Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage

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Platelets contain both pro- and antiangiogenic factors, but their regulatory role in angiogenesis is poorly understood. Although previous studies showed that platelets stimulate angiogenesis in vitro, the role of platelets in angiogenesis in vivo is largely uncharacterized. To address this topic, we used two in vivo approaches, the cornea micropocket assay and the Matrigel model, in four animal models: thrombocytopenic, Lyst^bg^ (platelet storage pool deficiency), glycoprotein (GP) Ibα/IL4R transgenic (lacking extracellular GPIbα, the receptor for von Willebrand factor as well as other adhesive and procoagulant proteins), and FcγR^−/−^ (lacking functional GPVI, the collagen receptor) mice. Adult mice were rendered thrombocytopenic by i.p. administration of an antiplatelet antibody. The number of growing vessels in the thrombocytopenic mice was lower in the cornea assay, and they showed significantly increased appearance of hemorrhage compared with mice treated with control IgG. The thrombocytopenic mice also showed more protein leakage and developed hematomas in the Matrigel model. GPIbα/IL4R transgenic mice presented increased hemorrhage in both assays, but it was less severe than in the platelet-depleted mice. FcγR^−/−^ and Lyst^bg^ mice showed no defect in experimental angiogenesis. Intravital microscopy revealed a >3-fold increase in platelet adhesion to angiogenic vessels of Matrigel compared with mature quiescent skin vessels. Our results suggest that the presence of platelets not only stimulates angiogenic vessel growth but also plays a critical role in preventing hemorrhage from the angiogenic vessels. The adhesion function of platelets, as mediated by GPIbα, significantly contributes to the process.

Although the best-defined function of platelets is in hemostasis and thrombosis, platelets also participate in other processes, such as inflammation and atherosclerosis (1). Platelets are anucleated cellular fragments rich in organelles including three types of secretory vesicles: dense granules, α-granules, and lysosomes (2). Under physiological conditions, circulating platelets do not interact with the vessel walls. However, in response to endothelial activation or vascular injury when underlying extracellular matrix (ECM) is exposed, platelet adhesion and subsequent thrombus formation occur. Two major adhesion receptors, glycoprotein (GP) Ib-IX-V and GPVI, are primarily responsible for regulating this initial platelet adhesion (3–6). The binding of GPIb-IX-V to von Willebrand factor (VWF) establishes a transient bond, which reduces platelet velocity and thus facilitates their adhesion and activation (7, 8). A rapid conversion to stable platelet adhesion is required to promote thrombus formation. This process is primarily mediated by the interaction of platelet integrins and GPVI with collagen. Platelet activation also leads to secretion of platelet agonists, such as ADP and thromboxane A2 (secreted from dense granules), to reinforce the platelet aggregation, and of adhesion molecules, such as VWF, and growth factors from α-granules.

Thrombocytopenic mice | blood vessel | α-granule | cornea | collagen receptor

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Abbreviations: bFGF, basic FGF; ECM, extracellular matrix; GP, glycoprotein; VWF, von Willebrand factor; MP, microparticle.

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support angiogenesis and prevent excessive leakage and hemorrhage from newly formed vessels.

**Results**

**Platelet Depletion Led to the Formation of Fewer Vessels and, Most Notably, Highly Hemorrhagic Vessels in a Cornea Angiogenesis Model.** To determine whether platelet depletion affects experimental angiogenesis, we used the cornea micropocket assay. Pellets containing the slow-release polymer hydron and bFGF were surgically implanted into the micropockets of mouse corneas, which are avascular. Thrombocytopenia was induced 1 h after implantation of the pellets. A single i.p. injection of antiplatelet antibody in the mice resulted in a profound thrombocytopenia within 1 h, with a >95% reduction in the number of circulating platelets. The thrombocytopenia induced by this single injection was transient, and platelet levels started to return to normal by day 3. Sustained thrombocytopenia was induced by a repeated injection of the antibody on the third day. Sprouting of the limbal vessel into the cornea was observed. Newly formed vessels in the corneas of the control mice were clearly visible and nonhemorrhagic at 72 and 96 h (Fig. 1A Left) and also at 5 days (data not shown). The angiogenic vessels in the corneas of the platelet-depleted animals were less well defined and were surrounded by extravascular RBCs (Fig. 1A Right). The length of new vessels was therefore difficult to define in corneas of the platelet-depleted animals. However, there was a significant decrease in clock hours (length of the limbal vessel showing sprouts) 5 days after pellet implantation in the platelet-depleted group when compared with the control group (2.21 ± 0.16 for IgG and 1.80 ± 0.11 for antiplatelet group, P < 0.05). Animals treated with the antiplatelet antibody also had lower numbers of corneal vessels when compared with the control animals (Fig. 1B). The number of vessels was significantly decreased in the antiplatelet antibody group at 72 and 96 h after pellet implantation (P < 0.05), and the difference was close to significant at 120 h after pellet implantation (P = 0.057). To compare the RBC leakiness of the newly formed vessels, a blinded observer assigned a hemorrhagic score to the eyes. The score was significantly different in the two sets of animals at all time points (P < 0.001) (Fig. 1C).

**Platelet Depletion Caused Hemorrhage and Fragility of the Implanted Matrigel.** To test the role of platelets in angiogenesis further, we used the Matrigel assay (38). One group of mice was injected with the antiplatelet antibody 1 h after Matrigel implantation and reinjected on the third day, and the control group was injected with IgG. The Matrigels were dissected 7 days later. We found readily discernable differences between the two groups in the gross morphology of the Matrigel (Fig. 2A). Matrigel plugs of platelet-depleted mice were very fragile, and various degrees of hemorrhage were found in every plug in the antiplatelet group, but no obvious hemorrhage was detected in the plugs from the control group (Fig. 2B). Infusion of 2% Evans blue dye (binds to albumin) intravenously 3 h before Matrigel isolation showed greater protein leakage in platelet-depleted angiogenic vessels (OD per g was 13.5 ± 0.7 for the antiplatelet group and 10.2 ± 0.6 for the IgG group; n = 7–8, P < 0.02). Hematoxylin and eosin staining of Matrigel sections showed a marked presence of extravascular RBCs and many hematomas in the antiplatelet antibody-treated gels as compared with those from IgG-treated mice (Fig. 2B). To evaluate the RBC content in the
Matrigel more quantitatively, we determined the Hb level, which
was significantly increased in the Matrigel implants after anti-
platelet antibody treatment compared with mice treated with
IgG (Fig. 3, *P < 0.002).

Platelet Storage Pool Deficiency Did Not Alter Angiogenesis. To study
whether the leakiness of the angiogenic vessels after platelet
depletion is due to absence of dense granular and lysosomal
secretion, we used Lyst<sup>bg</sup> mice. The phenotype closely resembles
Chediak–Higashi disease in man. These mice have platelet
storage pool deficiency, leading to a prolonged bleeding time and
mild-to-moderate mucocutaneous bleeding. Formation of new
blood vessels and the area of neovascularization in the corneas
of Lyst<sup>bg</sup> mice were similar to those observed in controls, and
they showed no hemorrhage (Fig. 4A Upper). In addition, we did
not find a difference in the level of Hb between Lyst<sup>bg</sup> and
controls (Fig. 3). These results indicate that platelet-dense
granular and lysosomal secretion is not essential for the forma-
tion of stable angiogenic vessels.

Platelet Adhesion Deficiency (GPIbα Replacement) Led to Abnormal
Angiogenesis. The adhesion function of platelets is another factor
that may play a role in the angiogenic process. To evaluate the
role of adhesion, we used a GPIbα/IL4R transgenic mouse
model where the extracellular domain of GPIbα is replaced with
that of the IL-4 receptor (39). In the cornea model, GPIbα/IL4R
transgenic mice did not show significant differences in the area
of neovascularization at 72, 96, and 120 h after pellet implan-
tation (data not shown). However, we found increased leakage
of the angiogenic vessels in the corneas of GPIbα/IL4R when
compared with the controls (Fig. 4A Lower). The hemorrhage
appeared in 63% of animals from the GPIbα/IL4R group 72 h
after pellet implantation (Fig. 4B) and in 38% 120 h after pellet
implantation. Hemorrhage was rare in the control animals of
similar genetic background. This finding indicates that the
platelet adhesion plays a role in preventing hemorrhage from
angiogenic vessels in the cornea micropocket model.

Hb Levels Were Increased in Matrigels of GPIbα/IL4R Transgenic Mice
but Not in FcγR<sup>−/−</sup> Mice. Seven days after Matrigel implantation,
the plugs of GPIbα/IL4R transgenic mice were removed and
used for hematoxylin and eosin staining and for Hb content
determination. The dissected Matrigels showed only small signs
of hemorrhage when compared with platelet-depleted mice;
therefore, we analyzed the plugs for Hb levels as a reflection of
RBC content. Hb levels in Matrigels from GPIbα/IL4R transgen-
ic mice were significantly increased when compared with
matched WT animals (Fig. 3, *P < 0.02). This increase could also
mean that there was elevated neovascularization in the mutant
mice. However, when we quantified the number of vessels and
their density in the Matrigel sections, there were no significant
differences in either parameter between GPIbα/IL4R mice and
WT (25 ± 1.58 for WT and 24 ± 1.03 for GPIbα/IL4R; n = 8,
*P = 0.8). This finding indicates that, although we did not see any
large hematomas in the Matrigel sections, there was increased
hemorrhage from the mutant vessels. Because the Matrigel
model gives a better readout of hemorrhage, we tested the
importance of another adhesion receptor mediating platelet
adhesion to ECM: GPVI. We did not find a significant difference
in Hb levels in FcγR<sup>−/−</sup> mice lacking functional GPVI when
compared with WT mice (Fig. 3, *P > 0.05).

Platelets Adhered Preferentially to Angiogenic Vessels. To study platelet
behavior in the newly formed vessels in the Matrigel and
compare it with that in mature quiescent vessels, we used
intravital microscopy and the skin chamber (40). Fluorescently
labeled platelets were injected intravenously, and skin chambers
with or without implanted Matrigel were observed. Whereas
platelets rarely interacted with endothelium of skin microcircu-

Fig. 3. Hb levels in Matrigels of antiplatelet, GPIbα/IL4R, FcγR<sup>−/−</sup>, and Lyst<sup>bg</sup>
mice. Seven days after s.c. implantation, the Matrigels were harvested and
homogenized. Hb concentration was measured in the supernatants. *, *P < 0.002 compared with the control group (n = 6–8).

Fig. 4. Mouse cornea assay in GPIbα/IL4R and Lyst<sup>bg</sup> mice. (A) Corneas of WT and Lyst<sup>bg</sup> mice 96 h after pellet implantation (Upper) and corneas of WT and
GPIbα/IL4R mice 72 h after pellet implantation (Lower). Significant hemorrhage is seen only in the GPIbα/IL4R mice. (B) Percentage of eyes showing hemorrhage
in the corneas of GPIbα/IL4R mice (gray bar) and WT (black bar) implanted with bFGF hydron pellets. *, *P < 0.02 compared with the control group.
employed the hypoxia-induced retinal angiogenesis model in newborn mice, whereas in the current study we used two different models of angiogenesis in adult mice. These results also corroborate the study by Ma et al. (13), demonstrating that thrombocytopenia in rats caused a significant inhibition of gastric ulcer healing, a process known to depend on angiogenesis similar to wound healing. Recently Brill et al. (43) showed that infusion of platelet-derived microparticles (MPs) generated from thrombin-activated platelets induced angiogenesis and improved revascularization after chronic ischemia in vivo. This work extends the in vitro study by Kim et al. (44) in which platelet-derived MPs promoted proliferation and survival of endothelial cells, as well as tube formation. Although GPIbα, which we show to be important, is present and ready for adhesion on resting platelets, the results suggest that, in addition, platelet activation generating MPs is involved in the process of angiogenesis. GPIbα could be involved also in platelet activation and generation of MPs. There is evidence that GPIbα binds α-thrombin and modulates its function (45). Interestingly, the binding site for thrombin on GPIbα was shown to play a role in the exposure of negatively charged phospholipids on the platelet surface (46), a step in MP generation.

The above results indicate that platelets' overall effect on angiogenesis is stimulatory. In our experiments, the occurrence of hemorrhage and excessive protein leakage in platelet-depleted animals also shows platelets’ role in the stabilization of newly formed vessels. It is possible that the platelet–endothelial interaction or platelet adhesion to exposed ECM during the endothelial cell sprouting is essential to prevent leakage and hemorrhage from the angiogenic vessels. Our intravital microscopy results indicate that platelets preferentially adhere to the newly formed vessels in Matrigel in the skin chamber angiogenesis assay (Fig. 5). It will be critical to establish whether platelets adhere to endothelial cells activated by the angiogenic process (47) or to ECM components exposed during angiogenesis. Platelets adhere to collagen mainly through GPVI. However, our results indicate that, in contrast to GPIbα, GPV1-mediated adhesion does not affect angiogenesis (Fig. 3). Rhee et al. (42) found platelet remnants and microvesicles at the sites of angiogenic sprouts, and platelet microthrombi were also seen accumulating in the retinal neovascularization of the diabetic rats (11). These microthrombi were necessary to suppress the breakdown of the blood–retinal barrier. Taken together, these results suggest the importance of platelets (or their MPs) in the stabilization of angiogenic vessels.

The effects of platelets in angiogenesis might be mediated by platelet granular growth factors and cytokines released from platelets in addition to platelet–vessel wall interactions. Using a model for platelet-dense granule and lysosomal-secretion deficiency, we investigated their role in angiogenesis. We did not find any defects in angiogenesis in LysOTΔ mice, suggesting that the dense granule contents and lysosomal secretion may not be critical for the formation of new blood vessels. Although our study demonstrates that the dense granules and secretory lysosomes of platelets are not essential in angiogenesis, it is likely that α-granules play an important role in angiogenesis. Because thrombocytopenia produced a stronger phenotype than lack of platelet adhesion, activation of platelets after adhesion may trigger secretion of α-granule contents such as growth factors (e.g., VEGF, TGFβ, and platelet-derived growth factor), which stimulate endothelial sprouting and formation of new vessels. At present, however, no model for α-granule deficiency is available to test this hypothesis directly.

Platelets are surrounded by a membrane that consists of phospholipids. Three angiogenic phospholipids (lysophosphatidate, phosphatidic acid, and sphingosine-1-phosphate) have mitogenic activities and stimulate migration, proliferation, adhesion, junction assembly, liberation of endothelial cells from

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**Fig. 5.** Visualization of platelets in vivo. Platelet–endothelium interactions were investigated in angiogenic and mature quiescent vessels in a dorsal skin fold chamber by in vivo fluorescence microscopy. (A) Two representative images taken 3 s apart show the same field within the Matrigel. Arrows indicate platelets that remained adherent during this period. Asterisks in Upper indicate examples of nonadherent platelets (moved away in Lower). It is important to note that only ~5% of the circulating platelets were fluorescent. Thus, the actual number of adherent platelets is possibly much higher than shown. (Scale bars, 50 μm.) (B) Quantitative analysis of platelet adherence. Percentage of adherent platelets from total number of platelets observed in the field was determined as described in Materials and Methods. n = 4 animals in each group.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% platelets adhering for ≥3 s</th>
<th>p-value</th>
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<tr>
<td>Matrigel</td>
<td>29.63 +/- 0.04</td>
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<tr>
<td>Skin</td>
<td>8.74 +/- 0.04</td>
<td>&lt;0.004</td>
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**Discussion**

Based on clinical and preclinical findings, Folkman and colleagues (9) proposed that tumor angiogenesis depends not only on endothelial cells and tumor cells but also on platelet–endothelium interaction. The basis of this hypothesis was that platelets are a rich source of stimulators and inhibitors of angiogenesis and that they interact with the endothelium to change its properties. This hypothesis appears to apply to other angiogenic processes given that our current study indicates that the platelets and their adhesive function are critical because they stimulate the growth of new blood vessels while preventing excessive hemorrhage.

Recently, it was shown that platelet releasate promotes endothelial cell migration and that the addition of platelets into the Matrigel solution before injection induces angiogenesis in a dose-dependent manner (41). Our result showed that, when the platelets were depleted in vivo, there was a significant reduction of neovascularization as determined by the cornea micropocket assay (Fig. 1). This finding is in agreement with the result reported by Rhee et al. (42) showing a reduction of retinal neovascularization by thrombocytopenia, although excessive hemorrhage was not noted in that study. Rhee et al. (42)
established monolayers, and morphogenesis into capillary-like structures (24, 25, 48). Among these phospholipids, especially sphingosine-1-phosphate was shown to be important in the stabilization of angiogenic vessels (25, 48), where the pericytes may be involved (49). Sphingosine-1-phosphate could also be provided by the activated platelets.

Platelet–vessel wall interactions may involve platelet adhesion to ECM, to intact activated endothelium, or both. Resting platelets interact with endothelium by binding to P-selectin or VWF (50, 51). Both proteins can bind to GPIbα (51, 52). However, P-selectin knockout mice exhibited normal angiogenesis without hemorrhage (53), and, using the cornea model, we did not find any defect in VWF/−/− mice (our unpublished observations). This finding is in line with a previous report of normal angiogenesis in the VWF/−/− mice (42). Our study showed the critical role for the platelet GPIbα, because the replacement of its extracellular domain resulted in abnormal angiogenesis. Beside VWF and P-selectin, GPIb has other ligands such as the leukocyte integrin Mac-1 (54) and thrombospondin I (55). In addition, GPIb is a thrombin receptor and binds other coagulation factors (56). The hemostatic phenotype of the GPIbα/IL-4R mice is significantly more severe (W. Bergmeier, J.W., Z.M.R., and D.D.W., unpublished observations) than that of VWF/−/− mice that can form thrombi (57). Therefore, it is likely that several GPIb ligands could be involved in the various aspects of the angiogenic process.

In summary, we demonstrate that the absence of platelets inhibits the early stages of angiogenesis and leads to the formation of a decreased number of new vessels in vivo. Platelets are also required to prevent leakage and hemorrhage from the angiogenic vessels. Our working model is that the expression of GPIbα ligands in the sprouting vessel recruits platelets to this site. Their adhesion prevents excessive hemorrhage from the remodeling vessel. Platelet activation leads to release of active factors from α-granules, MP formation, and the secretion of angiogenic phospholipids. These promote endothelial migration, survival, and vessel stabilization. Taken together, these findings point to a central role of platelets, and their hemostatic function, in angiogenesis.

Materials and Methods

Mice. Eight- to 12-week-old, age-matched C57BL/6J, C57BL/6J-Lystk6, FcγR−/− (purchased from The Jackson Laboratory), and GPIbα/IL-4R (39) mice were used in the study. The Institutional Animal Care and Use Committees of the CBR Institute for Biomedical Research, Children's Hospital, and Massachusetts General Hospital approved the experimental procedures.

Induction of Thrombocytopenia. Mice were injected i.p. with 50 μg of pAb4 (58) (rat anti-mouse GPIbα) or control IgG2a [kind gifts from Bernhard Nieswandt (University of Würzburg, Würzburg, Germany) and later purchased from Emfret Analytics, Würzburg, Germany], and platelet counts were monitored by flow cytometry.

Mouse Cornea Micropocket Angiogenesis Assay. Central, intrastromal linear keratotomy was performed in the topically anesthetized eyes, and the micropocket was dissected toward the temporal limbus. The pocket was extended to 1.0 mm of the temporal limbus, and a single slow-release polymer Hydron pellet containing 80 ng of recombinant bFGF was placed into the pocket in each eye (59). The corneas and induced vascular response were examined by slit-lamp microscopy on days 3, 4, and 5 after implantation. We measured the number of vessels, clock hours, area of neovascularization, and hemorrhage.


Matrigel Plug Angiogenesis Assay. The method described by Pasaniti et al. (38) was used with minor modifications. Mice (7–8 weeks old) were injected s.c. with 300 μl of Matrigel (BD Biosciences) mixed with 80 ng of bFGF. Each mouse received two Matrigel implants. Seven days later, animals were killed and Matrigel plugs were carefully dissected away from the surrounding adherent tissue. Plugs were used for histological studies, determination of Hb levels, and Evans blue dye leakage.

Hb Determination in Matrigel. Matrigel implants were homogenized, and the Hb content of the implants was determined by a Drabkin reagent kit (Sigma). The OD at 540 nm was measured by using SpectraFluor.

Vascular Permeability Evaluation. Evans blue dye (2%; Sigma) in PBS was injected intravenously. Three hours after injection, the Matrigel plugs were isolated from the surrounding tissue, weight was determined, and plugs were put into 0.5 ml of formamide. Three days later the color intensity of the solutions was evaluated by spectrophotometer at 620 nm (Beckman-DU 65) in a masked fashion. OD per g was calculated.

Intravital Microscopy. Dorsal skin-fold chambers were implanted as described in ref. 40. After 2 days, 50 μl of Matrigel enriched with 80 ng of recombinant bFGF was placed on the upper tissue layer. The Matrigel implant became highly vascularized after 2–3 weeks. Mouse platelets were isolated as described in ref. 60, labeled with calcine-AM (Molecular Probes), and injected intravenously immediately before observation. Anesthetized mice were placed in left lateral decubital position on a Plexiglas pad; skin-fold chamber was locked into a fixed position, and vessels were visualized using an intravital microscope (Zeiss Axioplan) equipped with an intensified charge-coupled device video camera as described in ref. 61 with a water immersion objective (magnification ×20). Platelet adhesion to angiogenic vessels in Matrigel vs. quiescent vessels in control skin (n = 4 mice) was analyzed blinded to site. For each mouse, five to eight random fields were evaluated at three different time points. The result was expressed as percentage of platelets in the field that were adherent to the vessel wall for three or more seconds (immobile). All values were averaged to obtain one value per each animal.

Statistics. The values are presented as mean ± SEM. Group differences were evaluated by one-way ANOVA followed by a Kruskal–Wallis test. Corneal hemorrhage was scored on a graded scale of 0–4 (0 for minimum and 4 for a maximum). The grading was performed in a blinded manner and one-way ANOVA followed by Dunnett’s test by using STATVIEW 5.0 software. The differences in platelet adhesion observed by intravital microscopy were analyzed by the paired, one-tailed Student t test. P values of 0.05 or less were regarded as statistically significant.