

expanded this section of the trace to show the ABT-737–induced calcium signal. This small signal might not be sufficient to directly activate platelets, although it could reflect a larger but more local subcellular signal, or a subpopulation of platelets that respond differently compared with most platelets.

We also found that incubation with ABT-737 induced calcium store depletion. However, this was not a rapid event, but rather required prolonged incubation with ABT-737. To assess Ca^{2+} store content, we treated platelets with $10\mu\text{M}$ ABT-737 for either a short time (5 minutes) or a long time (120 minutes). The extracellular Ca^{2+} was then chelated by EGTA (1.2mM) and ionomycin ($1\mu\text{M}$) added to artificially release the intracellular calcium stores (Figure 1B). Brief treatment with ABT-737 (5 minutes) did not substantially affect the ionomycin-induced Ca^{2+} signal, indicating that the filling state of the intracellular Ca^{2+} stores was unaffected. This is consistent with the observations reported by Schoenwaelder and Jackson.⁵ However, ionomycin-induced Ca^{2+} release was substantially reduced after longer treatment with ABT-737. This suggests that the ionomycin-sensitive intracellular Ca^{2+} stores are slowly depleted after inhibition of Bcl-x_L , consistent with the data of Vogler et al.¹

Taken together, our data indicate that BH3 mimetics such as ABT-737 modify intracellular Ca^{2+} handling in platelets. Whether this contributes to platelet apoptosis and BH3 mimetic–induced thrombocytopenia requires further investigation.

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To the editor:

Platelet tissue factor is not expressed transiently after platelet activation

Blood coagulation is initiated by the binding of plasma factor VII/VIIa to cell surface–expressed tissue factor (TF). Normally, TF expression is confined to subendothelial tissues like fibroblasts¹ and pericytes² to limit blood coagulation to sites of vascular injury. Consistent with this notion, our laboratory demonstrated previously that platelets do not express active TF constitutively,^{3,4} or as a result of de novo synthesis, transfer from monocytes, or α -granule release.⁵ Recently, Camera et al suggested that expression of TF by platelets occurs rapidly after activation, and is transient,⁶ explaining our inability to observe detectable TF antigen and activity after a 2-hour platelet stimulation.⁵ In that study,⁶ 3 different anti-TF antibodies (2 obtained from commercial sources and another obtained from Dr Y. Nemerson, Mt Sinai School of Medicine, New York, NY [deceased]) were used and TF antigen was detected on the platelet surface after activation with a 6-amino acid protease activated receptor-1 (PAR1) agonist peptide for 15 minutes. Using a commercially available antibody (American Diagnostica; 45-07CJ), an $\sim 50\%$ increase in the mean fluorescence intensity (MFI) of the immunostained, activated platelets was observed when compared with unactivated platelets, or when activated platelets were stained with an isotype-matched control antibody. In contrast, no TF expression ($< 10\%$ increase in MFI) was observed after a 2-hour stimulation, consistent with our previous observation.⁵ In an

attempt to duplicate these results, we performed analogous experiments using 2 different fluorophore-conjugated anti-TF antibodies including a specific, inhibitory anti-TF monoclonal antibody described previously (anti-TF-5)⁷ and the same commercially available anti-TF antibody used by Camera and colleagues (American Diagnostica, 45-07CJ).⁶ By flow cytometry, nearly identical levels of anti-TF-5 immunoreactivity with platelets, activated for 15 minutes (728 ± 118.3 MFI; dotted line) or 2 hours (625.3 ± 62.3 MFI; black line) with PAR 1 agonist peptide (SFLLRN), were obtained (Figure 1A). Interestingly, we observed similar results using the same anti-TF antibody used by Camera and colleagues.⁶ No difference in MFI between 15 minutes (5263 ± 683.5 MFI) and 2 hours (5426 ± 664.5 MFI) of platelet activation was observed (Figure 1B dotted and black lines, respectively). In both instances, the MFI were similar to those obtained when platelets were immunostained using isotype-matched control antibodies (753.8 ± 78.9 MFI and 4543 ± 568.6 MFI, respectively; shaded histograms). Control experiments performed using a lipopolysaccharide-stimulated monocyte-like cell line (THP-1) versus unstimulated cells confirmed positive immunoreactivity of the antibodies. In the absence of LPS-stimulation, few cells expressed TF ($4.3 \pm 0.1\%$) using anti-TF-5 (Figure 1C dotted line). Subsequent to LPS-stimulation, $49.3 \pm 0.0\%$ of the cells

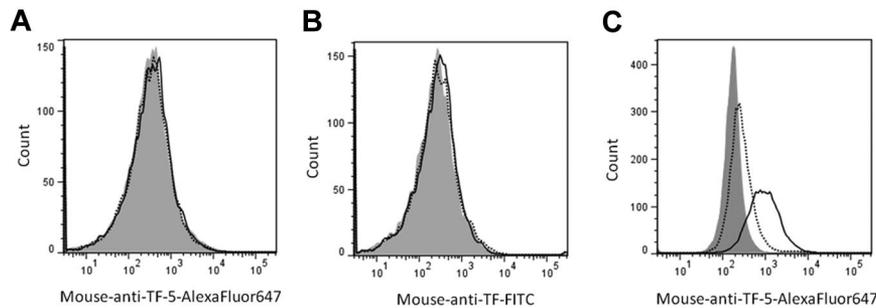


Figure 1. No platelet tissue factor expression: short or long activation. Washed platelets were activated with PAR1 (100 μ M) agonist peptide for 15 minutes (dotted lines) or 2 hours (black lines) at 37°C. TF expression on platelets was determined by immunostaining with (A) anti-TF-5 conjugated to AlexaFluor647 (0.5 μ M); (B) a 1:10 dilution of an anti-TF antibody conjugated to FITC (American Diagnostica 45-07CJ), or by equivalent concentrations of appropriate control antibodies (shaded histograms; 15 minutes activation period), in 20mM Hepes, 0.15M NaCl (pH 7.4) containing 10 μ g/mL human Fc. (C) Nonstimulated (dotted line) or LPS-stimulated (1 μ g/mL, 4 hours, 37°C; black line) THP-1 cells were immunostained with anti-TF-5 or an isotype-matched control antibody (shaded histogram; nonstimulated cells) as described. Platelets or THP-1 cells (10 000), identified by their forward and side scatter, were analyzed by flow cytometry using a BD LSR II Flow Cytometer. Identical results were obtained whether platelets, stained with isotype-matched control antibodies, were activated for 15 minutes or 2 hours. Similarly, LPS-stimulation of THP-1 cells was without effect on control antibody reactivity. Representative data are shown (n = 3).

were positive for TF expression (Figure 1C black line) similar to our previous observations.³ Similar results were also obtained using the anti-TF antibody from American Diagnostica (data not shown).

These combined data confirm our previous observation indicating the absence of TF on/in platelets⁵ and challenge the notion that expression of TF by activated platelets is a dynamic event.⁶ Recently, a report by Aass and colleagues⁸ demonstrated that many commonly used, commercially available immune reagents, including the TF antibody used in the study by Camera et al, can contain fluorescent antibody aggregates that form particles large enough to be quantifiable as monocyte-derived microparticles by flow cytometry, yet possess no measurable TF activity. Centrifugation of these antibodies before use resulted in disappearance of these fluorescent events.⁸ It is also possible that some of these aggregates may be mistaken for platelets, which are typically 2–3 μ m in diameter. These combined observations support our previous contention that differences in the assays and the quality of reagents used to quantify TF between laboratories, specifically, differences in antibody handling, may explain some of the apparent discrepancies.

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Response

Functionally active platelets do express tissue factor

At the beginning of the 2000, Rauch et al proposed that thrombosis occurring on plaque rupture does not necessarily require the exposure of vessel wall-derived tissue factor (TF).¹ Platelets can be a source of active TF, the so-called “blood-borne” TF or “circulating” TF, which can sustain activation of the blood coagulation on the edge of a growing thrombus. Since then more than 10 papers have documented the presence of functionally active TF in human platelets,^{2–12} with at least

3 mechanisms implicated for the presence of TF in platelets: (1) the microparticle transfer mechanism, as firstly proposed by Rauch¹; (2) the storage within the α -granule, as suggested by Muller⁴; and (3) the de novo protein synthesis from the specific TF mRNA, as proved by Schwertz et al and Panes et al.^{7,8} We believe that these pathways are not mutually exclusive, and one mechanism may dominate over the other depending on the pathophysiologic conditions.