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Cofilin-1-induced actin reorganization in stored platelets

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Abstract

BACKGROUND: During platelet storage, there are extensive changes in cytoskeleton and phosphatidylserine exposure. The intrinsic mitochondrial pathway of apoptosis, activated in stored platelets, is a major mediator these changes. Cofilin-1 is an effector of actin reorganization. We examined the effect of cofilin-1 deficiency on cytoskeleton and phosphatidylserine exposure during storage and following activation of apoptosis.

METHODS AND RESULTS: We assessed actin filaments by Alexa-647-phalloidin and phosphatidylserine exposure by fluorescein isothiocyanate–lactadherin by fluorescence microscopy. In fresh platelets, actin filaments are distributed in the subcortical region, and they do not express phosphatidylserine in the outer surface. In stored platelets, there is retraction of actin filaments from the subcortical region with increased phosphatidylserine expression. These changes are seen in 20% of platelets of 6 days old and increases further with storage. Treatment with ABT-737, which activates the mitochondrial apoptosis, induces similar cytoskeletal changes in actin filaments with increased phosphatidylserine. Cofilin-1 is activated in stored platelets as well as in ABT-737 treated platelets by dephosphorylation. In cofilin-1 deficient murine platelets actin filaments are abnormal and ABT-737 induces less phosphatidylserine. Despite these changes in vitro, platelet survival of cofilin-1 deficient platelets in mice was not significantly different from their wild-type controls.

CONCLUSION: These results show that cofilin-1 plays a role in apoptosis-induced actin rearrangement and phosphatidylserine exposure during storage. Despite the defects in platelet cytoskeleton and phosphatidylserine exposure in cofilin-1–deficient platelets, the in vivo life span of platelets is similar to littermate controls, indicating multiple redundant pathways for the clearance of platelets in vivo.

In resting platelets, as in most eukaryotic cells, anionic phospholipids such as phosphatidylserine are localized exclusively to the inner cytoplasmic leaflet of the membrane bilayer.^{1,2} During platelet activation, there is transbilayer movement of

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

phosphatidylserine from inner to outer layer. In addition to their procoagulant activity, exposure of phosphatidylserine on the cell surface is also a well-known tag for clearance of cells from the circulation by the macrophage phosphatidylserine receptors.³ A senescence-induced mechanism for phosphatidylserine exposures, dependent on the mitochondrial pathway of apoptosis, has been described in platelets.^{4,5} The BCL family of proteins mediates this pathway.⁶ The BCL family has three functional groups: antiapoptotic proteins (e.g., Bcl-2, Bcl-1xL), proapoptotic effectors (e.g., Bax, Bak), and proapoptotic activators (e.g., Bid, Bad).⁷ During mitochondrial pathway of apoptosis, proapoptotic Bax and Bak translocate to the mitochondrial membrane and induce the permeabilization of the mitochondrial membrane and release of cytochrome c. Bcl-xL prevents apoptosis by sequestering Bax. Bcl-xL deficiency causes thrombocytopenia by shortening platelet life span in vivo.⁶ Deletion of Bak (and to a lesser extent Bax) can reverse thrombocytopenia induced by Bcl-xL deficiency by extending the platelet life span in vivo.⁶ Relative proportions of Bcl-xL and Bak constitute the major components of a “molecular clock” that determines platelet life span. As platelets age, degradation of Bcl-xL triggers Bak-mediated phosphatidylserine exposure and clearance from the circulation in mice.⁸ ABT-737, a synthetic Bcl-xL antagonist, induces apoptotic changes and phosphatidylserine exposure by preventing the antiapoptotic effect of Bcl-xL,⁹ thus mimicking the mitochondrial pathway of apoptosis.⁶

In resting platelets, actin filaments are at the periphery, as cortical actin in the marginal zone in close proximity to the membrane and provide a framework for platelet shape and structure.¹⁰ Activation of apoptosis during storage induces reorganization of actin filaments and phosphatidylserine exposure.^{11–14} Reorganization of actin filaments is necessary for the transbilayer movement of phosphatidylserine. Cofilin-1 is a major mediator of actin reorganization.¹⁵ Western blot analysis of stored platelets revealed activation of cofilin-1 by dephosphorylation, indicating its potential role in cytoskeletal reorganization and in mediating the phosphatidylserine exposure. Therefore, we examined the effect of platelet cofilin-1 deficiency on cytoskeletal integrity and the phosphatidylserine exposure in stored platelets.

EXPERIMENTAL PROCEDURES

Reagents

Anticofilin-1 antibody was from Cell Signaling Technology. Antiphospho-cofilin-1 (ser 3) antibody and Alexa-488 conjugated antiactin antibody were from Santa Cruz Biotechnology. Alexa-647-phalloidin was purchased from Life Technologies. Prostaglandin E1, apyrase, and sulfo-NHS-LC biotin were from ThermoFisher Scientific. ABT-737 was from Selleck chemicals. Phycoerythrin (PE)-conjugated streptavidin is from Invitrogen. Fluorescein isothiocyanate (FITC)-lactadherin was generated as described previously.¹⁶ All animals were treated in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Isolation of platelets

Outdated 5- or 6-day-old platelet concentrates were obtained from American Red Cross. Platelet-rich plasma was also obtained by centrifugation of blood collected in 1/10 volume of citrate (3.8% trisodium citrate, pH 6.5) at 180 g for 10 minutes from human volunteers after an informed written consent under a protocol approved by the Institutional Review Board of Baylor College of Medicine. Platelets were isolated from the platelet-rich plasma as previously described¹⁷ and suspended in modified Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 5 mM Hepes, 1 mM MgCl₂, 3 mM NaH₂PO₄, and 5.5 mM dextrose, pH 7.4) containing 0.5% bovine serum albumin. To isolate mouse platelets, blood was drawn from the inferior vena cava into 1/10 volume of citrate from 4-month-old or older mice under isoflurane anesthesia. Blood was diluted with an equal volume of modified Tyrode's buffer and platelet-rich plasma was obtained by centrifugation at 80 g for 10 minutes. Prostaglandin E1 (1 μ M) and apyrase (0.5 unit) were added, and platelets were sedimented by centrifugation at 1000 g for 10 minutes, washed once in Tyrode's buffer containing apyrase and suspended in Tyrode's buffer. Platelets were counted in a coulter counter and adjusted to 2×10^8 platelets/mL.

Immunofluorescence microscopy

Resting, 6-day-old, and ABT-737 treated platelets were immobilized on a polylysine-coated cover slip. To analyze the effect of activation, platelets were allowed to adhere to a collagen-coated surface as described before.¹⁸ The immobilized platelets were gently washed and incubated with FITC-lactadherin, which binds to the surface exposed phosphatidylserine.^{16,19} The platelets were then fixed in 4% formaldehyde and incubated with Alexa-647-phalloidin. Images are taken in a microscope (DeltaVision OMX, GE Healthcare) at 1000 $\times$ magnification and deconvolved with computer software (softWoRX 6.5.2) that applies a 3D iterative constrained deconvolution algorithm.

Detection of phosphatidylserine or actin exposure by flow cytometry

Expression of phosphatidylserine and actin were quantified on a flow cytometer (Coulter FCC 500, Beckman-Coulter) using the CXP software as described previously.²⁰ The gates for platelets were set using a fluorescein-conjugated anti-CD42b antibody and light scatter, and fluorescence channels were set at a logarithmic gain. We used FITC-lactadherin, as it preferentially binds to phosphatidylserine in regions of sharp curvature in apoptotic blebs.^{16,21} Platelets were incubated with FITC-lactadherin (5 μ g/mL) only¹⁶ for 30 minutes at room temperature for phosphatidylserine expression. For actin exposure, platelets were incubated with Alexa 488-conjugated antiactin antibodies (5 μ g/mL) for 30 minutes at room temperature as described before.¹⁶

Cofilin-1-deficient mice

We obtained breeder mice (strain Cfl1tm1a [Knockout Mouse Project (KOMP)]) harboring a modified Cfl1<tm1a> allele with LACZ-LoxP-neomycin resistance cassette flanked by flippase recognition target sites and the second exon flanked by loxP sites from Jackson Laboratories. The mice were crossed with flippase deleter mice and the homozygous floxed mice were crossed with platelet factor 4 (PF4) Cre mice to obtain the platelet-specific

cofilin-1 knockout mice. Polymerase chain reaction (PCR) amplification using primers 5'-ccactcatggaagcaggaccagtaaggacc-3' and 5'-actgtatttagcccacatgtggaaaggggc-3') gave 318 bp for wildtype allele and 380 bp for loxed allele. The presence of PF4 promoter was confirmed by PCR. The null allele was further confirmed by immunoblot of cofilin-1 in platelet lysate. Cofilin-1-deficient mice had normal platelet count $1051 \pm 135 \times 10^9/\text{mL}$ compared to $974 \pm 207 \times 10^9/\text{mL}$ platelets in cofilin-1^{flox/flox} mice. The platelets are slightly bigger in cofilin-1-deficient mice 6.9 ± 0.2 fl compared to 5.68 ± 0.58 fl in cofilin-1^{flox/flox} mice.

Platelet survival in vivo

The circulating platelets in the bloodstream of mouse was biotinylated (at $t = 0$) by tail vein injection of 100 μL of 15 mg/mL sulfosuccinimidyl 6-(biotinamido) Hexanoate (sulfo-NHS-LC-biotin) as described by before.^{22,23} For flow cytometric analysis, approximately 5 μL of blood is obtained from the tail vein or at various intervals. The biotinylated platelet fraction is detected in a flow cytometry by incubating with 2 μg per mL of PE-conjugated streptavidin, and fluorescein-labeled CD41.

Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD) of three biologic replicates with different donors for each experiments. Comparisons between individual groups were performed using the Student t test with paired and unpaired samples. A p value of 0.05 or below was considered statistically significant.

RESULTS

Distribution of F-actin and exposure of phosphatidylserine in stored platelets

In resting platelets, F-actin (Alexa-688-phalloidin) is distributed in the subcortical region as a thin submembranous network, consistent with previously described cortical actin network (red fluorescence in Fig. 1A). In 6-day-old platelets, the actin filaments have retracted from the subcortical region toward the center of the platelet (compare Fig. 1A and B). These changes are seen in up to 20% of 6-day-old platelets in five separate donors. These changes are even more marked in platelets treated with ABT-737 (10 μM), which activates the mitochondrial pathway of apoptosis. Almost all platelets show the dramatic morphological changes with complete centripetal retraction of the cortical actin (Fig. 1C), similar to stored platelets (Fig. 1B).

Resting platelets display minimal phosphatidylserine on the surface when probed with FITC-lactadherin (minimal green fluorescence, Fig. 1A, E, and F). In 6-day-old stored platelets, there is a significant increase in platelet surface phosphatidylserine (Fig. 1B, E, and F). The topographic expression of phosphatidylserine in stored platelets is different from that seen during platelet activation (Fig. 1B and D). As previously described, activated platelets form balloon-like phosphatidylserine-positive membrane structures from the center and break up into procoagulant microvesicles.²⁴ In contrast, the phosphatidylserine in stored platelets is seen in the peripheral rim, similar to phosphatidylserine exposure during apoptosis in nucleated cells.¹⁹ ABT-737 recapitulates the morphological appearance of

storage-induced phosphatidylserine exposure (in addition to the cytoskeletal changes) consistent with the concept that apoptotic pathway initiates phosphatidylserine exposure in stored platelets.

Exteriorization of actin in stored platelets

We also observed that actin, the major cytoplasmic protein in platelets, is also detectable on the surface of stored platelets, when analyzed by flow cytometry. While fresh platelets express small basal level of actin on their surface, an increased amount of actin is seen on the outer surface of stored platelets (Fig. 1G and H), suggesting that storage induces actin exposure on platelet surface. When platelets are treated with ABT-737, a similar increase in cell surface actin was seen, suggesting that the mitochondrial apoptotic pathway may be responsible for the actin exposure (Fig. 1I and J).

Activation of cofilin-1 by dephosphorylation in stored platelets

Because cofilin-1 is a major reorganizer of platelet cytoskeleton and we have previously noticed that cofilin-1 activation is associated with increased phosphatidylserine exposure in ROCK1-deficient mice,²⁰ we examined the phosphorylation status of cofilin-1 in stored platelets by a phosphospecific cofilin antibody. As shown in Fig. 2, there was decreased phosphorylation of cofilin-1 in stored platelets. These results are the mean and SD of four independent analyses of four separate platelet preparations. Under similar conditions, there is no significant alteration in the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; loading control).

Actin network and phosphatidylserine exposure in cofilin-1-deficient mouse platelets

We generated platelet specific cofilin-1-deficient mice by breeding the nonexpressive allele construct from KOMP as shown in Fig. 3A–C. The null allele was confirmed by the absence of cofilin-1 expression in platelet lysate by immunoblot (Fig. 3D). Cofilin-1-deficient mice had normal platelet count of $1051 \pm 135 \times 10^9/\text{mL}$ compared to $974 \pm 207 \times 10^9/\text{mL}$ platelets in cofilin-1^{flox/flox} mice. The platelets are slightly bigger in cofilin-1-deficient mice, 6.9 ± 0.2 fl compared to 5.68 ± 0.58 fl in cofilin-1^{flox/flox} mice ($p = .014$). We next examined the F-actin network in these mice. In resting cofilin-1-deficient platelets, the F-actins are aberrantly localized. While in normal platelets F-actin is present in the submembranous zone, in cofilin-1-deficient mouse platelets F-actin is distributed diffusely throughout the cytoplasm without preferential localization to the submembranous zone (Fig. 4A and B). Following ABT-737 treatment, phosphatidylserine exposure (green lactadherin fluorescence) is markedly decreased in cofilin-1-deficient platelets compared to controls (Fig. 4C and D and Fig. 5A–C). In control platelets, actin filaments retract from the subcortical region toward the center of the platelet as seen in human platelets. In contrast, in cofilin-1-deficient mouse platelets, there is poor centripetal retraction of actin. Furthermore, there is diminished cell surface expression of actin in cofilin-1-deficient mouse platelets (Fig. 5D–F). These results show that cofilin-1 plays a significant role in actin reorganization and phosphatidylserine exposure in mitochondrial pathway-mediated apoptotic changes.

Platelet life span in cofilin-1-deficient mouse platelets

Sulfo-NHS-LC-biotin is administered intravenously to label the circulating platelets, and biotin-positive platelets were determined by flow cytometry and followed subsequently.²³ As shown in Fig. 6, the life span of cofilin-1-deficient platelets were similar to control mice platelets. The curves were mostly superimposable except there was a small divergence with a higher percentage in cofilin-1-deficient platelets that was not statistically significant. These results are consistent with the notion that there are multiple redundant pathways for the clearance of platelets in vivo.²⁵

DISCUSSION

Platelets lack the machinery to undergo the type of programmed cell death of nucleated cells but nevertheless appear to have similar changes in the cytoplasm consisting of cytoskeletal reorganization and surface exposure of phosphatidylserine during storage.^{11,26} The mitochondrial apoptotic pathway has been shown to be activated in stored platelets, and the Bcl-2 family of proteins regulates apoptosis by permeabilizing the mitochondria during storage.^{12,27} Our results show that in stored platelets, there is extensive reorganization of actin filaments with centripetal retraction of actin similar to the changes described in nucleated cells during apoptosis.²⁸ These changes can be induced by ABT-737, which induces the mitochondrial pathway of apoptosis in platelets.²⁹

Actin is a major protein in platelets, and it exists as both soluble G-actin and as fibrous F-actin.¹⁰ In resting platelets, the cortical F-actin network is present in the submembranous zone, with actin filaments radiating outward to attach to the membrane via the actin-binding proteins. A number of actin-binding proteins, including vimentin, spectrin, gelsolin, and vinculin, are present in this submembranous zone in close proximity with the inner leaflet of the membrane bilayer; they interact with anionic phospholipids (phosphatidylserine and/or phosphatidylinositol).^{10,30–33} In addition to these indirect interactions, F-actin can interact directly with phosphatidylserine, which constitutes 20% of phospholipids in the inner leaflet of membranes.³² During storage, there is reorganization of actin filaments.^{11,12} The centripetally contracting cortical actin ring detaches from the membrane. These changes in membrane cytoskeletal interaction may release the constraints on the plasma membrane bilayer, allowing the transbilayer movement of phosphatidylserine.

We also noted apoptosis-induced actin reorganization exteriorizes actin to the membrane. The presence of exofacial actin on platelets has been recognized as early as 1980³⁴). The identity of actin on the platelet surface was established by immunological probes and by binding to deoxyribonuclease I. The mechanism of actin exteriorization and its pathophysiological significance is not known. A variety of deleterious effects on hemostasis has been described, including vascular obstruction and inhibition of fibrinolysis due to extracellular actin.³⁵ On the other hand, extracellular actin may have an antimicrobial effect by inhibiting proteases from microorganisms.³⁶

Cofilin-1, a major actin-binding factor in platelets, is required for severing actin filaments during reorganization of actin cytoskeleton. We noticed that cofilin-1 is activated in stored platelets due to elaboration of apoptotic pathways. To define the role of cofilin-1 in storage-

induced lesion in platelets, we generated cofilin-1–deficient mice. As expected, cofilin-1–deficient mouse platelets have fewer marked cytoskeletal changes due to decreased actin reorganization upon induction of apoptosis and decreased exposure of phosphatidylserine and actin. Caspases, which are activated in stored platelets, may modulate the major phosphatases, including slingshot phosphatase.³⁷ Slingshot phosphatase is known to activate cofilin-1 by dephosphorylating serine 3.³⁸

What determines the life span of platelets in normal steady state in vivo is not well understood. It has been proposed that platelet life span is determined by the “injuries” due to activation or by senescence-induced changes according to “the multiple-hit model.”²⁵ As platelets age in the circulation, they become denser. The older and denser fractions of platelets express phosphatidylserine at higher density. However, P-selectin expression (which depends on platelet activation) in these senescent platelets is similar to younger, less dense platelets. These findings suggest phosphatidylserine exposure in stored platelets is induced by the apoptotic pathway rather than the activation-induced pathway. Surprisingly, despite the defect in platelet cytoskeletal architecture and in apoptosis-induced phosphatidylserine exposure in cofilin-1–deficient platelets, the in vivo life span of platelets is similar to littermate controls. These observations show that there are multiple redundant pathways for platelet clearance in vivo and phosphatidylserine exposure may have only a limited role.²⁵

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ABBREVIATIONS:

FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3–phosphate dehydrogenase
KOMP	Knockout Mouse Project
PCR	polymerase chain reaction
PE	phycoerythrin
PF4	platelet factor 4
sulfo-NHS-LC-biotin	sulfosuccinimidyl 6-(biotinamido) Hexanoate

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Fig. 1.

Actin network and phosphatidylserine exposure in platelets. Platelets immobilized on polylysine- (A-C) or collagen-coated (D) coverslips, were gently washed and incubated with FITC-lactadherin (green fluorescence), which binds to the surface exposed phosphatidylserine. The platelets were washed, fixed, and incubated with Alexa-647-phalloidin to visualize F-actin (red fluorescence). Images were taken in a DeltaVision OMX microscope (GE Healthcare) at 1000× magnification and deconvolved using SoftWoRX 6.5.2 that applies a 3D iterative constrained deconvolution algorithm. (E-H) Washed

platelets were incubated with FITC-lactadherin (E and F) or Alexa-488-antiactin (G-J) antibodies. (I and J) Untreated resting and ABT-737 treated platelets were incubated with Alexa-488-antiactin. Fluorescence above background of resting unstimulated platelets, as defined by the gate G1 in panels E, G and I were considered positive. The bar diagrams represent the mean and standard deviation of three biologic replicates. * $p < 0.001$. [Color figure can be viewed at wileyonlinelibrary.com]

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Fig. 2. Cofilin-1 activation in stored platelets. (A) Lysates from fresh and 6-day-old stored platelets were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane and immunoblotted with antibodies specific to cofilin-1. The blots were stripped and reprobed with antibodies specific for phosphocofilin-1. The antibodies to GAPDH are shown for loading control. (B) The relative intensities of phosphocofilin-1 in fresh platelets were considered as 1 for comparison. The bars represent the mean and SD of four separate biologic replicate experiments. * $p < 0.05$.

Fig. 3. Generation of platelet-specific cofilin-1 deficiency. (A) Engineered allele of cofilin-1 from KOMP. The nonexpressive form is converted to a conditional allele via flippase recombination. Cross breeding with PF4-Cre generates platelet-specific cofilin-1 knockout. (B) PCR with appropriate primers gives a 180-bp fragment for the wild-type allele and 380 bp with the flippase recognition target deleted allele. (C) PCR of the *Cfl1*^{flx/flx} allele gives 380 bp for the homozygote, while the heterozygote gives 380-bp and 318-bp fragments. (D) Immunoblot of platelets from *Cfl1*^{fox/fox} and *Cof1*^{PF4Cre-flx/flx} mice shows the absence of cofilin-1.

Fig. 4. Actin organization and phosphatidylserine exposure in cofilin-1-deficient mice platelets. Untreated resting (A and B) and ABT-737 treated (panels C and D) platelets from (control) cofilin-1^{flox/flox} mice (A and C) and platelet-specific cofilin-1 deficient mice (B and D) were immobilized on polylysine-coated plates, gently washed, incubated with FITC-lactadherin (green fluorescence) for phosphatidylserine expression and Alexa-647-phalloidin (red fluorescence), and analyzed as in Fig. 1. [Color figure can be viewed at wileyonlinelibrary.com]

Fig. 5. Phosphatidylserine and actin expression in coflin-1-deficient mouse platelets. Platelets from control (cofilin-1^{flox/flox}) (A and D) and platelet-specific cofilin-1-deficient mice (B and E) were treated with ABT-737 (10 μ M) and the exposure of phosphatidylserine (A and B) or actin (D and E) were measured as in Fig. 1. In C and F, the bar diagram represents the mean and SD of three biologic replicates. * $p < 0.01$.

Fig. 6.

Platelets survival in platelet-specific cofilin-1-deficient mice. Cofilin-1^{flox/flox} control and platelet-specific cofilin-1-deficient mice were intravenously infused with biotin to label platelets in vivo. On subsequent days, mice were bled and the percentages of labeled platelets were determined by flow cytometry with PE-streptavidin. The Day 0 was determined by extrapolation of Day 2-4 labeled platelets and considered 100% for comparison.