Schwertz et al., http://www.jem.org/cgi/content/full/jem.20061302/DC1

SUPPLEMENTAL MATERIALS AND METHODS

Platelet isolation and activation

For all of the platelet studies, residual leukocytes were removed from the washed preparations by $CD45^+$ bead selection as previously described (1). Flow cytometric gating of the entire cell population demonstrated that CD45-depleted platelet preparations did not contain CD45, P-selectin glycoprotein-1 (PSGL-1), or CD14-positive cells (Fig. S1 and unpublished data). Platelets were resuspended in M199 serum-free culture medium (1, 2). Platelets were left quiescent or allowed to adhere to immobilized human fibrinogen (Calbiochem) in the presence of thrombin (0.05 U/ml; Sigma-Aldrich). Select studies were also conducted with suspended platelets that were activated with ADP, collagen, or thrombin. For splicing inhibition, platelets were preincubated for 30 min with Tg003 (Calbiochem) or DMSO. For translational inhibition, platelets were isolated and preincubated for 2 h in the presence of vehicle (water) or puromycin as previously described (2).

mRNA detection systems

As described in the main text, primers that targeted sequences in exon four (5′-CTCGGACAGCCAACAATTCAG-3′) and five (5′-CGGGCTGTCTGTACTCTTCC-3′), were used to determine endogenous splicing of tissue factor (TF) pre-mRNA in quiescent and activated platelets. Primers that detected alternatively spliced human TF (asHTF) were used as previously described (3). To detect full-length mature mRNA for human TF (mHTF) in activated platelets, primers were designed that targeted sequences in exon one (5′-CCAACTGGTAGACATGGAGAC-3′) and exon six (5′-CAGTAGCTCCAACAGTGCTTCC-3′).

Indirect in situ hybridization or direct in situ PCR was used to detect TF pre-mRNA in megakaryocytes and platelets, respectively (1). Primers specific for intron four (5′-ACCCATTTCTTCCCCAATTC-3′ and 5′-GTGCCTGGGATCCTCAATAG-3′) were used to generate DIG-labeled intronic probes for the indirect in situ PCR and direct in situ PCR experiments. For platelets that were adherent to fibrinogen in the presence of thrombin, the generated cDNA was amplified in the presence of DIG-labeled dNTP using primers that targeted exons three (5′-CTCCCCAGAGTTCACACCTTAC-3′) and five (5′-CGGGCTGTCTGTACTCTTCC-3′), respectively. These exonic primers allowed us to detect the spliced product (331 bp), but not the unspliced product (3,635 bp), by normal PCR methods.

Protein detection systems

A polyclonal antibody against human TF (pAb CL 20150A; Affinity Biologicals) was used under nonreducing conditions to detect TF protein by Western analysis. Antibodies directed against TF (mAb 550252; BD Biosciences), Clk family members (Abgent), or β-tubulin (Sigma-Aldrich) were used for immunocytochemical studies. For these in situ experiments, polymerized actin was detected with Alexa fluor 488 phalloidin (Molecular Probes). Specificity was confirmed by parallel studies with nonimmune IgG or deletion of the primary antibody. Detailed strategies for protein detection by Western analysis and immunocytochemistry have been previously published (1, 2).

Flow cytometric analysis of surface-expressed CD14, CD45, and CD162 (PSGL-1) was performed on washed platelet preparations before and after CD45 depletion, on CD45 positively selected cells, and on purified monocytes (Fig. S1 and unpublished data).

Measurement of TF-dependent procoagulant activity

TF-dependent procoagulant activity was calculated with an Actichrome TF assay (American Diagnostica) as previously described (4). A total of 2×10^9 freshly isolated CD45-depleted platelets, a value that approximates the number of platelets found in 10 ml of whole blood obtained from healthy subjects, was resuspended in M199 media. At the end of each experimental point, the platelets were immediately centrifuged at 15,500 *g* for 4 min at 4°C. The supernatants were collected and recentrifuged at 100,000 *g* for 90 min at 4°C to pellet microparticles. In parallel, the platelet pellets were resuspended in ice-cold 250 mM sucrose that was suspended in 10 mM of PBS that contained a broad band protease inhibitor cocktail. After a brief sonication to disrupt the cells, the platelets were centrifuged for 15 min at 420 *g* (4°C) to separate the sedimented cellular components from the supernatant-rich membranes. The supernatants were recentrifuged at 20,800 *g* for 30 min (4°C) to pellet the membrane proteins. Intact cellular membranes and microparticles were immediately placed in 25 μl of kit assay buffer and TF was calculated. In separate studies, we found that disruption of platelet membranes or microparticles by standard detergent lysis markedly reduced activity (unpublished data). To demonstrate the specificity of the assay for TF procoagulant activity, some samples were preincubated with a neutralizing TF antibody (pAb 4502; American Diagnostica). Factor VIIa was also eliminated from the reaction. The data are displayed as pM of TF per 2×10^9 platelets.

www.jem.org/cgi/doi/10.1084/jem.20061302

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