

The Clearance Mechanism of Chilled Blood Platelets

Karin M. Hoffmeister,^{1,*} Thomas W. Felbinger,^{3,4}
Hervé Falet,¹ Cécile V. Denis,^{3,4}
Wolfgang Bergmeier,^{3,4} Tanya N. Mayadas,²
Ulrich H. von Andrian,^{3,4} Denisa D. Wagner,^{3,4}
Thomas P. Stossel,¹ and John H. Hartwig¹

¹Division of Hematology

Department of Medicine

²Department of Pathology

Brigham and Women's Hospital
Boston, Massachusetts 02115

³The Center for Blood Research

⁴Department of Pathology

Harvard Medical School

Boston, Massachusetts 02115

Summary

Platelet transfusion is a very common lifesaving medical procedure. Not widely known is the fact that platelets, unlike other blood cells, rapidly leave the circulation if refrigerated prior to transfusion. This peculiarity requires blood services to store platelets at room temperature, limiting platelet supplies for clinical needs. Here, we describe the mechanism of this clearance system, a longstanding mystery. Chilling platelets clusters their von Willebrand (vWf) receptors, eliciting recognition of mouse and human platelets by hepatic macrophage complement type 3 (CR3) receptors. CR3-expressing but not CR3-deficient mice exposed to cold rapidly decrease platelet counts. Cooling primes platelets for activation. We propose that platelets are thermosensors, primed at peripheral sites where most injuries occurred throughout evolution. Clearance prevents pathologic thrombosis by primed platelets. Chilled platelets bind vWf and function normally *in vitro* and *ex vivo* after transfusion into CR3-deficient mice. Therefore, GPIb modification might permit cold platelet storage.

Introduction

Platelets are anucleate bone marrow-derived blood cells that protect injured mammals from blood loss by adhering to sites of vascular injury and by promoting the formation of plasma fibrin clots. Circulating platelets are smooth-surfaced discs that react to thrombin and other mediators associated with vascular damage by adhering to endothelial and subendothelial surfaces. They utilize actin cytoskeletal remodeling reactions to acquire complex shapes that facilitate adhesion and interactions with fibrin polymers formed at the injury sites.

Over 40 years ago, investigators noted that discoid platelets undergo similar extensive shape changes when exposed to temperatures < 15°C (Zucker and Borrelli, 1954), and more subtle morphological alterations are detectable even at higher temperatures (Maurer-

Spurej et al., 2001). Based on studies linking signaling to the mechanisms leading to platelet shape changes induced by agonists (Hartwig et al., 1995), we predicted that chilling, by inhibiting calcium extrusion, could elevate calcium levels to a degree consistent with the activation of the F actin severing and barbed end capping protein gelsolin. We also reasoned that a membrane lipid phase transition at low temperatures would cluster phosphoinositides. Phosphoinositide clustering uncaps F actin barbed ends to create nucleation sites for filament elongation (Janmey and Stossel, 1989). We produced experimental evidence for both mechanisms, documenting gelsolin activation, actin filament barbed end uncapping, and actin assembly in cooled platelets (Hoffmeister et al., 2001; Winokur and Hartwig, 1995). Others have reported myosin activation in chilled platelets, consonant with increased free intracellular calcium levels (Kawakami et al., 2001) and spectroscopic changes in chilled platelets interpreted as a membrane phase transition (Tablin et al., 1996). This information suggested a method for preserving the discoid shape of chilled platelets, using a cell-permeable calcium chelator to blunt the calcium rise and cytochalasin B to prevent barbed end actin assembly, and these compounds keep human platelets discoid in the cold (Winokur and Hartwig, 1995).

Since most cells round up in the cold, the response of platelets to chilling is an anomaly, and its physiological purpose unknown. Moreover, the effects of cooling platelets are of more than academic importance. Humans depleted of platelets by bone marrow failure suffer from life-threatening spontaneous bleeding, and less severe deficiencies of platelets contribute to bleeding complications following trauma or surgery. A major advance in medical care half a century ago was the development of platelet transfusions to correct such platelet deficiencies, and over four million platelet transfusions take place annually in the United States alone (Jacobs et al., 2001). Platelets, however, unlike all other transplantable tissues, do not tolerate refrigeration, because they disappear rapidly from the circulation of recipients if subjected to even very short periods of chilling, and the cooling effect that shortens platelet survival is irreversible (Becker et al., 1973; Berger et al., 1998). The resulting need to keep these cells at room temperature (RT) prior to transfusion has imposed a unique set of costly and complex logistical requirements for platelet storage. The major practical problem with RT storage, leading to its short (5 day) limitation, is the risk of bacterial infection. Bacterial contamination of blood components is currently the most frequent complication of their use exceeding by far that of viral agents (Engelfriet et al., 2000). The short shelf life of procured platelets coupled to fluctuating demands for platelet transfusions mean that many units of procured platelets cannot be used because of outdated. This wastage rate may be as high as 50% and may cost the American health care system over a billion dollars annually.

Evidence that a discoid shape was the best predictor of viability for platelets stored at room temperature (Schlichter and Harker, 1976) led to the conclusion that

*Correspondence: khoffmeister@rics.bwh.harvard.edu

the cold-induced shape change per se was responsible for the rapid clearance of chilled platelets. The belief is that irregularly shaped platelets, deformed by cooling, become entrapped in the microcirculation. We report here, however, that even discoid platelets clear rapidly from the circulation of mice. This information and other evidence that misshapen platelets can circulate fairly normally (Berger et al., 1998; Michelson et al., 1996) led us to seek other mechanisms responsible for the rapid clearance of chilled platelets.

We have shown that cooling platelets rearranges the surface configuration of glycoprotein Ib (GPIb), a major platelet adhesion receptor that binds activated forms of von Willebrand factor (vWf) deposited at sites of vascular injury. In response to cooling, the GPIb α subunit of the vWf receptor complex clusters and becomes targeted for recognition by CR3 highly expressed on liver macrophages, leading to platelet phagocytosis and clearance. Cooling, however, does not grossly impair the interaction between GPIb and activated vWf, implying that the hemostatic and clearance functions of GPIb are distinct.

As the temperature falls below 37°C, platelets become more susceptible to activation by thrombotic stimuli, a phenomenon we term "priming" (Faraday and Rosenfeld, 1998; Hoffmeister et al., 2001). Such priming may be an adaptation to limit bleeding at lower temperatures of body surfaces where most injuries occurred throughout mammalian evolution. We propose that the hepatic clearance system's purpose is to remove repeatedly primed platelets. Consistent with this theory, platelet counts of WT but not of CR3 null mice exposed to cold temperatures rapidly fall. Since the conformational changes in GPIb α that promote this clearance do not affect GPIb α 's hemostatically important binding to vWf, selective modification of GPIb α may accommodate cold storage of platelets for transfusion.

Results

The Clearance of Chilled Mouse Platelets Occurs Predominantly in the Liver and Is Independent of Platelet Shape

Mouse platelets kept at RT and infused into syngeneic mice disappear at a fairly constant rate over time for about 80 hr (Figure 1A). In contrast, approximately two-thirds of platelets chilled at ice bath temperature (Cold) before injection rapidly disappear from the circulation as observed previously (Becker et al., 1973; Berger et al., 1998). Chilled platelets treated with the cell-permeable calcium chelator EGTA-AM and the actin filament barbed end capping agent cytochalasin B (Cold + CytoB/EGTA) to preserve their discoid shape (Winokur and Hartwig, 1995), left the circulation as rapidly as chilled, untreated platelets despite the fact that these chemically treated platelets were fully functional as determined by thrombin-, ADP- or collagen related peptide-(CRP) induced aggregation *in vitro* (Figure 1B). The recoveries of infused platelets immediately following transfusion were 60%–70%, and the kinetics of disappearance were indistinguishable whether we used ¹¹¹Indium or CMFDA to label platelets. The relative survival rates of RT and chilled mouse platelets resemble the values reported previously for mice (Berger et al., 1998) and human plate-

lets (Becker et al., 1973). Also consistent with the earlier reports, we observed that the relatively small number of chilled transfused platelets remaining in the circulation after the initial clearance phase disappear at the same rate, or even somewhat slower, than RT platelets. We have no definite explanation for these findings, but possibilities are that the clearance mechanism becomes saturated or else that a small number of platelets are resistant to cold modification.

Figure 1C shows that the organ destinations of RT and chilled mouse platelets differ. Whereas RT platelets primarily end up in the spleen, the liver is the major residence of chilled platelets removed from the circulation. A greater fraction of radionucleotide detected in the kidneys of animals receiving ¹¹¹Indium-labeled chilled compared with RT platelets at 24 hr may reflect the degradation of chilled platelets and delivery of free radionuclide to the urinary system. One hour after injection, the organ distribution of CMFDA- and ¹¹¹Indium-labeled platelets was comparable. In both cases, 60%–90% of the chilled platelets were deposited in the liver, 10%–20% in the spleen, and 10%–25% in the lung. In contrast, 25% of the infused RT platelets distributed equally among the liver, spleen, and lung.

Chilled Mouse Platelets Colocalize with Liver Kupffer Cells

The clearance of chilled and rewarmed platelets by the liver and the evidence for platelet degradation is consistent with their ingestion by Kupffer cells, the major phagocytic scavenger cells of the liver. Figure 1D shows the location of Kupffer cells and adherent chilled CMFDA-labeled platelets in a representative confocal-micrograph of a mouse liver section 1 hr after transfusion. Kupffer cells were visualized by the injection of microspheres marked with Nile-red. Colocalization of chilled platelets and macrophages is shown in yellow in a merged micrograph of both fluorescence emissions. The chilled platelets localize with macrophages preferentially in the periportal and midzonal domains of liver lobules, sites rich in sinusoidal macrophages (Bioulac-Sage et al., 1996).

CR3^{-/-} Mice Do Not Rapidly Clear Chilled Platelets

CR3 ($\alpha_M\beta_2$ integrin; CD11b/CD18; Mac-1) is a major mediator of antibody-independent clearance by hepatic macrophages. Figure 2A shows that chilled platelets circulate in CR3^{-/-} mice with the same kinetics as RT platelets, although the clearance of platelets is faster in the CR3^{-/-} mouse compared to that in wild-type (WT) mice (Figure 1A). Chilled and rewarmed platelets also clear rapidly from complement factor 3^{-/-} mice (Figure 2C), missing a major opsonin that promotes phagocytosis and clearance via CR3, and from vWf^{-/-} mice (Figure 2B).

Chilled Mouse Platelets Adhere Tightly to Kupffer Cells *In Vivo*

The ratio between chilled and RT stored adherent platelets infused simultaneously was determined. Figure 3 shows that both chilled and RT platelets attach to sinusoidal regions with high Kupffer cell density (Figure 3A and 3B), but that 2.5 to 4 times more chilled platelets

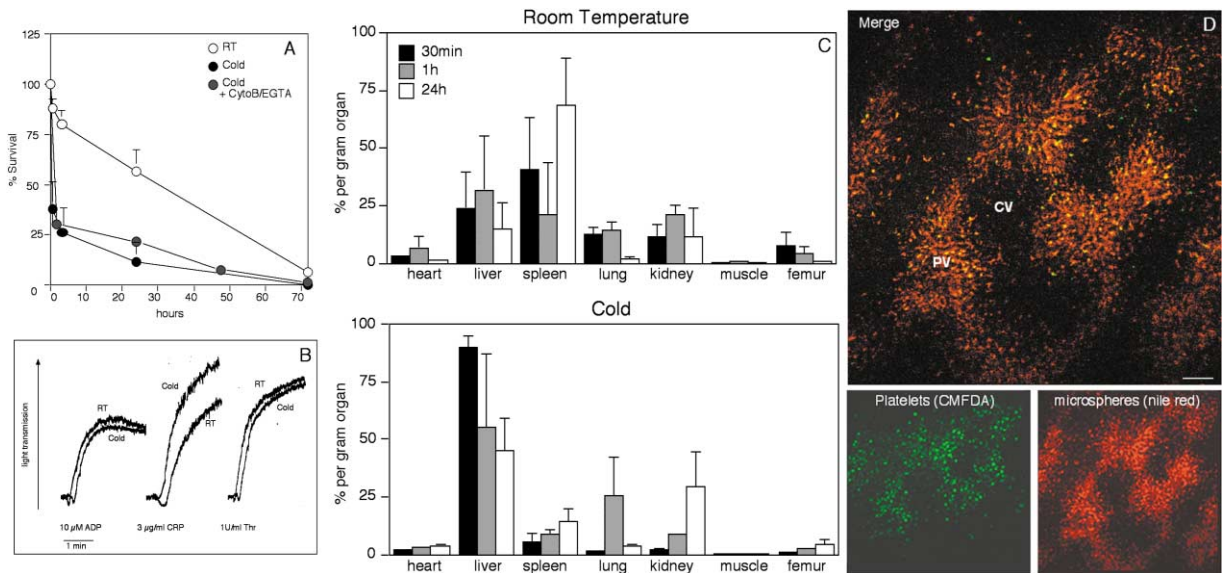


Figure 1. Clearance and Function of RT and Chilled Murine Platelets

(A) Circulation time in mice of RT platelets and of platelets chilled in the presence or absence of EGTA-AM and Cytochalasin B. The curves depict the survival of CMFDA-labeled, RT platelets, platelets chilled at ice bath temperature (Cold), and rewarmed to RT before injection and chilled and platelets treated with EGTA-AM and cytochalasin B (Cold + CytoB/EGTA) to preserve their discoid shape. Each curve represents the mean \pm SD of 6 mice. Identical clearance patterns were observed with 111 Indium-labeled platelets.

(B) Chilled mouse platelets aggregate normally in vitro. Washed, chilled-rewarmed (Cold), or RT WT platelets were stimulated by the addition of the indicated agonists at 37°C. Aggregation responses of chilled platelets treated with EGTA-AM and cytochalasin B were identical to untreated chilled platelets.

(C) Cold-induced clearance occurs predominantly in the liver of mice. The liver is the primary clearance organ of chilled platelets, containing 60%–90% of injected platelets. In contrast, RT platelets are cleared more slowly in the spleen. 111 Indium-labeled platelets were injected into syngeneic mice and tissues were harvested at 0.5, 1, and 24 hr. Data are expressed per gram of tissue. Each bar depicts the mean values of 4 animals analyzed \pm SD.

(D) Chilled mouse platelets colocalize with hepatic sinusoidal macrophages (Kupffer cells). This representative confocal-micrograph shows the hepatic distribution of CMFDA-labeled, chilled-rewarmed platelets (green) after 1 hr of transfusion, which preferentially accumulate in periportal and midzonal fields of liver lobules. Kupffer cells were visualized after injection of Nile red-labeled microspheres. Colocalization of chilled platelets and macrophages is in yellow. The lobule organization is indicated (CV: central vein; PV: portal vein, bar: 100 μ M).

attach to Kupffer cells in the WT mouse than RT platelets (Figure 3C). In contrast, the number of platelets adhering to Kupffer cells in $CR3^{-/-}$ mice was independent of chilling or RT exposure (Figure 3C).

Chilled Mouse Platelets Lacking GPIb α Circulate Normally

Because GPIb α , a component of the GPIb-IX-V receptor complex for vWf, can bind CR3 under certain conditions

in vitro (Simon et al., 2000), we investigated GPIb α as a possible counter receptor on chilled platelets for CR3. We stripped the extracellular domain of GPIb α from mouse platelets with O-sialoglycoprotein endopeptidase (Bergmeier et al., 2001) (Figure 4A, inset) and examined their survival in mice following RT or cold incubation. Figure 4A shows that chilled platelets no longer exhibit rapid clearance after removal of GPIb α . In fact, GPIb α -depleted RT-treated platelets have slightly elon-

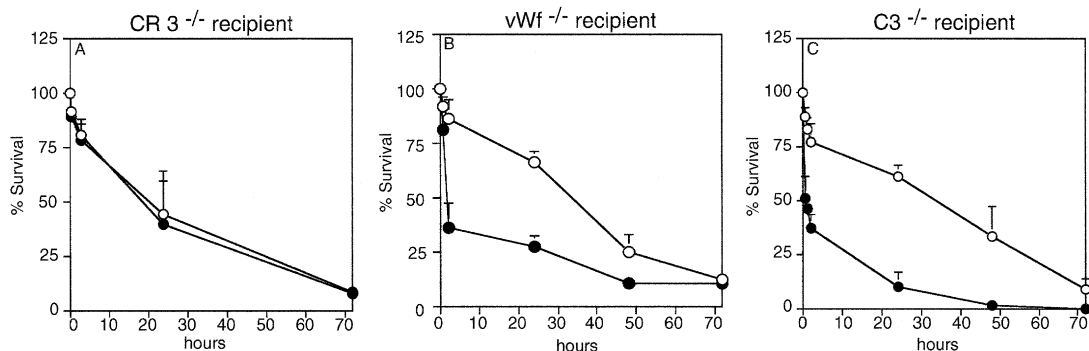


Figure 2. Chilled Platelets Circulate Normally in $CR3^{-/-}$ Mice, but Not in Complement 3 (C3) or $vWf^{-/-}$ Mice

CMFDA-labeled chilled-rewarmed (Cold, filled circles) and RT (open circles) WT platelets were transfused into six each of syngeneic $CR3^{-/-}$ (A), $vWf^{-/-}$ (B), and $C3^{-/-}$ (C) recipient mice and their survival times determined. Chilled platelets circulate in $CR3^{-/-}$ animals with the same kinetics as RT platelets, but are cleared rapidly from the circulation of C3 or $vWf^{-/-}$ mice. Data are mean \pm SD for 6 mice.

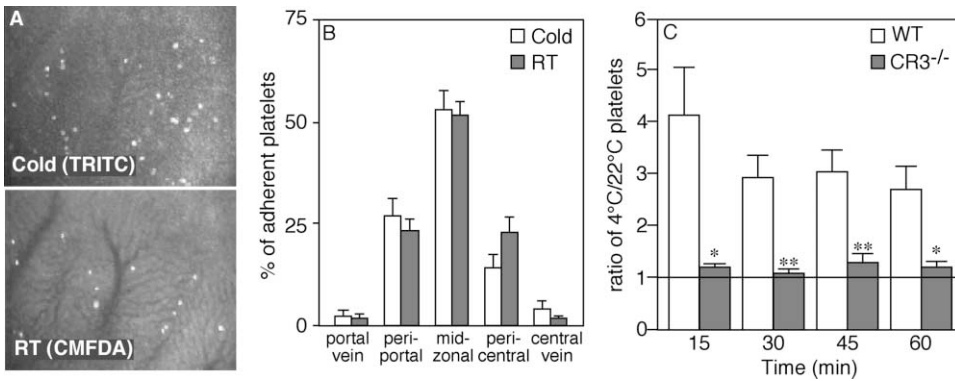


Figure 3. Chilled Platelets Adhere Tightly to CR3-Expressing Mouse Macrophages In Vivo

(A) Chilled-rewarmed TRITC-labeled platelets adhere with a higher frequency to liver sinusoids than RT CMFDA-labeled platelets. 30 min after the infusion of the platelets (B) chilled-rewarmed (Cold, open bars) and RT platelets (filled bars) adhere to sinusoidal regions with high macrophage density (midzonal) with similar distributions in WT mice. (C) Chilled-rewarmed platelets adhere 3–4 × more than RT platelets to macrophages in the WT liver (open bars). In contrast, chilled-rewarmed or RT platelets have identical adherence to macrophages in CR3^{-/-} mice (filled bars). Nine experiments with WT mice and 4 experiments with CR3^{-/-} mice are shown (mean ± SEM, * *p* < 0.05; ** *p* < 0.01).

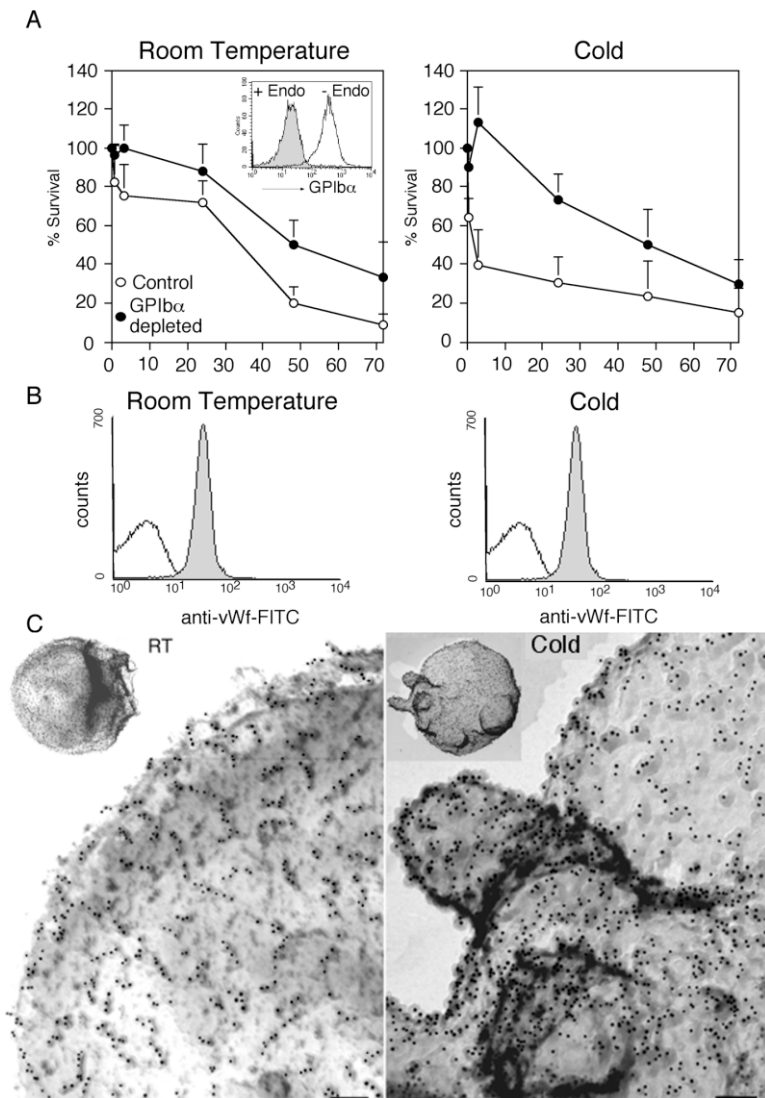


Figure 4. GPIIb α Mediates Chilled Mouse Platelet Clearance but Binds Activated vWf; vWf-Receptor Aggregates on the Surface of Chilled Platelets

(A) CMFDA-labeled mouse platelets enzymatically cleared of GPIIb α (left image, inset, filled area) or control platelets were kept at RT (left image) or chilled-rewarmed (right image) infused into syngeneic WT mice, and platelet survivals were determined. (Mean values ± SD for 6 mice). (B) Chilled, or RT platelet rich plasma was treated with (shaded area) or without (open area) botrocetin. vWf bound was detected using FITC-labeled anti-vWf antibody. (C) The vWf receptor redistributes from linear arrays (RT) into aggregates on the surface of chilled murine platelets (Cold). GPIIb α is visualized with 10 nm immunogold. The bars are 100 nm. Inset: low magnification of platelets.

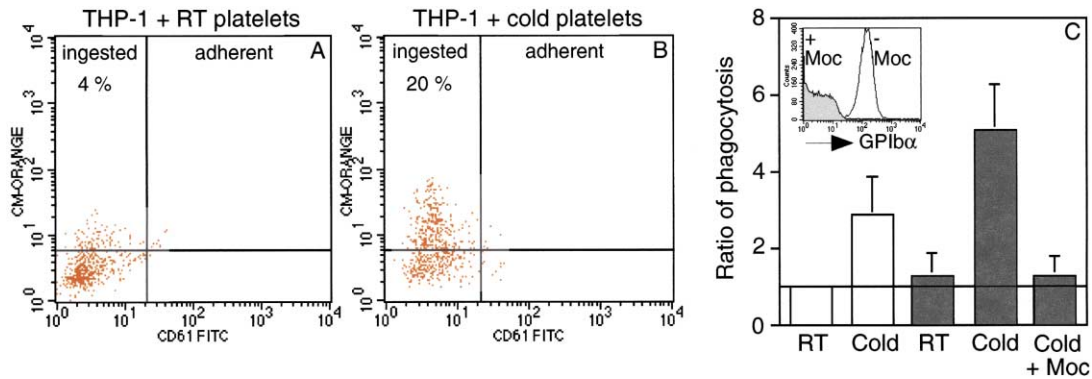


Figure 5. GPIIb/IIIa-CR3 Interaction Mediates Phagocytosis of Chilled Human Platelets In Vitro

THP-1 cells were incubated with RT (A) or chilled-rewarmed (Cold) platelets (B). CM-Orange-labeled platelets associated with macrophages shift in orange fluorescence up the y-axis. Chilling of platelets increases their phagocytosis from ~4% to 20%. Platelets were ingested, because they do not dual label with the FITC-mAb to CD61.

(C) Undifferentiated (open bars) THP-1 cells express ~50% less CR3 than differentiated cells and ingest half as many chilled-rewarmed platelets. Differentiation (filled bars) of CR3 expression leads to a 2-fold increase in chilled-rewarmed platelet phagocytosis, but has no significant effect on the uptake of RT platelets. Treatment of human platelets with mocarhagin (Moc), which removes GPIIb/IIIa from the surface (inset; control: solid line, mocarhagin treated platelets: shaded area), reduced phagocytosis of chilled platelets by ~98%. The mean percentage of the CM-Orange positive native macrophages incubated with RT kept platelets was normalized to 1. Data shown are means \pm SD of 5 experiments.

gated survival times (~5%–10%) when compared to the GPIIb/IIIa-containing RT controls.

Chilling Clusters GPIIb/IIIa on the Platelet Surface but Does Not Affect Binding of Activated vWf to Mouse Platelet vWf-Receptors

Figure 4B shows that botrocetin-activated vWf binds platelets equally well at RT and in the cold. However, chilling of platelets redistributes GPIIb/IIIa on the murine platelet surface. GPIIb/IIIa molecules, identified by anti-GPIIb/IIIa immunogold, form linear aggregates on the surface of resting platelets at RT (Figure 4C, RT), an arrangement consistent with their connection to underlying F actin by filamin A (Hartwig and DeSisto, 1991). After chilling (Figure 4C, Cold), many GPIIb/IIIa molecules organize as clusters over the platelet membrane deformed by internal actin rearrangements (Hoffmeister et al., 2001). Cooling of human platelets also caused clustering of GPIIb/IIIa molecules (data not shown).

Recognition of GPIIb/IIIa by CR3-Mediates Phagocytosis of Chilled Human Platelets In Vitro

Differentiation of monocytoic THP-1 cells using TGF- β 1 and 1,25-(OH) $_2$ vitamin D3 increases expression of CR3 by 2-fold (Simon et al., 2000). Chilling resulted in an ~3-fold increase of platelet phagocytosis by undifferentiated THP-1 cells and an ~5-fold increase by differentiated THP-1 cells (Figures 5B and 5C), consistent with mediation of platelet uptake by CR3. In contrast, the differentiation of THP-1 cells had no significant effect on the uptake of RT stored platelets (Figures 5A and 5C). To determine if GPIIb/IIIa is the counter receptor for CR3-mediated phagocytosis of chilled human platelets, we removed the extracellular domain of GPIIb/IIIa using mocarhagin (Ward et al., 1996). Removal of GPIIb/IIIa from the surface of human platelets reduced their phagocytosis after chilling by ~98% (Figure 5C).

Exclusion of Other Mediators of Cold-Induced Platelet Clearance

Table 1 shows results of experiments that examined whether cooling affected the expression of human and mouse platelet receptors other than GPIIb/IIIa or their interaction with ligands. These experiments revealed no detectable effects on the expression of P selectin, $\alpha_{IIb}\beta_3$ -integrin density, or fibrinogen binding, a marker of $\alpha_{IIb}\beta_3$ activation. Chilling also did not increase phosphatidylserine (PS) exposure, an indicator of apoptosis, nor did it change platelet binding of IgG or IgM immunoglobulins.

Circulating Chilled Platelets Have Hemostatic Function in CR3 $^{-/-}$ Mice

Despite their rapid clearance in WT mice, chilled platelets were functional 24 hr after infusion into CR3 $^{-/-}$ mice, as determined by three independent methods. First, chilled platelets incorporate into platelet aggregates in shed blood emerging from a standardized tail vein-bleeding wound (Figure 6). CMFDA-positive RT platelets

Table 1. Effect of Chilling on Binding of Various Antibodies or Ligands to Platelet Receptors.

Platelet receptor (ligand)	Binding ratio 4°C: 22°C	
	Human platelets	Murine platelets
P Selectin	1.01 \pm 0.06	1.02 \pm 0.03
Platelet associated IgGs	1.05 \pm 0.14	1.06 \pm 0.03
Platelet associated IgMs	0.93 \pm 0.10	1.01 \pm 0.02
Phosphatidylserine (annexin V)	0.95 \pm 0.09	1.04 \pm 0.02
$\alpha_{IIb}\beta_3$ (anti-CD61 mAb)	1.03 \pm 0.05	1.04 \pm 0.10
$\alpha_{IIb}\beta_3$ (fibrinogen)	1.05 \pm 0.10	1.06 \pm 0.06

The binding of fluorescently labeled antibodies or ligands against various receptors on chilled-rewarmed or RT human and murine platelets was measured by flow cytometry. The data are expressed as the ratio of fluorophore bound to the surface of chilled versus RT platelets (mean \pm SD, n = 3–4).

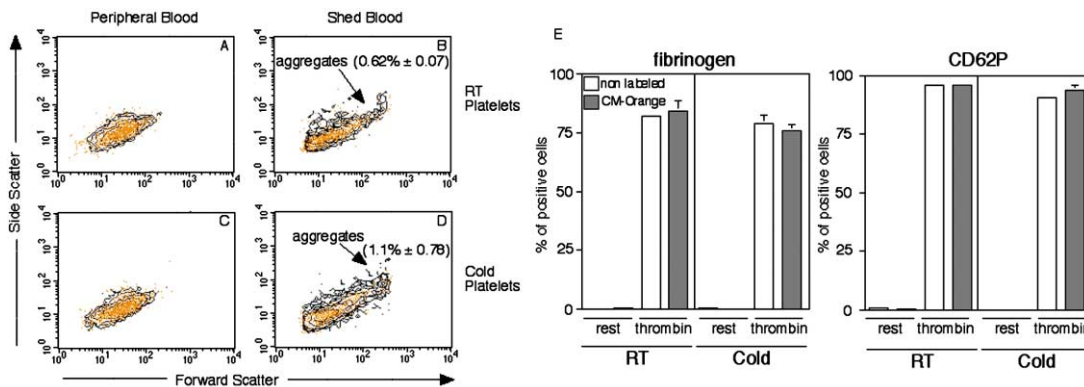


Figure 6. Circulating, Chilled Platelets Have Hemostatic Function in CR3^{-/-} Mice

Normal *in vivo* function RT platelets transfused into WT mice (A and B) and of chilled (Cold) platelets transfused into CR3^{-/-} mice (C and D), as determined by their equivalent presence in platelet aggregates emerging from the wound 24 hr after infusion of autologous CMFDA labeled platelets. Platelets in peripheral blood (A and C) and the blood emerging from the wound (shed blood, B and D) were identified by forward light scatter characteristics and binding of the PE-anti-GPIIb α mAb (pOp4). The infused platelets (dots) were CMFDA fluorescence positive, non-infused platelets (contour lines) were fluorescence negative. The percentages refer to the number of aggregates formed by CMFDA-positive platelets (n = 4).

(E) Ex vivo function of CM-Orange, RT platelets transfused into WT mice and CM-Orange, chilled-rewarmed (Cold) platelets transfused into CR3^{-/-} mice, as determined by exposure of CD62P (P selectin) and fibrinogen binding following 1 U/ml thrombin activation of blood drawn from the mice after 24 hr post infusion. Transfused platelets were identified by CM-Orange fluorescence (filled bars); non-transfused (unlabeled) platelets are the open bars. Results are expressed as the percentage of cells present in the P selectin and fibrinogen positive regions (mean \pm SD, n = 4).

transfused into WT mice (Figure 6B) and CMFDA-positive chilled platelets transfused into CR3^{-/-} mice (Figure 6D) formed aggregates in shed blood to the same extent as endogenous platelets. Second, as determined by platelet surface exposure of the fibrinogen binding site on $\alpha_{IIb}\beta_3$ 24 hr after transfusion of CM-Orange-labeled chilled and rewarmed platelets into CR3^{-/-} mice following ex vivo stimulation by thrombin. Third, CM-Orange labeled platelets chilled and rewarmed were fully capable of upregulation of P selectin in response to thrombin activation (Figure 6E). In addition to these experiments in which we reacted platelets with maximal concentrations of agonists, we have used 10- and 100-fold lower doses and have obtained comparable results (data not shown).

Mice with CR3 Receptors Rapidly Decrease Their Platelet Counts When Exposed to Cold Temperatures; CR3^{-/-} Mice Have Increased Basal Platelet Counts that Do Not Fall in the Cold

At RT CR3^{-/-} mice had platelet counts 20% higher ($2.26 \pm 0.18 \times 10^9/\text{ml}$) than their heterozygous littermates ($1.88 \pm 0.16 \times 10^9/\text{ml}$, Mean \pm SD, n = 8 for both groups). The higher platelet count seen in the CR3^{-/-} mice follows a similar trend ($\sim 10\%$) as previously described (Coxon et al. 1996). Within one half-hour of residence in a 4°C room, the platelet counts of control mice were detectably lower than baseline and fell steadily to less than 80% of the initial value by 2 hr ($1.4 \pm 0.16 \times 10^9/\text{ml}$). By contrast, the platelet counts of CR3^{-/-} mice did not change in the cold ($2.24 \pm 0.39 \times 10^9/\text{ml}$) ($p < 0.01$) (Figure 7). During this interval the mice were unrestrained and active, indicating maintenance of their core temperature despite exposure to the cold.

Discussion

Cold-Induced Platelet Shape Change Alone Does Not Lead to Platelet Clearance *In Vivo*

Cooling rapidly induces extensive platelet shape changes mediated by intracellular cytoskeletal rearrangements (Hoffmeister et al., 2001). These alterations are partially but not completely reversed by rewarming, and rewarmed platelets remain more spherical than discoid. The idea that preservation of discoid shape is a major requirement for platelet survival has been a dogma, despite evidence that transfused murine and baboon platelets activated ex vivo by thrombin circulate normally with extensive shape changes (Berger et al., 1998; Michelson et al., 1996). Here, we have shown that chilling leads to specific changes in the platelet surface that mediate their removal independently of shape change, and that the shape change per se does not lead to rapid platelet clearance. Chilled and rewarmed platelets, preserved as discs with pharmacological agents, clear with the same speed as untreated chilled platelets, and misshapen chilled and rewarmed platelets circulate like RT maintained platelets in CR3^{-/-} mice. The small size of platelets may allow them to remain in the circulation, escaping entrapment despite these extensive shape deformities.

Receptors Mediating Clearance of Chilled Platelets: CR3 and GPIIb α

The normal platelet life span in humans and mice is ~ 7 days and 4 days, respectively (Aas and Gardner, 1958; Berger et al., 1998). The incorporation of platelets into small blood clots engendered by continuous mechanical stresses undoubtedly contributes to platelet clearance, because massive thrombotic reactions, such as those

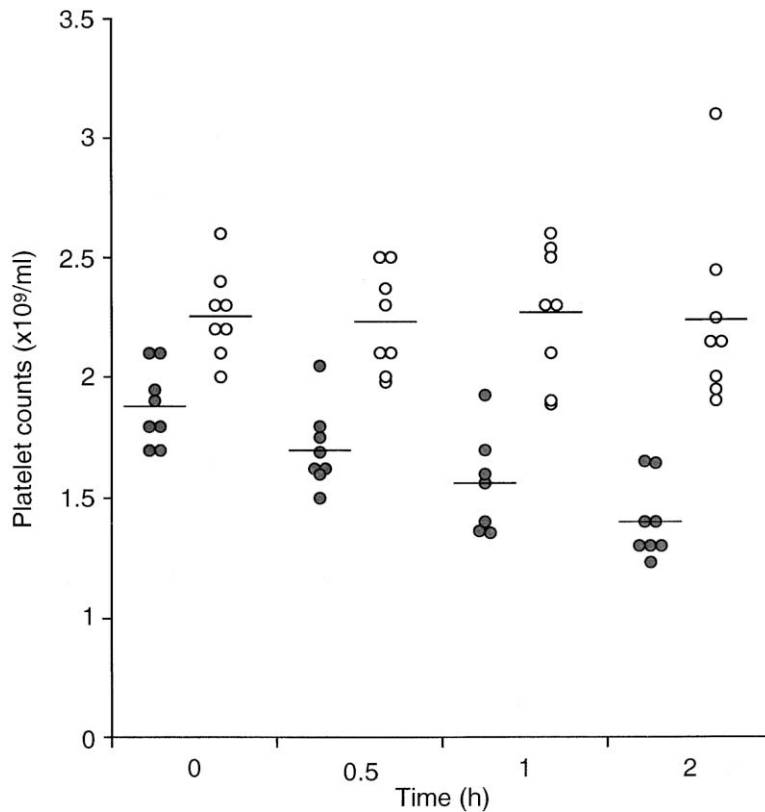


Figure 7. Platelet Counts of CR3^{-/-} and Control Mice at Room Temperature and after Exposure to the Cold

Platelets counts of CR3^{-/-} mice (open circles) and their heterozygote littermates (filled circles) were determined at RT and after their residence in a 4°C room for the indicated time points. The bar represents the mean platelet count value of 8 mice.

that occur during disseminated intravascular coagulation, cause thrombocytopenia (Seligsohn, 1995). The fate of platelets in animals exposed to thrombotic stimuli differs from that of infused *ex vivo*-activated platelets, because *in vivo* platelet stimulation occurs on injured vessel walls, and the activated platelets rapidly sequester at these sites.

Isoantibodies and autoantibodies accelerate the phagocytic removal of platelets by Fc-receptor-bearing macrophages in individuals sensitized by immunologically incompatible platelets or in patients with autoimmune thrombocytopenia, but otherwise little information exists regarding mechanisms of platelet clearance. We showed however, that the quantities of IgG or IgM bound to chilled or RT human platelets are identical, implying that binding of platelet-associated antibodies to Fc-receptors does not mediate the clearance of cooled platelets. We also demonstrated that chilling of platelets does not induce detectable PS exposure on the platelet surface *in vitro* militating against PS exposure and the involvement of scavenger receptors in the clearance of chilled platelets.

Although publications have referred to effects of cold on platelets as "activation", aside from cytoskeletally mediated shape changes, chilled platelets do not resemble platelets activated by stimuli such as thrombin or ADP. Normal activation markedly increases surface P selectin expression, a consequence of secretion from intracellular granules (Berman et al., 1986). Chilling of platelets does not lead to upregulation of P selectin, and the clearance of chilled platelets isolated from WT or P selectin^{-/-} mice is equally rapid (Berger et al., 1998).

Activation also increases the amount of $\alpha_{IIb}\beta_3$ -integrin and its avidity for fibrinogen (Shattil, 1999), but chilling is without these effects. The normal survival of thrombin-activated platelets is consistent with our findings.

We have shown that CR3 on liver macrophages is primarily responsible for the recognition and clearance of chilled platelets. The predominant role of CR3 bearing liver macrophages in clearance of chilled platelets, despite abundant CR3-expressing macrophages in the spleen, is consistent with the principally hepatic clearance of IgM-coated erythrocytes (Yan et al., 2000) and may reflect blood filtration properties of the liver that favor binding and ingestion by macrophage CR3. The extracellular domain of GPIb α , isolated by proteolysis from intact platelets, binds avidly to CR3 *in vitro*, and when immobilized on a surface, supports the rolling and firm adhesion of THP-1 cells (Simon et al., 2000). Cleavage of the extracellular domain of murine GPIb α results in normal survival of chilled platelets transfused into mice. GPIb α depletion of human chilled platelets greatly reduces phagocytosis of the treated platelets by macrophage-like cells *in vitro*. The finding that two different enzymes, O-sialoglycoprotein endopeptidase and mocarhagin, that target the extracellular domain of mouse and human GPIb α respectively, had identical effects on CR3-mediated platelet recognition that supports the specificity of the enzyme treatment approach for removal of GPIb α . We propose, therefore, that GPIb α is the counter-receptor on chilled platelets for liver macrophage CR3 leading to their clearance by phagocytosis.

The normal clearance of chilled platelets lacking the

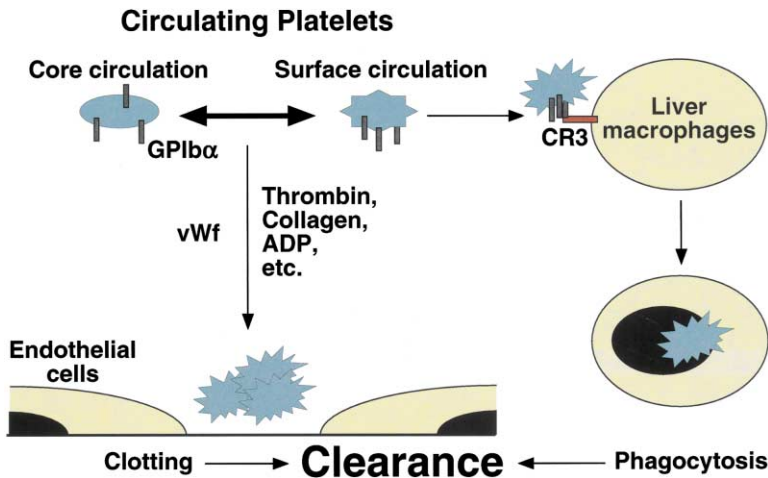


Figure 8. Pathways Contributing to Basal Platelet Clearance

Scheme depicting two platelet clearance pathways. Platelets traverse central and peripheral circulations, undergoing reversible priming at lower temperatures at the body surface. Repeated priming leads to irreversible vWf receptor complex reconfiguration and clearance by CR3 bearing hepatic macrophages. Platelets are also cleared after they participate in microvascular coagulation.

N-terminal portion of GPIb α rules out the many other CR3 binding partners, including molecules expressed on platelet surfaces as candidates for mediating chilled platelet clearance. These ligand candidates include ICAM-2, fibrinogen bound to the platelet integrin $\alpha_{IIb}\beta_3$, iC3b, P selectin, glucosaminoglycans, and high molecular weight kininogen. We excluded deposition of the opsonic C3b fragment iC3b as a mechanism for chilled platelet clearance using mice deficient in complement factor 3, and the expression level of $\alpha_{IIb}\beta_3$ and fibrinogen binding are also unchanged after chilling of platelets.

Binding to Activated vWf and Cold-Induced Binding to CR3 Appear to Be Separate Functions of GPIb α

GPIb α on the surface of the resting discoid platelet exists in linear arrays (Kovacs and Hartwig, 1996) in a complex with GPIb β , GPIX, and V, attached to the submembrane actin cytoskeleton by filamin A and filamin B (Lopez et al., 1998; Stossel et al., 2001). Its role in hemostasis is to bind the activated form of vWf at sites of vascular injury. GPIb α binding to activated vWf is constitutive and requires no active contribution from the platelet since activated vWf binds equally well to GPIb α on resting or on stimulated platelets. Stimulation of platelets in suspension by thrombin and other agonists causes GPIb α to redistribute in part from the platelet surface into an internal membrane network, the open canalicular system, but does not lead to platelet clearance in vivo (Berger et al., 1998; Michelson et al., 1996) or to phagocytosis in vitro (K.M.H., unpublished data). Cooling of platelets however, causes GPIb α clustering rather than internalization. This clustering is independent of barbed end actin assembly, because it occurs in the presence of cytochalasin B. Presumably, isolated GPIb α extracellular domain fragments that bind CR3 in vitro achieve this clustered conformation (Simon et al., 2000).

Despite cold's promoting recognition of platelet GPIb α by CR3, it has no major effect on the interaction between GPIb α and activated vWf in vitro, and chilled platelets transfused into vWf $^{-/-}$ mice disappear as rapidly as in WT mice. The separability of GPIb α 's interaction with vWf and CR3 suggests that selective modifi-

cation of GPIb α might inhibit cold-induced platelet clearance without impairment of GPIb α 's hemostatically important reactivity with vWf. Should the clearance function of GPIb α be subject to inhibition, tests of more subtle non-equilibrium interactions between GPIb and vWf, such as shear-induced binding and platelet rolling on vWf-coated surfaces, will be warranted (Goto et al., 1995). Nevertheless, since all assays we performed of function of cooled platelets in vitro and after infusion into CR3 $^{-/-}$ mice yielded grossly normal results, suitably modified platelets might be reasonably effective in mediating hemostasis. Previous studies have shown that platelets stored in the cold have better in vitro function than platelets stored at RT (Becker et al., 1973; Snyder et al., 1989). The markedly shortened survival of chilled platelets unfortunately offsets their functional advantages.

Physiological Importance of Cold-Induced Platelet Clearance

Although gross platelet shape changes become obvious only at temperatures below 15°C, accurate biochemical analyses show that cytoskeletal alterations and increased responsiveness to platelet-activating mediators are detectable as the temperature falls below 37°C (Faraday and Rosenfeld, 1998; Hoffmeister et al., 2001). We refer to those changes as "priming," because cooling does not by itself lead to changes associated with hemostatic platelet activation other than alterations in morphology (Faraday and Rosenfeld, 1998). We have previously speculated that platelets are thermosensors, designed to be less responsive to thrombogenic stimuli at the core body temperature of the central circulation where coronary or cerebral thrombosis could be lethal, but become primed for activation at the lower temperatures of external body surfaces, sites most susceptible to bleeding throughout evolutionary history (Hoffmeister et al., 2001). The findings that prolonged (Deveci et al., 2001), and, as we report here, brief exposure to cold diminishes the platelet counts of animals, support this hypothesis. The lack of a drop in platelet count in CR3 $^{-/-}$ mice is completely consistent with the operation of the clearance pathway we have defined based on irreversible changes in GPIb α , unrelated to its interaction with

vWf. Rather than allowing chilled platelets to circulate, the organism clears low temperature-primed platelets by CR3-mediated phagocytosis.

A system involving at least two clearance pathways, one for removal of locally activated platelets and another for taking out excessively primed platelets (Figure 8), can possibly explain why chilled platelets circulate and function normally in CR3^{-/-} mice and have a slightly prolonged circulation following removal of GPIb α . We propose that some primed platelets enter microvascular thrombi on a stochastic basis. Others are susceptible to repeated exposure to body surface temperature, and this repetitive priming eventually renders these platelets recognizable by CR3-bearing macrophages. Platelets primed by chilling are capable of relatively normal hemostatic function in CR3^{-/-} mice, and coagulation contributes to their clearance. Since the platelets of CR3^{-/-} mice have a somewhat shortened circulation time, possibly resulting from increased clearance of primed platelets in microvascular clots, increased platelet production must account for the slightly higher basal platelets counts in these animals. If, as it has been proposed (Sungaran et al., 2000), platelet contents stimulate thrombopoietin production, an increase in the number of platelets being cleared by microvascular thrombosis might result in elevated platelet production.

Experimental Procedures

Materials

We obtained: FITC-annexin V, PE-anti-human CD11b monoclonal antibodies (mAb), FITC-anti-mouse and anti-human IgM mAb, FITC-anti-mouse and anti-human CD62P mAb from Pharmingen, San Diego, CA; FITC-rat anti-mouse and human IgG mAb from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Cytochalasin B, human thrombin, PGE₁, PMA from Sigma, St. Louis, MO.

Animals

For assays of clearance and survival studies, we used age-, strain- and sex-matched C57BL/6J WT mice (Jackson Laboratory, Bar Harbor, ME). Mice lacking complement component C3 (Wessels et al., 1995) (provided by Dr. M.C. Carroll, Center for Blood Research, Boston, MA) and CR3 (Coxon et al., 1996) were of the C57BL/6J 129/sv genetic background. vWf^{-/-} mice were of the genetic C57BL/6J background (Denis et al., 1998). Heterozygote littermates were used as controls. Mice were maintained and treated as approved by Harvard Medical Area Standing Committee on Animals according to NIH standards as set forth in The Guide for the Care and Use of Laboratory Animals.

Platelet Preparation

Platelets were isolated from normal human volunteers as described (Hartwig and Desisto, 1991). Human platelets used in the in vitro phagocytosis assay were labeled with 1.8 μ M CellTracker Orange CMTMR (CM-Orange) (Molecular Probes, Inc., Eugene, OR) for 20 min at 37°C (Brown et al., 2000). Unincorporated dye was removed by centrifugation (850 \times g, 5 min.) with 5 volumes of 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, and 1 μ g/ml PGE₁, [pH 6.0] (buffer A). Platelets were suspended at 3×10^9 /ml in 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl₂, 5 mM NaHCO₃, 10 mM glucose, and 10 mM HEPES, [pH 7.4] (buffer B).

Murine blood was obtained by retro orbital eye bleeding into 0.1 volume of Aster-Jandl anticoagulant from anesthetized mice using 3.75 mg/g of Avertin (Fluka Chemie, Steinheim, Germany) and centrifuged at 300 \times g for 8 min at RT to obtain PRP. Platelets were separated from plasma proteins by centrifugation at 1200 \times g for 5 min and washed in buffer A two times by centrifugation (1200 \times g for 5 min). Before injection into mice, platelets were suspended at a concentration of 1×10^9 /ml in buffer B.

GPIb α was enzymatically removed from the surface of chilled or

RT maintained human or mouse platelets in buffer B, also containing 1 mM Ca²⁺ and 10 μ g/ml of the snake venom metalloprotease mo-carhagin (provided by Dr. M. Berndt, Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3168, Australia) (Ward et al., 1996) or 100 μ g/ml O-sialoglycoprotein endopeptidase (Cerladane, Hornby, Canada) (Bergmeier et al., 2001), respectively. After the enzymatic digestion, human and mouse platelets were washed by centrifugation. GPIb α removal from platelet surface was monitored by flow cytometry analysis (FACScalibur Flow Cytometer, Becton Dickinson Biosciences, San Jose, CA), using 5 μ g/ml of the FITC-conjugated anti-human GPIb α (SZ2) mAb (Immunotech, Marseille, France) or 5 μ g/ml of the PE-anti-mouse GPIb α mAb pOp4, (Bergmeier et al., 2000).

Radioactive platelet clearance studies were performed with ¹¹¹Indium (NEN Life Science Products, Boston, MA) loaded mouse platelets (Kotze et al., 1985). Platelets, at a concentration of 2×10^9 /ml in 0.9% NaCl, [pH 6.5] were incubated with 500 μ Ci ¹¹¹Indium for 30 min at 37°C, washed and suspended in buffer B.

For intravital microscopy or other platelet survival experiments, platelets were labeled with 2.5 μ M CellTracker Green CMFDA (Baker et al., 1997) (Molecular probes, Inc. Eugene, OR) or with 0.15 μ M TRITC (Sigma, St. Louis, MO) for 20 min at 37°C in buffer B also containing 0.001% DMSO (Sigma, St. Louis, MO), and 20 mM HEPES (GIBCO, Invitrogen Corp., Grand Island, NY). Unincorporated dye was removed by centrifugation and platelets were suspended in buffer B.

To inhibit cold-induced platelet shape changes, 10⁹/ml platelets in buffer B were loaded with 40 μ M EGTA-AM (Molecular probes, Inc. Eugene, OR) followed by 2 μ M cytochalasin B as previously described (Winokur and Hartwig, 1995), labeled with 2.5 μ M CMFDA for 30 min at 37°C, and then chilled or maintained at RT.

Platelet Temperature Protocols

To study the effects of temperature on platelet survival or function of unlabeled, radioactively, or fluorescently labeled mouse or human platelets were incubated for 2 hr at RT (~25°C) or else at ice bath temperatures and then rewarmed for 15 min at 37°C before transfusion into mice or in vitro analysis. Platelets subjected to these treatments are designated as chilled or RT platelets.

Murine Platelet Recovery, Survival, and Fate

CMFDA labeled chilled or RT murine platelets (10⁸) were injected into syngeneic mice via the lateral tail vein. For recovery and survival determination, blood samples were collected immediately (< 2 min) and 0.5, 2, 24, 48, and 72 hr after transfusion into 0.1 volume of Aster-Jandl anticoagulant. Whole blood analysis using flow cytometry was performed and the percentage of CMFDA positive platelets determined (Baker et al., 1997). A total of 50,000 events were collected in each sample. CMFDA positive platelets measured at a time < 2 min was set as 100%. The recoveries were 72.8% \pm 5.53 SD for RT stored platelets and 65.5 \pm 4.39 SD for chilled platelets. The mean platelet count per mouse was 1.55 \pm 0.23 SD \times 10⁹/ml. The maximal percent of circulating labeled platelets, determined at 2 min was 5.3% \pm 1.4 SD.

To evaluate the fate of platelets, tissues were harvested at 0.5, 1, and 24 hr after the injection of 10⁸ ¹¹¹Indium labeled chilled or RT platelets into mice. Organ weight and radioactivity were determined by γ -count. Results are expressed as % of radioactivity/per gm organ relative to the maximal radioactivity injected. For recovery and survival determination of radioactive platelets, blood samples were collected into 0.1 volume of Aster-Jandl anticoagulant immediately (< 2 min), 0.5, 1, and 24 hr after transfusion and their γ -count determined (Kotze et al., 1985).

Platelet Aggregation

Samples of 0.3 ml murine platelets were stirred and exposed to 1 U/ml thrombin, 10 μ M ADP, or 3 μ g/ml CRP (Falet et al., 2002) at 37°C. Light transmission was recorded over 3 min (Bio/Data aggregometer, Horsham, PA).

Activated vWf Binding

Platelet rich plasma was treated with 2 U/ml botrocetin (Centerchem Inc., Norwalk, CT) for 5 min at 37°C (Bergmeier et al., 2001). Bound vWf was detected by flow cytometry using a FITC-anti-vWf antibody (DAKOCytomation, Glostrup, Denmark).

Surface Labeling of Platelet GPIb α

Resting mouse platelets maintained at RT or chilled were applied to glass coverslips by centrifugation in PBS containing 0.05% glutaraldehyde, fixed with 0.5% glutaraldehyde in PBS for 10 min., quenched with 0.1% sodium borohydride in PBS, and washed with PBS containing 1% BSA. GPIb α was labeled with a mix of three rat anti-mouse GPIb α monoclonal antibodies (provided by Dr. B. Nieswandt, Witten/Herdecke University, Wuppertal, Germany), each at 10 μ g/ml for 1 hr, followed by one incubation with 10 nm gold conjugated goat anti-rat IgG. The coverslips were washed, fixed with 1% glutaraldehyde, and prepared for electron microscopy as described (Kovacs and Hartwig, 1996).

In Vitro Phagocytic Assay

Monocytic THP-1 cells (ATCC, Manassas, VA) were cultured for 7 days in RPMI 1640 cell culture media supplemented with 10% fetal bovine serum, 25 mM HEPES, and 2 mM glutamine and differentiated using 1 ng/ml TGF- β 1 and 50 nM 1,25-(OH) $_2$ vitamin D3 (Calbiochem, San Diego, CA) for 24 hr, which is accompanied by increased expression of CR3 (Simon et al., 2000). CR3 expression was monitored by flow cytometry using a PE-anti-human CD11b mAb. Undifferentiated or differentiated THP-1 cells (2×10^5 /ml) were plated onto 24-well plates and allowed to adhere for 45 min at 37°C. The adherent macrophages were activated by the addition of 15 ng/ml PMA for 15 min. CM-Orange-labeled, chilled, or RT platelets (10 7 /well), previously subjected to different treatments were added to the phagocytes in Ca $^{2+}$ - and Mg $^{2+}$ -containing HBSS (GIBCO Invitrogen, Grand Island, NY) and incubated for 30 min at 37°C. The phagocyte monolayer was washed for 3 times with HBSS, and adherent platelets were removed by treatment with 0.05% trypsin/0.53 mM EDTA in HBSS (GIBCO Invitrogen, Grand Island, NY) at 37°C for 5 min followed by 5 mM EDTA at 4°C to detach the macrophages (Brown et al., 2000) for flow cytometry analysis. Human CM-Orange-labeled, chilled, or RT platelets expressed the same amount of the platelet specific marker CD61 (not shown). Platelets were resolved from the phagocytes according to their forward and side scatter characteristics. The macrophages were gated and 10,000 events acquired for each sample. CM-Orange-labeled platelets that associate with the phagocyte population shift in orange fluorescence (Figure 6A and Figure 6B, ingested, y axis). Platelets adherent to macrophages were not present because the macrophages failed to dual label with the FITC-mAb to human-CD61 (Accurate Scientific, Westbury, NY).

Immunolabeling and Flow Cytometry of Platelets

Washed murine or human platelets were analyzed for surface expression of CD62P, CD61, or surface bound IgM and IgG after chilling or RT storage by staining with fluorophore-conjugated Abs (5 μ g/ml) for 10 min at 37°C. Phosphatidylserine exposure by chilled or room temperature platelets was determined by FITC-conjugated annexin-V. As a positive control for PS exposure, platelet suspensions were stimulated with 1 μ M A23187 (Sigma, St. Louis, MO). Fibrinogen binding was determined by the addition of 50 μ g/ml Oregon Green-fibrinogen (Molecular probes, Eugene, MO) for 20 min at RT. All platelet samples were analyzed immediately by flow cytometry.

Intravital Microscopy Experiments

Animal preparation, technical, and experimental aspects of the intravital microscopy setup have been described (von Andrian, 1996). Six to eight-week-old mice of both sexes were anesthetized and the right jugular vein was catheterized with PE-10 polyethylene tubing. The lower surface of the left liver lobe was surgically prepared and covered by a glass cover slip for in vivo microscopy (Menger et al., 1991). Chilled platelets (10 8) and RT platelets labeled with CMFDA and TRITC respectively were mixed 1:1 and administered intravenously. The circulation of labeled platelets in liver sinusoids was followed by video triggered stroboscopic epi-illumination. Ten video scenes were recorded from 3 centrilobular zones at each time point. The ratio of chilled (CMFDA)/RT (TRITC) adherent platelets in the same visualized field was calculated. Confocal microscopy was performed using a Radiance 2000 MP confocal-multiphoton imaging system connected to an Olympus BX 50 WJ upright microscope

(Biorad, Hercules, CA) Images were captured and analyzed with Laser Sharp 2000 software (Biorad).

Platelet Aggregation in Shed Blood

CMFDA labeled RT murine platelets (10 8) were injected into heterozygote littermate mice and 10 8 CMFDA labeled, chilled platelets into CR3 $^{-/-}$ mice. Twenty-four hrs after the platelet infusion, a standard bleeding time assay was performed, severing a 3 mm segment of a mouse tail (Denis et al., 1998). The amputated tail was immersed in 100 μ l of saline at 37°C containing 0.1 volume of Aster-Jandl anticoagulant, shedding blood was collected for 2 min and fixed with 1% paraformaldehyde (final concentration). Peripheral blood was obtained by retroorbital eye plexus bleeding, diluted 1/10 with saline, and fixed with 1% paraformaldehyde. To analyze aggregates in vivo by flow cytometry, blood shed from the wound and blood obtained by eye bleeding were labeled with PE-anti-murine GPIb α mAb pOp4. The infused platelets were identified by their CMFDA labeling and discriminated from the CMFDA negative non-infused platelets. The number of platelet aggregates in shed blood was determined as described (Michelson et al., 1996).

Flow Cytometric Analysis of Murine Platelet Fibrinogen Binding and P Selectin Exposure of Circulating Platelets

RT and chilled CM-Orange-labeled platelets (10 8) were injected into WT mice or CR3 $^{-/-}$ mice. The mice were bled at 24 hr and platelets isolated. Resting or thrombin activated (1 U/ml, 5 min) platelets (2×10^8) were diluted in PBS and stained with FITC-anti-mouse CD62P mAb or Oregon Green-fibrinogen. Platelet samples were analyzed by flow cytometry. CD62P expression and fibrinogen binding were measured for each CM-Orange positive and negative population before and after stimulation with thrombin.

Effect of Exposure of Mice to Cold Temperatures on Their Platelet Counts

Eight-week-old mice $^{-/-}$ in CR3 and their WT littermates of body mass of \sim 25 g were exposed to 4°C air temperatures for two hours. The mice were freely mobile during that interval. Ten μ l of blood was obtained by a standard bleeding time assay, severing a 3 mm segment of each mouse tail. Emerging blood was collected after 0.5, 1, and 2 hr into 0.1 volume of Aster-Jandl anticoagulant. The platelet count was determined by flow cytometry using reference beads (SPHERO rainbow fluorescent beads, 5.5 μ m diameter, Spherotech Inc., Libertyville, IL) as described (Alugupalli et al., 2001).

Statistics

The intravital microscopy data are expressed as means \pm SEM. Groups were compared using the nonpaired t test. P values < 0.05 were considered significant. All other data are presented as the mean \pm SD.

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