Two Distinct Pathways Regulate Platelet Phosphatidylserine Exposure and Procoagulant Function

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Abstract

Procoagulant platelets exhibit hallmark features of apoptotic cells, including membrane blebbing, microvesiculation and phosphatidylserine (PS) exposure. Although platelets possess many well known apoptotic regulators, their role in regulating the procoagulant function of platelets is unclear. To clarify this, we investigated the consequence of removing the essential mediators of apoptosis, Bak and Bax, or directly inducing apoptosis with the BH3 mimetic compound ABT-737. Treatment of platelets with ABT-737 triggered PS exposure and a marked increase in thrombin generation in vitro. This increase in procoagulant function was Bak/Bax- and caspase-dependent, but was unaffected by inhibitors of platelet activation or by chelating extracellular calcium. In contrast, agonist-induced platelet procoagulant function was unchanged in Bak\(^{-/-}\)/Bax\(^{-/-}\) or caspase inhibitor-treated platelets, but completely eliminated by extracellular calcium chelators or inhibitors of platelet activation. These studies demonstrate the existence of two distinct pathways regulating the procoagulant function of platelets.
Introduction

The hemostatic and prothrombotic function of platelets is critically dependent on two key properties: (i) their ability to adhere and aggregate at sites of vascular injury; and (ii) their ability to support blood coagulation, necessary for thrombin generation and fibrin formation (platelet procoagulant function). The conversion of activated platelets to a procoagulant state is associated with specific biochemical and morphological changes, some of which are similar to those occurring in apoptotic cells, including caspase activation, proteolytic processing of cytoskeletal elements, surface exposure of phosphatidylserine (PS), and membrane contraction, blebbing and microvesiculation.

Platelets contain many well known apoptotic regulators, including members of the Bcl-2 protein family, and downstream effectors such as caspases. It was recently demonstrated that pro-survival Bcl-xL maintains platelet viability, primarily by restraining the pro-apoptotic function of Bak. Ex vivo treatment of platelets with the BH3 mimetic compound ABT-737, a potent inhibitor of Bcl-xL, triggers mitochondrial damage, caspase activation and membrane externalisation of PS. The relationship between platelet apoptosis, and the activation-dependent pathways that drive platelet procoagulant activity is unclear. A number of groups have suggested that Bcl-2 family proteins can be post-transcriptionally regulated in agonist-stimulated platelets. It has also been reported that Bcl-xL levels decline in stored platelets, and that this coincides with an increase in PS exposure and increased platelet procoagulant activity. Taken together, these findings suggest that the apoptotic machinery may regulate the procoagulant function of platelets.

In this study, we have investigated whether direct induction of apoptosis stimulates platelet procoagulant activity, independent of platelet activation, and whether the apoptotic machinery in platelets plays an important role in regulating agonist-induced platelet procoagulant function.
Materials and Methods

A detailed description of the materials and methods employed in this study are described in Supplementary Materials (available on the Blood website; see the Supplemental Materials link at the top of the online article). All animal studies were approved by the Alfred Medical Research and Education Precinct animal ethics committee. All procedures involving collection of human blood were approved by the Monash Committee on Ethics in Research and informed consent was obtained in accordance with the Declaration of Helsinki.
Results and Discussion

Caspases are Activated in Apoptotic Platelets Independent of Platelet Activation

Membrane fragmentation, microvesiculation and the exposure of phosphatidylserine (PS) on the surface of agonist-stimulated platelets requires calcium influx and is associated with the proteolytic cleavage of a range of cellular substrates. Prominent amongst these are the cytoskeletal proteins, filamin-1 and gelsolin that are cleaved by the calcium-activated thiol protease, calpain. Induction of apoptosis in washed human platelets with the BH3 mimetic compound ABT-737 resulted in the time-dependent cleavage of both gelsolin and filamin that paralleled PS exposure (Suppl. Fig. 1Ai, B) and platelet membrane blebbing (data not shown). However, in contrast to agonist-stimulated platelets (Suppl. figure 1Aii, filamin-1 and gelsolin cleavage induced by ABT-737 was not inhibited by chelators of extracellular calcium (Fig. 1A, EGTA), calpain inhibitors (Suppl. Fig. 1A, 737+CP) or by global inhibitors of platelet activation (PGE1 and theophylline) (Fig. 1B), but was completely blocked by the caspase inhibitor Q-VD-Oph (Suppl. Fig. 1A, 737+QVD).

Agonist-induced PS exposure and membrane fragmentation is associated with high sustained cytosolic calcium flux, collapse of the inner mitochondrial membrane potential ($\Delta\psi_m$), and caspase activation. To examine the effect of ABT-737 on $\Delta\psi_m$, platelets were loaded with tetramethylrhodamine-methyl ester (TMRM), a fluorescent dye retained in functional, intact mitochondria. The number of TMRM-positive platelets did not alter significantly following incubation of platelets with ABT-737 over a 3 hour time period (Suppl. Fig. 1C). Dual labelling of ABT-737 treated platelets with TMRM and Alexa 488-labelled-Annexin V revealed a high percentage of platelets staining positive for both fluorescent probes. In contrast, agonist stimulation of platelets resulted in a ~50% reduction in TMRM positive cells, with a shift in the population from single positive TMRM to single positive Annexin V staining, consistent with previous findings. Furthermore, inhibitors of the mitochondrial permeability transition pore (MPTP), cyclosporine and co-enzyme Q, had minimal effect on ABT-737-induced PS exposure, whilst significantly reducing PS exposure induced by CRP/thrombin (Suppl. Fig. 1D). These findings suggest that the mitochondrial changes in PS-positive apoptotic platelets are distinct from those occurring in agonist-stimulated procoagulant platelets.
Apoptotic Platelets Exhibit Procoagulant Activity

PS exposure on the surface of activated platelets is essential for the membrane assembly of coagulation factor complexes, including the Tenase and Prothrombinase complexes, necessary for thrombin generation. Whether PS exposure alone is sufficient to support blood coagulation, independent of other events linked to platelet activation, has not been defined. To investigate this, ABT-737-treated platelets were incubated with recalcified platelet-poor plasma and thrombin generation quantified in real time by monitoring cleavage of the fluorogenic thrombin substrate, Fluo-Substrate™. As demonstrated in Figure 1 and Supplementary Figure 2, ABT-737-treated platelets supported robust thrombin generation, similar in rate and extent to that induced by calcium ionophore A23187 or CRP (Fig. 1 C-E; Suppl. Fig. 2), in the absence of any apparent signs of platelet activation, as assessed by P-selectin expression, integrin αIIbβ3 activation (Suppl, Fig. 3) and increases in cytosolic calcium (data not shown). This increase in procoagulant activity was independent of platelet co-stimulation by enzymatically generated thrombin, as PAR4−/− platelets, which are completely unresponsive to thrombin, were equally effective at supporting thrombin generation following ABT-737 treatment (Fig. 1F). Furthermore, the potent platelet activation inhibitors, PGE1 and theophylline (PGE1/Theo) abolished CRP-induced thrombin generation, but had no significant inhibitory effect on ABT-737 induced thrombin generation (Fig. 1 C-E). In contrast, Q-VD-Oph abolished ABT-737-induced thrombin generation, but had no effect on CRP-stimulated thrombin generation (Fig. 2C, D).

PS Exposure by Apoptotic Platelets requires Bak and Bax

Since Bak and Bax are the central mediators of the intrinsic apoptotic pathway, we investigated their role in mediating platelet PS exposure and thrombin generation induced by ABT-737 or agonist. In mouse platelets, loss of Bak alone (Bak−/−Bax+/+) resulted in a 10% decrease in ABT-737-induced PS exposure, while additional loss of one Bax allele (Bak−/−Bax−/+) inhibited ABT-737-induced PS exposure by ~60%. Bak−/−Bax−/− platelets were completely resistant to the effects of ABT-737 (Fig. 2A). In contrast, stimulation of Bak−/−Bax−/−, Bak−/−Bax+/− or Bak−/−Bax−/− platelets with either calcium ionophore A23187 (data not shown) or a combination of CRP and thrombin was associated with normal levels of PS expression and thrombin generation (Fig. 2A,B). The caspase inhibitor Q-VD-Oph prevented ABT-737-induced PS exposure, consistent with a reduction in thrombin generation (Fig. 2C-E), but had no effect on effect on the procoagulant activity of platelets stimulated with ionophore A23187 or physiological agonists (data not shown and Fig. 2D).
stimulated and apoptotic platelets thrombin generation was dependent on surface exposure of PS, as it was completely inhibited by the addition of purified Annexin V (Suppl. Fig 2C).

**Conclusions**

Our findings demonstrate that two distinct pathways can regulate PS exposure and the procoagulant activity of platelets *in vitro*: (i) a calcium-dependent, caspase-independent pathway induced by physiological agonists; and (ii) a Bak/Bax-caspase-mediated pathway independent of platelet activation. Moreover, we have shown that although agonist-induced procoagulant (PS+ve) platelets exhibit characteristics of apoptotic cells, the processes regulating their formation are distinct from those regulating platelet apoptosis. Whether PS exposure on the surface of apoptotic platelets has any relevance to thrombin generation *in vivo*, or whether it represents a signal for the clearance of platelets at steady state, remains to be established. Notably, in preliminary studies we have demonstrated that ABT-737 treated platelets have reduced adhesive function and are rapidly cleared from the circulation following infusion into healthy mice, features that may limit any prothrombotic potential *in vivo*. Nonetheless, our demonstration that platelet procoagulant function can occur independently of platelet activation is of particular interest as BH3 mimetic compounds enter clinical trials for the treatment of malignant diseases.
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Footnotes

a Platelet procoagulant function refers to the ability of platelets to support thrombin generation.

b Membrane fragmentation refers to blebbing and loss of membrane integrity, as observed through DIC and SEM microscopy.
Author Contributions

S.M.S - designed research, performed research, analyzed data and wrote the paper; Y.Yuan - performed research and analyzed data; E.C.J. - performed research and analyzed data; M.J.W.- performed research and analyzed data; Y.Yao - performed research; K.D.M.- performed research; L.A.O'R.- performed research; K.J.H.- performed research; A.O - performed research; S.H. - performed research; A.W. - performed research; A.W.R. - designed research and analyzed data; D.C.S.H.- designed research, analyzed data and contributed vital new reagents; H.H.S. - Designed research and analyzed data; B.T.K. - designed research, analyzed data, and wrote the paper; S.P.J. - Designed research, analyzed data and wrote the paper. The authors have no conflicts of interest to declare.
References


Figure Legends

Figure 1. ABT-737 induces caspase activation and promotes thrombin generation independent of platelet activation.
Washed human platelets (3.0 x 10⁸/ml), or C57BL/6 (C57) or PAR4-deficient mouse platelets (PAR4⁻/⁻) (0.5 x 10⁸/ml) resuspended in Tyrode’s buffer in the absence (A,B) or presence (C-F) of BSA, were incubated with vehicle (DMSO), CRP (10 μg/ml, 20 min), calcium ionophore A23187 (A23187 - 1 μM, 20 min) or ABT-737 (737 - 1 μM, 90-180 min). In some experiments, platelets were preincubated with PGE₁ (2 μg/ml) and Theophylline (20 mM) [PGE₁/Theo], prior to ABT-737 treatment. (A,B) Human platelets were lysed for western blot analysis of pro-caspase-3, gelsolin and filamin cleavage, as described under ‘Materials and Methods’. Immunoblots are representative of 3 independent experiments. (C-F) The ability of human (C-E) or PAR4⁻/⁻ mouse platelets (F) to promote thrombin generation was assessed using a Thrombinoscope™, as described under ‘Materials and Methods’. Line graphs (C,E) are taken from one representative of 3 independent experiments. Histograms (D,F) represent the means ± SEM (n=3; **P<0.005).

Figure 2. Two distinct pathways regulate phosphatidylserine exposure and platelet procoagulant activity.
Washed Bak/Bax-deficient mouse platelets (A,B) or human platelets (C-E) (3.0 x 10⁸/ml) were resuspended in Tyrode’s buffer in the presence of BSA (5 mg/ml), then incubated with vehicle (DMSO), ABT-737 (1 μM) (90-180 min), calcium ionophore A23187 (A23187:1 μM, 20 min), or CRP (10 μg/ml) and thrombin (1 U/ml) (CRP & Thr, 20 min). In some experiments, platelets were preincubated with Q-VD-Oph (QVD, 50 μM) or calpeptin (CP, 100 μg/ml) prior to treatment with ABT-737/agonist. (A,E) The level of phosphatidylserine exposure (PS) in Bak/Bax-deficient mouse platelets (A) and human platelets (E), as measured by Alexa 488-conjugated Annexin V binding, quantified by flow cytometry as described under ‘Materials and Methods’. Histograms depict the means ± SEM (n=3). (B-D) The ability of platelets to promote thrombin generation in Bak/Bax deficient mouse platelets (B) or human platelets (C,D) was assessed as described in Figure 1. Line graphs are taken from one representative of 3 independent experiments. The histogram (D) represents the means ± SEM (n=3; **P<0.01) of three independent experiments.
Figure 1
Figure 2
Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function

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