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Platelet Senescence and Phosphatidylserine Exposure

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Abstract

Background—The exposure of phosphatidylserine occurs during platelet activation and during in vitro storage. Phosphatidylserine exposure also occurs during apoptosis following the release of mitochondrial cytochrome c. We have examined the role of cytochrome c release, mitochondrial membrane potential ($\Delta\Psi_m$), and cyclophilin D (CypD) in phosphatidylserine exposure due to activation and storage.

Study Design and Methods—The exposure of phosphatidylserine and the loss $\Delta\Psi_m$ were determined in a flow cytometer using FITC-lactadherin and JC-1, a lipophilic cationic reporter dye. The role of CypD was determined with cyclosporine A and CypD-deficient murine platelets. Cytochrome C induced caspase-3 and Rho associated kinase I (ROCK1) activation were determined by immunoblotting and using their inhibitors.

Results—Collagen and thrombin-induced exposure of phosphatidylserine was accompanied by a decrease in $\Delta\Psi_m$. Cyclosporin A inhibited the phosphatidylserine exposure and the loss of $\Delta\Psi_m$. CypD^{-/-} mice had decreased loss of $\Delta\Psi_m$ and impaired phosphatidylserine exposure. Collagen and thrombin did not induce the release of cytochrome c nor the activation of caspase-3 and ROCK1. In contrast, in platelets stored for more than 5 days, the phosphatidylserine exposure was associated with cytochrome c induced caspase-3 and ROCK1 activation. ABT737, a BH3 mimetic that induces mitochondrial pathway of apoptosis, induced cytochrome c release and activation of caspase-3 and ROCK1 and phosphatidylserine exposure independent of CypD.

Conclusion—These results show that in stored platelets cytochrome c release and the subsequent activation of caspase-3 and ROCK1 mediate phosphatidylserine exposure and it is distinct from activation-induced phosphatidylserine exposure.

Introduction

In resting platelets, phosphatidylserine and other anionic phospholipids are located on the inner leaflet of the membrane bilayer (1,2). Following platelet activation with thrombin, collagen, or shear-stress phosphatidylserine moves from the inner to the outer leaflet of platelet plasma membrane. The transbilayer movement of phosphatidylserine is responsible

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for the platelet procoagulant activity (3,4). In addition, exposure of phosphatidylserine is also a flag for macrophage recognition and clearance from the circulation (5,6)

Platelets, stored at room temperature for transfusion for more than 5 days, undergo changes collectively known as platelet storage lesions (7). These changes result not only in reduced responsiveness toward agonists but also in an accelerated clearance from the circulation following transfusion (8). The mechanism(s) of increased clearance is not known but increased phosphatidylserine expression and caspase-3 activity in stored platelets has been observed (9,10) and phosphatidylserine expressing platelets are rapidly cleared from the circulation (11). The mechanism of phosphatidylserine exposure in platelets is not known but a role for mitochondria has been proposed in collagen and thrombin-induced platelet activation (12).

Under normal physiological conditions, the inner mitochondrial membrane maintains a electrochemical gradient which drives the synthesis of ATP. Protons from the matrix are pumped to the intermembrane space by the oxidative phosphorylation complexes, resulting in a charge difference called mitochondrial transmembrane potential ($\Delta\Psi_m$). Loss of $\Delta\Psi_m$ can occur from opening of mitochondrial permeability transition pores (MPTP), which results in an increase in the permeability of the mitochondrial membranes to molecules of less than 1500 Daltons in molecular weight. The precise structure of the MPTP is not known, several proteins including cyclophilin D (CypD) constitutes the component of the pore (13). At a low conductance state, the MPTP functions as a mitochondrial Ca^{2+} release channel in the regulation of physiological cellular Ca^{2+} homeostasis (14). During apoptosis in most nucleated cells, loss of inner mitochondrial transmembrane potential also occurs through Bax/Bak mediated outer membrane permeabilization, which leads to mitochondrial swelling, rupture, and release of cytochrome c to the cytosol initiating the apoptotic pathways (13). The requirement of MPTP in cytochrome c release is not certain and in most nucleated cells two distinct pathways of cytochrome c release are present: one CypD and mitochondrial permeability transition pore dependent and other Bax/Bak dependent but independent of CypD (15).

Platelets also express Bcl-2 family of proteins Bax and Bak in the mitochondria (16-18). The Bax/Bak-mediated phosphatidylserine exposure plays a role in in vivo senescence (16-18). Genetic ablation of antiapoptotic Bcl-x(L) reduces platelet half-life and causes thrombocytopenia and deletion of proapoptotic Bak corrects these defects, and platelets from Bak-deficient mice live longer than normal (17). ABT737, a BH3 mimetic, induces mitochondrial pathway of apoptosis by binding to Bcl-2 and Bcl-X_L thereby blocking their inhibitory interaction on the proapoptotic Bax and Bak. ABT737 has been used to mimic in vivo senescence and phosphatidylserine exposure in platelets (17).

In this report, we have examined the role of mitochondria in phosphatidylserine exposure in stored platelets and compared it to collagen and thrombin induced phosphatidylserine exposure.

Materials and Methods

Thrombin was purchased from Hematologic Technologies Inc, Essex Junction, VT and collagen was obtained Helena laboratories Corp., Beaumont, TX. ABT737 was synthesized as described previously (19). JC-1, was obtained from Peninsula lab Inc, San Carlos, CA. Calcium ionophore A23187, cyclosporin A and Y-27632 were obtained from Sigma/Aldrich, St. Louis, MO. Anticaspase-3 antibody and anticytochrome c antibody were obtained from Cell Signaling Technology Inc, Danvers, MA. Cytochrome c releasing apoptosis kit and z-DVED-fmk (caspase-3 inhibitor), anti-ROCK1 (Rho associated kinase I)

monoclonal antibody, Clone 12M03, were obtained from BioVision Inc, Mountain View, CA. FITC-lactadherin was generated as described before (20).

Breeding pairs of cyclophilin D (CypD) deficient mice (Stock Ppif^{tmt1.1Mmos}/J and control mice (B6₁₂₉SF₂/J) were purchased from Jackson laboratory. The CypD^{-/-} mice were viable, fertile and did not display any gross physical or laboratory abnormalities. The animals were bred and all the colonies were maintained in the vivarium of Michael E. DeBaakey Veterans Affairs Medical Center. All animal procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Isolation of platelets

Blood was drawn through 19-gauge needles into polypropylene syringes containing one tenth volume of 3.8% trisodium citrate, pH 6.5, after an informed consent approved by the committee for protection of human subjects at Baylor College of Medicine. The blood was immediately transferred to polypropylene tubes, and washed platelets were prepared as previously described (21) and resuspended in modified Tyrode's buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 5 mM Hepes, 1 mmol/L MgCl₂, 3 mmol/L NaH₂PO₄, 5.5 mmol/L Dextrose, pH 7.35) containing 1% bovine serum albumin. Platelets were stimulated with either with a combination of collagen (5 µg/ml) and thrombin (1 unit/ml) or ABT737 (10 µM). Six- day old outdated platelets were obtained from the Gulf-coast Blood Center, Houston.

To isolate mouse platelets, blood was drawn in EDTA (5 mM final concentration) from 4-month old mice under isoflurane anesthesia from the inferior vena cava. Blood was diluted with equal volume of HBS and the platelet rich plasma was obtained by centrifugation at 260 g for 10 minutes. Prostaglandin E1 (1 µM) was added and platelets were sedimented by centrifugation at 1000 g for 10 minutes and washed twice in modified Tyrodes buffer as described for human platelets.

Platelet activation

Platelet suspensions were stimulated with a combination of collagen (5µg/ml) and thrombin (1 unit/ml) or ABT737 (10 µM). For inhibition studies, platelets were incubated with buffer alone, cyclosporin A (2 µM), z-DVED-fmk (20 µM) or Y27632 (20 µM) for 20 minutes before stimulation.

Flow cytometry

Phosphatidylserine expression and $\Delta\Psi_m$ were measured on a flow cytometer (Coulter FCC 500, Beckman-Coulter, Fullerton, CA) using the CXP software. The gates for intact platelets were set using fluorescein antiCD42b antibody and light scatter and fluorescence channels were set at a logarithmic gain. For phosphatidylserine expression, washed platelets were incubated with FITC-lactadherin (5 µg/ml) and a PE-labeled antiCD42b (2.5 µg/ml) for 30 minutes at room temperature (20). Loss of $\Delta\Psi_m$ was determined by incubating activated platelet suspension with JC-1, a lipophilic cationic reporter dye, for 20 minutes (22). Collapse of $\Delta\Psi_m$ caused a large shift in emission spectrum from red to green that was detected by flow cytometry as a decrease in red fluorescence.

Mitochondrial cytochrome c release, caspase-3 and ROCK1 activation

The release of cytochrome c to the cytosol was evaluated by western blotting of the cytosolic fraction of resting and stimulated platelets. Extraction of the cytosol was performed by the cytochrome c releasing apoptosis kit according to the manufacturer's instruction (BioVision Inc). The proteolytic activation of caspase -3 and ROCK1 were determined by SDS-PAGE and electrophoretic transfer of platelet extracts (20 µg/well) to

PVDF membrane. The membranes were probed with a polyclonal antibody to caspase-3. Activation of procaspase-3 results in the appearance of ~17 kDa band. ROCK1 activation was probed with a monoclonal antibody specific to the cleaved 30 kDa carboxy terminal fragment. As a control, jurkat cells (5×10^5) were incubated with etoposide (10 μ M) for 5 h and the cytosolic extract was probed for cytochrome c as described above.

Statistical Analysis

All data are expressed as mean and standard deviations of triplicate measurements except when indicated otherwise. Comparison between individual groups were performed using the Student T-test with paired and unpaired samples. A probability value (p) of 0.05 or below was considered statistically significant.

Results

Transbilayer movement of phosphatidylserine and the role of cyclophilin D

Stimulation of washed human platelets with a combination of collagen (5 μ g/ml) and thrombin (1 unit/ml) induced transbilayer movement of phosphatidylserine as determined by the binding of fluorescein-lactadherin (Figure 1 panel A). Cyclosporin A (2 μ M) inhibited collagen and thrombin induced phosphatidylserine exposure (Figure 1 Panels A and C). Under similar conditions, ABT737 also induced an increased expression of phosphatidylserine (Figure 1, Panel B and C). ABT737 elicits apoptosis by binding to Bcl-2 and Bcl-X_L thereby blocking their inhibitory interaction with the proapoptotic Bax and Bak (23). In contrast to collagen and thrombin-induced phosphatidylserine exposure, cyclosporin A had no effect on ABT737-induced phosphatidylserine exposure. As in human platelets, the combination of thrombin and collagen and ABT737 induced an increase in phosphatidylserine expression in wild type murine platelets (figure 2, Panels A, B and C). In cyclophilin D deficient mice, phosphatidylserine exposure induced by the combination of collagen and thrombin was impaired (Figure 2 Panels A and C) while there was no impairment of ABT737-induced phosphatidylserine (Figure 2, Panels B and C). These results show that cyclophilin D is involved in collagen and thrombin-induced phosphatidylserine exposure while ABT737-induced phosphatidylserine exposure does not require cyclophilin D.

Transbilayer movement of phosphatidylserine and $\Delta\Psi_m$

The combination of collagen and thrombin caused a significant loss of $\Delta\Psi_m$ in normal human platelets. Cyclosporin A inhibited the loss of $\Delta\Psi_m$ (Figure 3, Panels A, B and E). Under similar conditions, ABT737 induced a more marked collapse of $\Delta\Psi_m$. In contrast to the collagen and thrombin-induced mitochondrial depolarization, cyclosporin A had no effect on ABT-induced depolarization (Figure 3, Panels C, D and E). As in human platelets, a combination of collagen and thrombin induced mitochondrial depolarization in wild type murine platelets and it was impaired in CypD deficient mice (figure 4 Panels A, B and E). In contrast there was no impairment in ABT737-induced mitochondrial depolarization (Figure 4, panels C, D and E), consistent with the results with cyclophilin D inhibition with cyclosporine A in human platelets.

Transbilayer movement of phosphatidylserine and cytochrome c release

In washed human platelets, stimulated with a combination of collagen and thrombin, there was no release of cytochrome c into the cytosol even after incubation for five hours (figure 5, Panel B). Under similar conditions, ABT 737, induced dramatic release of cytochrome c in platelets (figure 5, Panel C). These studies show that despite the loss of mitochondrial

potential, there is no release of cytochrome c when platelets were stimulated with a combination of collagen and thrombin.

Activation of caspase 3 and phosphatidylserine exposure

Cytochrome c, released from the mitochondria into cytosol in many nucleated cells, activates caspase cascade. To determine whether ABT737-induced caspase 3 activation, we determined the cleavage of procaspase 3 to its activated fragments. ABT737 treatment is associated with significant caspase-3 cleavage (Figure 6, Panel A). Under similar conditions, collagen and thrombin stimulation did not induce caspase-3 activation. To determine whether caspase-3 activation leads to phosphatidylserine exposure, we examined the effect of z-DEVD-fmk, a caspase 3 inhibitor. z-DEVD-fmk suppressed ABT737 induced phosphatidylserine exposure but had no significant effects on collagen and thrombin induced phosphatidylserine exposure (Figure 6 Panel B). These results show that in ABT737-treated platelets caspase-3 activation is upstream of phosphatidylserine exposure. In contrast, caspase-3 does not play a role in phosphatidylserine exposure induced by the combination of collagen and thrombin.

Activation of ROCK1 and phosphatidylserine exposure

The Rho associated kinase I (ROCK1) controls actin-cytoskeleton assembly by phosphorylating various substrates such as myosin light chain kinase (24) and in platelets contributes to shape change (25). Y-27632, a specific inhibitor of ROCK (26), had no effect on collagen and thrombin-induced phosphatidylserine exposure, while it inhibited ABT737 induced phosphatidylserine exposure (Figure 6, Panel C). Consistent with these results, there was no proteolytic cleavage of ROCK1 with collagen and thrombin, while in ABT737 treated platelets, there was a distinct 30 kDa fragment, which was generated only after proteolytic cleavage of intact ROCK1 (Figure 6, Panel D).

Phosphatidylserine exposure in stored platelet

As previously shown (27), platelets stored for over five days showed increased expression of phosphatidylserine (figure 7 panels A and B). The activation of caspase-3 and ROCK1 activation in 6-day old platelets was examined by immunoblots using antibodies to caspase-3 and a monoclonal antibody specific to the carboxy-terminal fragment of ROCK1 generated by proteolytic cleavage of intact ROCK1. As shown in figure 7, Panels C there is clear evidence of caspase-3 activation of six day-old stored platelets. Furthermore, ROCK1 activation is also seen in stored platelets (figure 7, panel D) while in fresh platelets neither caspase-3 nor ROCK1 activation is detected.

Discussion

The mean life span of platelets in vivo is about 7-10 days. Recovery of platelets following transfusion is shorter and is influenced by platelet injury during collection and storage (28). Prolonged storage beyond five days further decreases the recovery (7). The relatively short 'shelf-life' of stored platelets leads to constant over collection and outdating. Several changes occur in platelets stored in vitro and among them, the exposure of phosphatidylserine may account for the short survival. To prolong the survival it is necessary to understand the molecular mechanism(s) leading to phosphatidylserine exposure as phosphatidylserine can be exposed due to platelet activation during storage or due to elaboration of apoptotic pathways (3,12,29). Our results suggest that in stored platelets, phosphatidylserine exposure is not due to activation but due to the elaboration of the mitochondrial apoptotic pathways.

At the end of the life span, circulating platelets are destroyed by the macrophages, primarily in the liver and spleen. Recent studies show the Bcl family of proteins is the major regulator of platelet survival (17). In senescent platelets, as the antiapoptotic Bcl-xL degrades the pro-apoptotic Bak is freed from inhibition and initiates the apoptotic pathways. Genetic ablation of Bcl-xL reduces platelet life span (16-18). Further, deletion of Bak corrects these defects in Bcl-xL deficient mice, and platelets from Bak-deficient mice live longer than normal (17). However, agonist-induced phosphatidylserine exposure is unaffected in Bak^{-/-}/Bax^{-/-} mouse (30). The importance of cytochrome c release in platelet turnover is also shown in a recent report of a family with a mutation in cytochrome c resulting in an enhanced apoptotic activity (31). Most remarkably, this family has no other phenotypic indication of abnormal apoptosis, except thrombocytopenia caused by dysregulated platelet formation.

Our results show that a similar mechanism is also active during in vitro storage of platelets. We have used ABT737, a BH3 mimetic to trigger Bak-mediated cytochrome c release, which results in the activation of caspase-3 and ROCK1 and the subsequent exposure of phosphatidylserine. Both caspase-3 and ROCK1 inhibitors decreases the phosphatidylserine exposure while CypD inhibition or deficiency has no effect. Both caspase-3 and ROCK1 activation is apparent during prolonged storage. This is in contrast to thrombin and collagen induced phosphatidylserine exposure which is dependent on CypD as shown previously (12,29) and independent of activation of caspase-3 and ROCK1.

In summary, our results suggest that release of cytochrome c from mitochondria and activation of caspases-3 and ROCK1 plays an essential role in phosphatidylserine exposure in stored platelets. Strategies to inhibit this process may prevent phosphatidylserine exposure and improve the recovery of stored platelets following transfusion.

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Figure 1. Effect of cyclosporine A on platelet phosphatidylserine expression

Washed human platelets ($2 \times 10^8/\text{ml}$) were incubated with cyclosporine A ($2 \mu\text{M}$) or buffer alone and stimulated with a combination of collagen ($5 \mu\text{g}/\text{ml}$) and thrombin ($1 \text{ unit}/\text{ml}$) in **Panel A** or ABT737 ($10 \mu\text{M}$) in **Panel B**. The phosphatidylserine expression was measured by FITC-lactadherin. **Panel C**. The percentages of platelets with fluorescence above background levels (as defined in the gate G in panels A and B) were plotted with the means and standard deviations of a representative triplicate measurements.

Figure 2. Effect of cyclophilin D deficiency on phosphatidylserine expression in murine platelets Washed platelets from control or CypD^{-/-} mice were treated with a combination of collagen and thrombin (**Panel A**) or ABT737 (**Panel B**) as in figure 1. The phosphatidylserine expression was measured by FITC-lactadherin. **Panel C**. The percentages of platelets with fluorescence above background levels (as defined in the gate G in panels A and B) were plotted with the means and standard deviations of a representative triplicate measurements.

Figure 3. Effect of cyclosporine A on $\Delta\Psi_m$

Washed human platelets ($2 \times 10^8/\text{ml}$) were incubated with buffer alone (**Panels A and B**) or cyclosporine A ($2 \mu\text{M}$) (**Panels C and D**) for 20 minutes at room temperature followed by activation with a combination of collagen and thrombin (Panels A and B) or ABT737 (**Panels B and D**). The samples were labeled with JC-1 and the loss of $\Delta\Psi_m$ was measured as a decrease in the red fluorescence. The gate P shows the platelet population with loss of $\Delta\Psi_m$. **Panel E**. The percentage of platelets with loss of $\Delta\Psi_m$ in gate P were plotted with means and standard deviations of a representative triplicate measurements.

Figure 4. Effect of cyclophilin D deficiency on $\Delta\Psi_m$ in murine platelets

Washed platelets ($2 \times 10^8/\text{ml}$), from CypD^{+/+} (control mice) in Panels A and C or CypD^{-/-} mice in Panels B and D, were stimulated a combination of collagen and thrombin (**Panels A and B**) or ABT737 (**Panels C and D**) and then labeled with JC-1. The loss of $\Delta\Psi_m$ was determined as in Figure 3. **Panel E** shows the means and standard deviations of a representative triplicate measurements of platelets with loss of $\Delta\Psi_m$ in gate P.

Figure 5. Release of cytochrome c

Panel A. Washed Jurkat cells (5×10^5) were incubated with buffer alone or etoposide ($10 \mu\text{M}$) for 5 h and release of cytochrome c to the cytosol was determined by SDS-PAGE of the extract followed by electrophoretic transfer to PVDF membranes and immunoblotting with a polyclonal antibody to cytochrome c. **Panels B and C.** Washed platelets ($2 \times 10^8/\text{ml}$) were incubated with a combination of collagen and thrombin (**Panel B**) or ABT737 (**Panel C**). Release of cytochrome c to the cytosol was determined as in Panel A.

Figure 6. Phosphatidylserine exposure and cleavage of caspase-3 and ROCK1

Panel A. Washed platelets (2×10^8 /ml) were treated with a combination of collagen and thrombin or ABT737 and the activation of caspase-3 were determined by immunoblotting following SDS-PAGE with a polyclonal antibody to caspase-3. Activation of caspase-3 results in the cleavage of intact molecule (35 kDa) into a 17 kDa fragment. **Panel B.** Washed platelets were incubated with caspase 3 inhibitor zDEVD fmk (20 μ M) for 20 minutes and stimulated with combination of collagen and thrombin or with ABT737 and the phosphatidylserine exposure was measured with fluorescein-lactadherin. Shown are the means and standard deviations of a triplicate measurement. **Panel C.** Washed platelets were incubated with ROCK1 inhibitor Y-27632 (20 μ M) for 20 minutes, stimulated with combination of collagen and thrombin or with ABT737 and the phosphatidylserine exposure was measured with fluorescein-lactadherin. Shown are the means and standard deviations of a triplicate measurement. **Panel D.** Washed platelets were treated with a combination of collagen and thrombin or ABT737. Platelet extracts (20 μ g/well) were subjected to SDS-PAGE and immunoblotted with an antibody specific to the carboxy-terminal fragment of ROCK1.

Figure 7. Phosphatidylserine exposure and caspase-3 and ROCK1 cleavage in stored human platelets

Panel A. Phosphatidylserine expression on fresh and 6-day old stored platelets was determined with FITC-lactadherin as in figure 1. **Panel B.** The percentages of platelets with fluorescence above background levels as defined in the gate G in figure 1. **Panel C and D.** Platelet extracts (20 µg/well) from fresh and 6 day old stored platelets were subjected SDS-PAGE, transferred to PVDF membranes and blotted with a polyclonal antibody to caspase-3 (**Panel C**) or monoclonal antibody to activation fragment 1 (**Panel D**). Lane 1, fresh platelets treated with ABT737; Lane 2, fresh platelets and Lanes 3-5, six-day old platelets from three different individuals.