were washed and probed with biotinylated burro anti-human antithrombin at 5 µg/mL in HBS, 1% BSA, and incubated at room temperature on an orbital rocker for 1 hour. Plates were then washed and binding was detected using HRP-Streptavidin and a chromogenic substrate on a BioTek (Winooski, VT) EL 312e microplate reader. The αTAT standard was generated by reacting 20 µM αIIa with 40 µM AT in HBS/PEG/Ca^{2+} for 30 min at 37°C. The mTAT standard was generated by reacting 7 µM rMZ, 14 µM AT and 14 µM fondaparinux with 2 U/mL Ecarin in HBS/PEG/Ca^{2+} for 30 min at 37°C. The rMZ mutant was used for the mTAT standard as it only forms one covalent species (mTAT) with antithrombin. The inactivation reactions were monitored with the chromagenic substrate Spectrozyme TH, and the extent of the reaction verified by SDS-PAGE. Both standards were quenched with FPRck and stored at 4°C and routinely monitored for stability via SDS-PAGE.

Supplemental Figure 1



PMA treatement of washed RBCs. Washed RBCs were diluted to 2% in wash buffer and mixed with an equal volume of wash buffer containing 4, 8, 12 or 16 µM PMA (2, 4, 6, 8 µM Final) and incubated at room temperature for 30 minutes. The PMA treated cells were then centrifuged at 1100g for 5 minutes prior to being washed 3x. Treated cells were diluted to $2x10^6$ cell/mL in RBC wash buffer and PS exposure was detected with FITC-bovine lactadherin on a Beckman Coulter FC500 flow cytometer.

Supplemental Figure 2



Reticulocyte detection in washed RBC populations. Whole blood (**A**) or washed untreated RBCs (**B**) diluted to $2x10^6$/mL and treated with FITC-anti cd71 on a Beckman Coulter FC500 flow cytometer.

References

1. Cote HC, Stevens WK, Bajzar L, Banfield DK, Nesheim ME, MacGillivray RT. Characterization of a stable form of human meizothrombin derived from recombinant prothrombin (R155A, R271A, and R284A). J Biol Chem. 1994;269:11374-11380.
2. Griffith MJ, Noyes CM, Church FC. Reactive site peptide structural similarity between heparin cofactor II and antithrombin III. J Biol Chem. 1985;260:2218-2225.
3. Katzmann JA, Nesheim ME, Hibbard LS, Mann KG. Isolation of functional human coagulation factor V by using a hybridoma antibody. Proc Natl Acad Sci U S A. 1981;78:162-166.
4. Bajaj SP, Mann KG. Simultaneous purification of bovine prothrombin and factor X. Activation of prothrombin by trypsin-activated factor X. J Biol Chem. 1973;248:7729-7741.
5. Nesheim ME, Taswell JB, Mann KG. The contribution of bovine Factor V and Factor Va to the activity of prothrombinase. J Biol Chem. 1979;254:10952-10962.
6. Kisiel W, Hermodson MA, Davie EW. Factor X activating enzyme from Russell's viper venom: isolation and characterization. Biochemistry. 1976;15:4901-4906.
7. Lundblad RL, Kingdon HS, Mann KG. Thrombin. Methods Enzymol. 1976;45:156-176.
8. Lawson JH, Krishnaswamy S, Butenas S, Mann KG. Extrinsic pathway proteolytic activity. Methods Enzymol. 1993;222:177-195.
9. Barenholz Y, Gibbes D, Litman BJ, Goll J, Thompson TE, Carlson RD. A simple method for the preparation of homogeneous phospholipid vesicles. Biochemistry. 1977;16:2806-2810.
10. Hojima Y, Pierce JV, Pisano JJ. Hageman factor fragment inhibitor in corn seeds: purification and characterization. Thromb Res. 1980;20:149-162.
11. Williams EB, Krishnaswamy S, Mann KG. Zymogen/enzyme discrimination using peptide chloromethyl ketones. J Biol Chem. 1989;264:7536-7545.
12. Higgins DL, Mann KG. The interaction of bovine factor V and factor V-derived peptides with phospholipid vesicles. J Biol Chem. 1983;258:6503-6508.