Platelet Granule Secretion Continuously Prevents Intratumor Hemorrhage

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Abstract

Cancer is associated with a prothrombogenic state capable of platelet activation. Platelets, on the other hand, can support angiogenesis, a process involved in the progression of tumor growth and metastasis. However, it is unclear whether platelet/tumor interactions substantially contribute to tumor physiology. We investigated whether platelets stabilize tumor vessels and studied the underlying mechanisms. We induced severe acute thrombocytopenia in mice bearing s.c. Lewis lung carcinoma or B16F10 melanoma. Intravital microscopy revealed that platelet depletion led to a rapid destabilization of tumor vessels with intratumor hemorrhage starting as soon as 30 min after induction of thrombocytopenia. Using an inhibitor of glycoprotein Ibα (GPIbα) and genetically engineered mice with platelet adhesion defects, we investigated the role of platelet adhesion receptors in stabilizing tumor vessels. We found that a single defect in either GPIbα, von Willebrand factor, P-selectin, or platelet integrin activation did not lead to intratumor hemorrhage. We then compared the ability of transfused resting and degranulated platelets to prevent intratumor hemorrhage. Whereas resting platelets prevented thrombocytopenia-induced tumor bleeding, circulating degranulated platelets did not. This suggests that the prevention of intratumor hemorrhage by platelets relies on the secretion of the content of platelet granules. Supporting this hypothesis, we further found that thrombocytopenia dramatically impairs the balance between propermeability and antipermeability factors in tumor-bearing animals, in particular depleting blood of angiopoietin-1 and serotonin. Our results show a crucial contribution of platelets to tumor homeostasis through continuous prevention of severe intratumor hemorrhage and consequent cell death. The study also suggests platelet function as a reasonable target for specific destabilization of tumor vessels. [Cancer Res 2008;68(16):6851–8]

Introduction

By 1865, Armand Trousseau had already reported the association between cancer and thrombosis (1). The thrombogenic properties of tumors have since been widely studied. Tumor cells promote coagulation and inflammation through various mechanisms, including overexpression of tissue factor (2), the initiator of the coagulation cascade, and secretion of proinflammatory cytokines (3, 4) and of metalloproteinases (5), which lead to endothelial activation, an important step in thrombosis. In addition, sluggish blood flow, hyperpermeability, and discontinuous endothelial lining are recognized features of tumor microcirculation (6, 7) that may also contribute to the tumor thrombogenic environment. This thrombogenic environment could activate platelets, the major orchestrators of coagulation and thrombosis.

Platelets may influence cancer progression. Both depletion of platelets and antiplatelet treatments have been shown to reduce the number of experimental metastases, indicating that platelets support the metastatic process (8–10). Various mechanisms have been proposed to explain this effect. Coating of circulating cancer cells with platelets may protect cancer cells from the immune response (8) and facilitation of cancer cell adhesion to leukocytes and endothelial cells by platelets may promote the essential step of extravasation in the metastatic process (8, 10, 11). Several studies have shown that platelets enhance the formation of capillary-like structures by endothelial cells *in vitro* (12) and angiogenesis *in vivo* (13), a process that is essential to tumor growth and metastasis. Platelets are a rich source of proangiogenic and antiangiogenic factors [vascular endothelial growth factor (VEGF; ref. 14), platelet-derived growth factor (15), basic fibroblast growth factor (16), epidermal growth factor (17), transforming growth factor (18), insulin-like growth factors (19), angiopoietin-1 (20), sphingosine-1-phosphate (21), matrix metalloproteinases (22), thrombospondin I (23), platelet factor 4 (24), plasminogen activator inhibitor I (25), endostatin (26), and angiostatin (27)] that are released on platelet activation. These proangiogenic and antiangiogenic factors are mostly organized into separate platelet α-granules that could be differentially released, suggesting that platelets may actively stimulate or inhibit angiogenesis (28).

Recently, we showed that platelets and their adhesion support angiogenesis *in vivo* in experimental models of angiogenesis. Platelets prevented excessive hemorrhage from the growing vessels in Matrigel and corneal micropocket assays (29). We now investigated the contribution of platelets to the function of tumor vessels and the mechanisms involved. We found that platelets continuously support tumor vascular homeostasis by regulating the stability of tumor vessels through the secretion of the content of their granules.

Materials and Methods

Reagents. Fetal bovine serum (FBS) was from the American Type Culture Collection (ATCC). Penicillin/streptomycin and high-glucose DMEM were from Life Technologies/Invitrogen. Medium titanium skinfold chambers were from APJ Trading Co. Polyclonal anti–glycoprotein Ibα (GPIbα) rat IgG R300 and polyclonal nonimmune rat IgG C301 were from emfret Analytics. Hematoxylin, eosin, prostacyclin (PGI2), trypsin-EDTA, and Drabkin's reagent

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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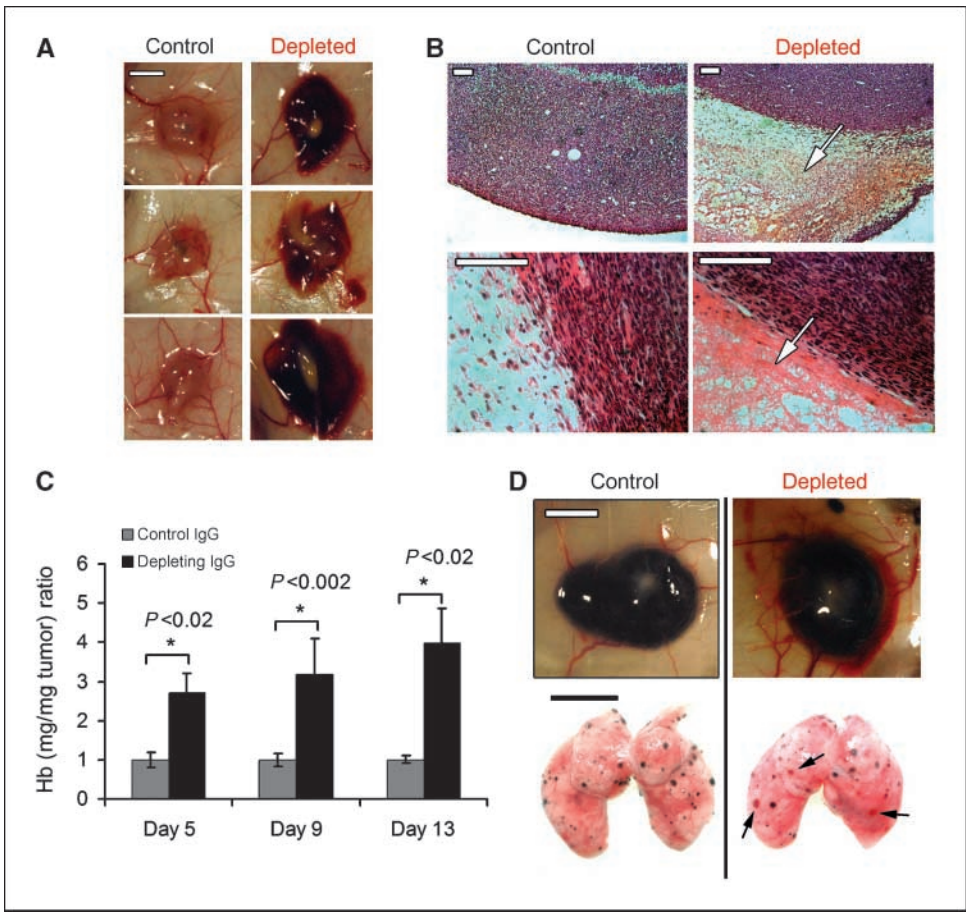

Figure 1. Acute thrombocytopenia induces tumor bleeding independently of the tumor type, age, and location. *A,* at day 8 after s.c. implantation of LLC tumor cells, mice were injected with either the control IgG (*Control*) or the platelet-depleting IgG (*Depleted*) and tumors were photographed 18 h later. *Bar,* 5 mm. *B, arrows,* H&E staining of the LLC tumors showed massive accumulation of RBCs in the tumor stroma only in platelet-depleted mice. *Bars,* 100 μm. *C,* thrombocytopenia was induced at days 4, 8, or 12 following s.c. LLC implantation. Eighteen hours later, intratumor hemoglobin content was determined and compared with control IgG-treated tumors ($n = 4$). *D,* 10 d following either s.c. (*top*) or i.v. (*bottom*) injection of B16F10 melanoma cells, mice were injected with either the control IgG (control) or the platelet-depleting IgG (depleted). Eighteen hours later, photographs of skin and lungs were taken. *Arrows,* hemorrhage was observed only in tumors from platelet-depleted mice. *Bars,* 5 mm.

were from Sigma-Aldrich. *O*-sialoglycoprotein endopeptidase was from Cedarlane Laboratories. 4′,6-Diamidino-2-phenylindole (DAPI) was from Molecular Probes Invitrogen. GPG-290, an inhibitor of the von Willebrand factor (vWF)/GPIbα interaction (30), was prepared by Wyeth Research and was a kind gift of Dr. Gray D. Shaw (Wyeth Research, Cambridge, MA).

Animals. All animal procedures described in this study were performed using 6- to 8-wk-old C57BL/6J female mice (purchased from The Jackson Laboratory) except in experiments using dorsal skinfold chamber for which 12-wk-old C57BL/6J male mice were used. Mice deficient in VWF, P-selectin, and CalDAG-GEFI were bred and housed in our animal facility. All experimental procedures involving the use of mice were approved by the Animal Care and Use Committee of the Immune Disease Institute.

Cell culture. Murine B16F10 melanoma cells and Lewis lung carcinoma cells (LLC) were purchased from ATCC. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2, high-glucose (4.5 g/L) DMEM supplemented with 10% FBS and 1% glutamine and were used by passage 10 for implantation into syngenic C57BL/6J recipient mice.

Tumor cell implantation. One hundred microliters of either LLC or B16F10 melanoma cells at 1.0×10^7/mL in Dulbecco's PBS were injected s.c. into the back of 6- to 8-wk-old C57BL/6J female mice.

Induction of thrombocytopenia. Thrombocytopenia was induced at indicated time points following tumor cell implantation by an i.v. injection of 2.5 μg/g mouse of the platelet-depleting antibody (polyclonal anti-mouse GPIbα rat IgG; emfret Analytics; ref. 8). Control mice were injected with a nonimmune rat polyclonal IgG (emfret Analytics). Thrombocytopenia was evaluated by flow cytometry.

The i.v. injection of the depleting antibody resulted in ≥9% reduction in circulating platelets at 1 h after injection in all mice.

Determination of intratumor hemoglobin content. Tumors were excised from the back of the sacrificed animals, weighed, homogenized in Drabkin's reagent (Sigma), and centrifuged ($2000 \times g$; 10 min) and hemoglobin content of supernatants was measured by absorbance reading at 540 nm.

Immunohistology of LLC tumors. S.c. LLC tumors were harvested from sacrificed animals, fixed in zinc fixative (100 mmol/L Tris-HCl containing 37 mmol/L zinc chloride, 23 mmol/L zinc acetate, and 3.2 mmol/L calcium acetate), paraffin embedded, and sectioned. Tumor sections were stained with H&E or with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL; Roche Applied Science) to visualize DNA fragmentation. Tumor cell mitosis was quantified by immunostaining of bromodeoxyuridine (BrdUrd) incorporation using a BrdUrd labeling and detection kit (Roche Applied Science). Tumor sections stained for BrdUrd incorporation or for TUNEL were counterstained with DAPI to visualize all nuclei. After washing, the slides were mounted with Gel/Mount aqueous mounting medium (Biomeda) and observed under an epifluorescence microscope. The proliferative and apoptotic indexes were calculated as the percentage of either BrdUrd- or TUNEL-positive nuclei relative to DAPI-stained nuclei, respectively. For H&E staining, slides were mounted with DPX Mountant (Fluka BioChemika) and observed in light microscopy.

Metastasis. Subconfluent B16F10 melanoma cells (70–80%) were washed with PBS and detached by brief exposure to 0.25% trypsin and 0.2% EDTA. Cells were washed twice with PBS, resuspended in serum-free medium, and kept on ice until injection. Tumor cell (100 μL, 1×10^5 cells) suspension was injected to the lateral tail vein of mice. Ten days later, mice were injected with either the control IgG or the platelet-depleting IgG. The day following the induction of thrombocytopenia, lungs were harvested, perfused, and washed with PBS and photographed.

Tail bleeding time. Vehicle (PBS) or GPG-290 (5 mg/kg mouse) was injected i.v. at day 8 following s.c. LLC cell implantation and tail bleeding time was assessed the following day before tumor excision. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) before cutting 3 mm of the distal tip of the tail using a sharp

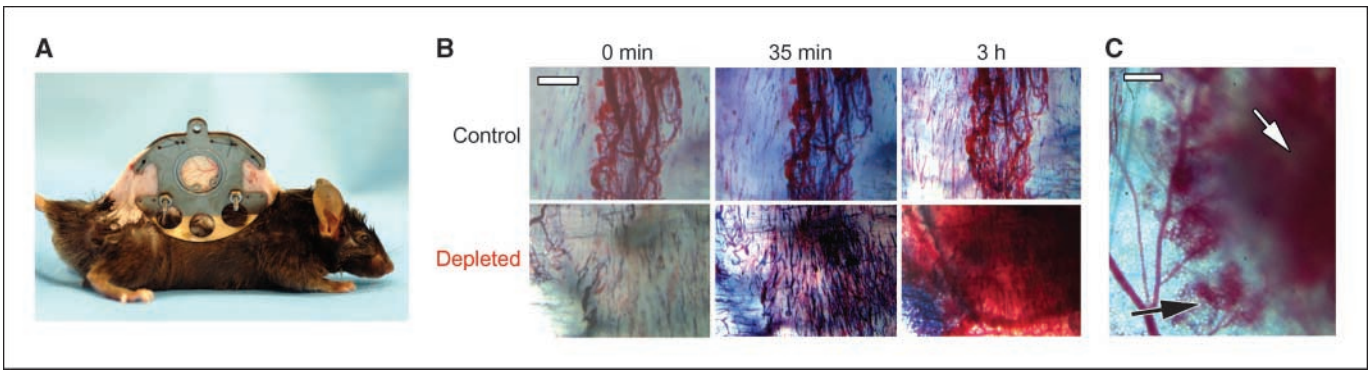

Figure 2. Kinetics and localization of tumor bleeding in platelet-depleted mice. *A,* mouse carrying a dorsal skinfold chamber. *B,* mice bearing 5-d-old LLC tumors were injected with Evans blue and either the control (*top*) or the platelet-depleting antibody (*bottom*) at time 0. Tumors were observed through the dorsal skinfold window for 3 h. Times after infusion are indicated. *Bar,* 500 μm. *C,* LLC tumor viewed 3 h after induction of thrombocytopenia. *White arrow,* intratumor hemorrhage; *black arrow,* hemorrhage occurring from vessels surrounding the tumor. *Bar,* 500 μm.

razor blade. The tail was immediately immersed in 37°C PBS and the time required for the bleeding to stop was determined. If bleeding did not recur within 15 s of cessation, it was considered to have stopped. Experiments were terminated after 15 min if no cessation of blood flow occurred.

***In vivo* imaging of LLC.** Dorsal skinfold chambers and surgical preparation were performed as described (31). After 2 d of recovery, 5×10^5 LLC cells were implanted in the conjunctive tissue below the striated skin muscle layer of the remaining skin layer and allowed to grow for 5 d.

Mice were then injected i.v. with 100 μL of 5% Evans blue and tumors were observed through the dorsal skinfold chamber for 3 h starting from the injection of either the control or the platelet-depleting antibody. During *in vivo* microscopy, mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Light microscopy imaging was performed using an upright microscope (Axioplan; Zeiss) with a ×2.5 magnification objective and recorded by a digital camera (AxioCam HSc) attached to it. Data acquisition was done with the time-lapse function in the software from the same manufacturer (Axiovision 4.6.3).

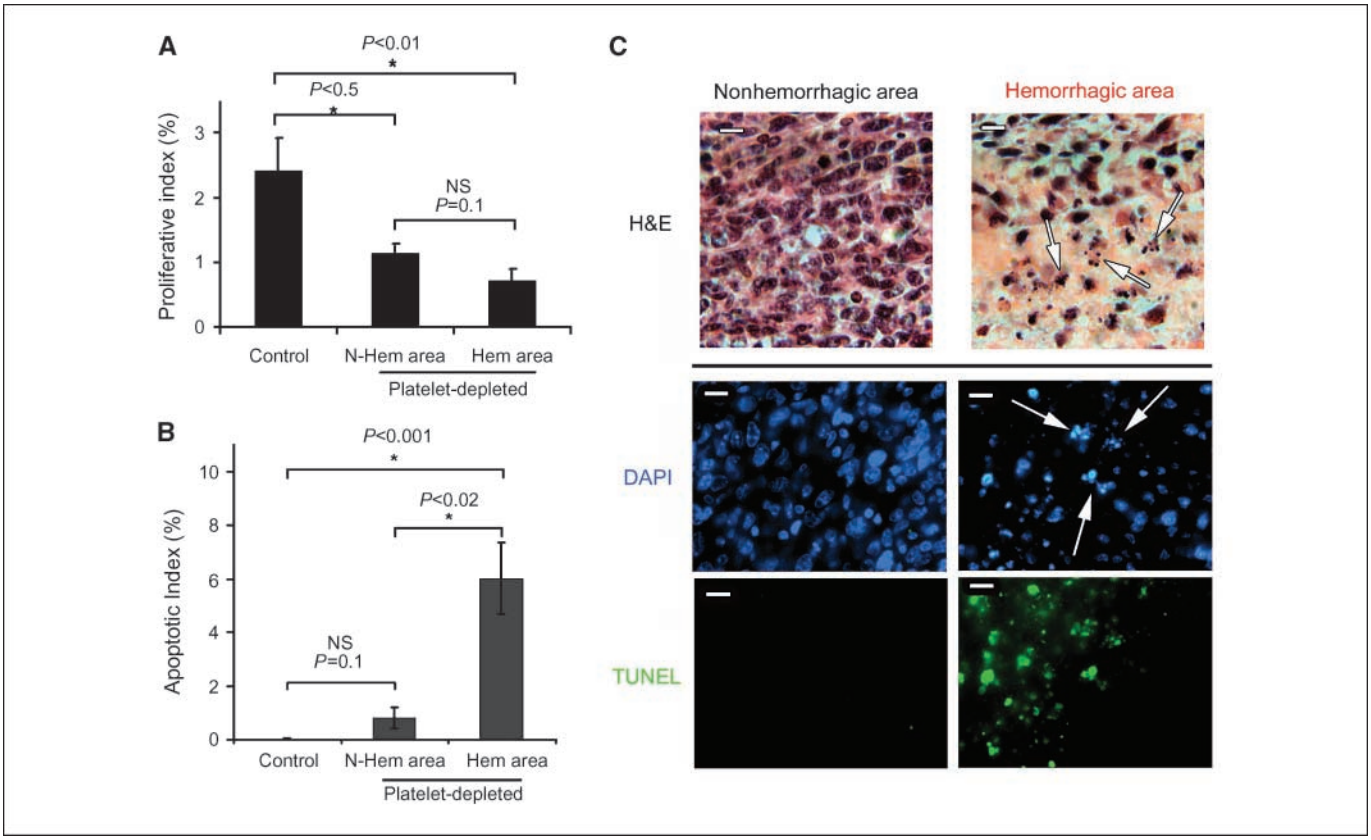

Figure 3. Thrombocytopenia reduces cancer cell proliferation and locally affects tumor viability. At day 8 after s.c. implantation of LLC tumors, thrombocytopenia was induced, and 48 h later, tumors were harvested and sectioned. *A,* mice were injected with BrdUrd 3 h before sacrifice and the proliferative index was calculated as the percentage of BrdUrd-positive nuclei relative to DAPI-stained nuclei (n = 10 microscopic fields out of four tumors for each). *B,* the apoptotic index was calculated as the percentage of TUNEL-positive nuclei relative to DAPI-stained nuclei (n = 10 microscopic fields out of five tumors for each). *C,* H&E, DAPI, and TUNEL staining of nonhemorrhagic and hemorrhagic areas of the LLC tumors. *Arrows,* fragmented and condensed nuclei. *Bars,* 20 μm.

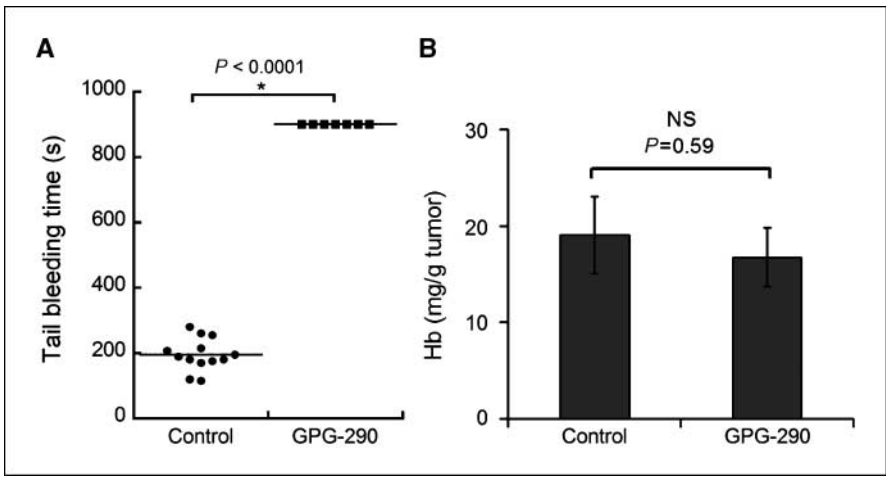

Figure 4. Prevention of tumor hemorrhage by platelets is independent of platelet GPIbα. PBS or the GPIbα chimera (GPG-290) was injected i.v. at day 8 following s.c. LLC tumor implantation. *A,* tail bleeding time was assessed 18 h after GPG-290 injection. *B,* comparison of hemoglobin (*Hb*) content of control and GPG-290–treated tumors ($n = 5$). No difference in hemoglobin content was found between the two groups.

Quantification of VEGF, angiopoietin-1, and serotonin levels. VEGF levels in platelet-poor plasma (PPP), serum, and tumor were assayed using an ELISA kit (R&D Systems) according to the manufacturer's instructions. Angiopoietin-1 levels were quantified by ELISA using glutaraldehyde-immobilized recombinant mouse Tie2-Fc chimera (R&D Systems) for capture and a goat polyclonal to angiopoietin-1 (Santa Cruz Biotechnology) for detection. In addition, PPP and serum samples from control and platelet-depleted mice were analyzed by SDS-PAGE (10%, reducing conditions) and Western blotting with a goat polyclonal antibody to angiopoietin-1. Serotonin levels were quantified by enzyme immunoassay according to the manufacturer's instructions (Labor Diagnostika Nord GmbH & Co. KG).

Platelet preparation. Donor mice were bled from the retro-orbital venous plexus under anesthesia. Blood was collected into polypropylene tubes containing 7.5 units/mL heparin. Platelet-rich plasma (PRP) was obtained by centrifugation at 200 × *g* for 5 min at room temperature. The PRP was incubated for 2 min with PGI2 (0.1 μg/mL) and platelets were isolated by centrifugation at 850 × *g* for 5 min. The resulting pellet was washed and resuspended in Tyrode's buffer [137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L $NaHCO_3$, 0.3 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 5 mmol/L HEPES (pH 7.3)] containing 0.35% bovine serum albumin.

Treatment of platelets with *O*-sialoglycoprotein endopeptidase. To prevent clearance of transfused platelets by the anti-GPIbα antibody, the external part of GPIbα was removed before transfusion by treating the platelets with *O*-sialoglycoprotein endopeptidase as previously described (32). Briefly, washed platelets were resuspended in Tyrode's buffer containing 1 mmol/L $CaCl_2$ and incubated at 37°C for 30 min with 250 μg/mL *O*-sialoglycoprotein endopeptidase. Aliquots of the platelet suspensions were analyzed by flow cytometry to assess the removal of the NH_2-terminal domain of GPIbα using a FITC-conjugated antibody directed against the extracellular domain of GPIbα (emfret Analytics).

Activation of platelets. For activation, platelets pretreated with *O*-sialoglycoprotein endopeptidase were stimulated with human thrombin (1 units/mL) for 10 min at 37°C in the presence of 2 mmol/L EDTA to avoid aggregation. Hirudin (2 units/mL) was added to stop the reaction. Platelet degranulation was confirmed by expression of P-selectin using FITC-conjugated anti-P-selectin antibody (BD PharMingen). EDTA and hirudin were also added to resting platelet preparations. Aliquots of resting and activated platelets supernatant were analyzed for angiopoietin-1 content by ELISA.

Statistical analysis. Data are presented as mean ± SE and were analyzed by ANOVA and by unpaired two-tailed Student's *t* test. *P* values of <0.05 were regarded as statistically significant.

Results

Acute thrombocytopenia induces severe tumor hemorrhage independent of the tumor type, age, and location. Platelet depletion was induced in mice bearing s.c. tumors at days 4, 8, and 12 following LLC tumor cell implantation. Eighteen hours after the induction of platelet depletion, the mice treated with the platelet-depleting antibody had less than 2.5 ± 0.9% of normal platelet count, whereas platelet number was unaffected in mice treated with the control IgG (94 ± 7% of normal platelet count) compared with non–tumor-bearing untreated control mice. Morphologic examination and H&E staining of the s.c. tumors revealed extensive hemorrhage in and around all tumors of platelet-depleted mice but not in mice treated with control IgG (Fig. 1*A* and *B*). As illustrated in Fig. 1*B*, massive accumulation of RBCs was observed at the interface of the tumor and the adjacent connective tissue in the platelet-depleted mice. Hemorrhage was not seen anywhere in areas distant from the tumor in the thrombocytopenic mice.

Thrombocytopenia-induced tumor bleeding was independent of the age of the tumor. Induction of thrombocytopenia at either day 4, 8, or 12 after tumor cell implantation invariably resulted 18 h later in a 2- to 3-fold increase in intratumor hemoglobin content compared with tumors from control mice with normal platelet counts (Fig. 1*C*). This indicates that platelets are required continuously to prevent hemorrhage from primary tumor vessels. Interestingly, induction of acute severe thrombocytopenia also resulted in hemorrhage in s.c. implanted B16F10 melanoma and in established B16F10 melanoma lung metastasis (Fig. 1*D*), indicating that the requirement of platelets for the prevention of intratumor hemorrhage was likely independent of the tumor type, age, and location.

Intravital observation of s.c. LLC tumors through a dorsal skinfold chamber (Fig. 2*A*) revealed that plasma protein leakage, detected by extravasation of Evans blue, occurs continuously and excessively in both control and thrombocytopenic mice (Fig. 2*B*; Supplementary Movies). In contrast, tumor hemorrhage was observed only in the thrombocytopenic mice. First signs of tumor hemorrhage occurred as soon as 35 min following the injection of the depleting antibody (Fig. 2*B*; Supplementary Movie 2). In depleted animals, plasma protein leakage and hemorrhage were observed both from intratumor vessels and from postcapillary venules directly surrounding the tumor (Fig. 2*B* and *C*). Altogether, these results show that platelets continuously help prevent excessive tumor vessel fragility.

Acute thrombocytopenia reduces tumor cell proliferation and increases tumor necrosis. Induction of acute thrombocytopenia leads to severe tumor hemorrhage, an event that is likely

to affect tumor cell viability. We therefore studied the effect of acute thrombocytopenia on tumor growth and survival. Mice bearing 8-day-old LLC tumors were injected with either the control or the platelet-depleting antibody and tumors were allowed to grow for 2 more days. No significant differences in wet and dry weight could be found between the excised tumors of thrombocytopenic and control mice (data not shown). Interestingly, however, quantitation of tumor cell mitosis by BrdUrd incorporation and *in situ* immunostaining revealed a decrease in the proliferative index of tumors from thrombocytopenic mice compared with tumors from control mice (0.9 ± 0.14% versus 2.3 ± 0.47%; $P < 0.004$). Less proliferation was observed in areas both distant and proximal to the hemorrhage (Fig. 3*A*).

H&E staining of LLC tumors from thrombocytopenic mice revealed that tumor necrosis could be observed in the area next to the hemorrhage as indicated by morphologic changes such as nuclear condensation and fragmentation (Fig. 3*C, top*). This observation was further confirmed by quantitation of apoptotic cells by TUNEL staining (Fig. 3*B* and *C*). The TUNEL apoptotic index in the nonhemorrhagic areas of tumors from platelet-depleted mice (0.8 ± 0.9%) was not significantly different ($P = 0.1$) from that of tumors from control mice (0.1 ± 0.05%). In contrast, the TUNEL apoptotic index in the hemorrhagic areas of tumors from platelet-depleted mice was significantly increased compared with nonhemorrhagic regions (6.01 ± 4.03%; $P < 0.001$; Fig. 3*B*). This indicates that thrombocytopenia-induced tumor bleeding is injurious to the cancer cells.

Prevention of tumor-associated hemorrhage by platelets does not rely on their ability to form thrombi. We asked whether the continuous requirement of platelets to prevent tumor hemorrhage was dependent on their capacity to adhere to the vessel wall and to form thrombi. To determine this, we first studied the effect of GPG-290, a soluble competitive inhibitor of the platelet GPIbα/VWF interaction (30), on intratumor hemoglobin content. GPIbα-mediated platelet adhesion was previously found by our group to play a role in preventing hemorrhage during experimental angiogenesis not associated with tumors (29). However, although the GPG-290–treated mice could not arrest their bleeding as indicated by their increased tail bleeding time (Fig. 4*A*), no increase in their tumor hemoglobin content was found compared with control mice (Fig. 4*B*). This indicates that prevention of tumor-associated hemorrhage by platelets is independent of platelet GPIbα/VWF interaction. This was further confirmed by the absence of severe hemorrhage in LLC tumors implanted in $VWF^{-/-}$ mice (zero hemorrhage in five tumors examined) that also have prolonged tail bleeding time (33). Besides VWF, P-selectin mediates platelet rolling on the activated vessel wall (34). As for $VWF^{-/-}$ mice, no severe hemorrhage was found in LLC tumors grown in P-$selectin^{-/-}$ mice (zero hemorrhage in six tumors examined), thus indicating that P-selectin was also not crucial for the prevention of intratumor hemorrhage by platelets.

Mouse platelets lacking CalDAG-GEFI are severely compromised in integrin-dependent platelet aggregation because CalDAG-GEFI is a key signal integrator in the cascade leading to the activation of the integrin αIIbβ3 (35). LLC tumors grown in $CalDAG\text{-}GEFI^{-/-}$ mice did not show hemorrhage (zero hemorrhage in seven tumors examined) compared with LLC tumors grown in wild-type mice. This suggests that platelet integrin activation is also not required to prevent hemorrhage from angiogenic tumor vessels. Moreover, immunofluorescent staining using anti-GPIbα and anti-αIIbβ3 antibodies as well as H&E staining of LLC sections did not reveal any platelet aggregates or significant numbers of platelets adhering to the lumens of tumor vessels (data not shown). Altogether, these results suggest that the classic mechanisms of platelet adhesion and aggregation involved in primary hemostasis/thrombus formation might not be required for the prevention of intratumor hemorrhage by platelets.

Thrombocytopenia leads to an altered balance between propermeability and antipermeability factors. Platelets are an important source of angiogenic factors and of regulators of vascular permeability, such as VEGF, angiopoietin-1, and serotonin (14, 20, 36). Both VEGF and angiopoietin-1 have been previously reported to affect blood vessel maturation and stability as well as

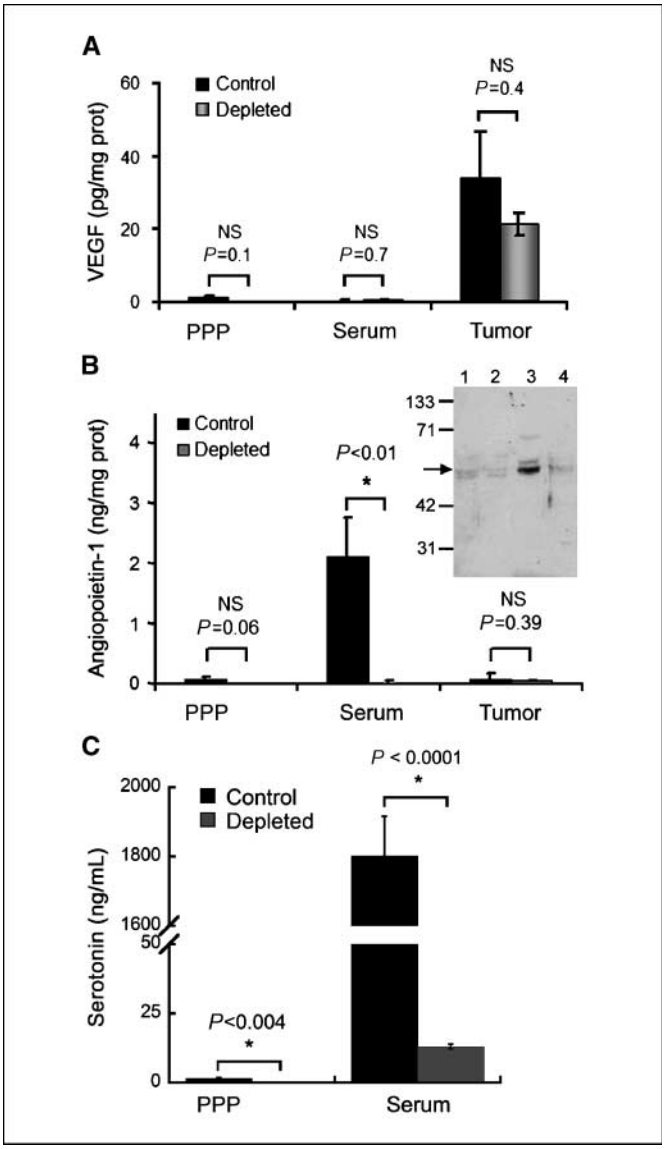

Figure 5. Platelet depletion effects on serum concentrations of VEGF, angiopoietin-1, and serotonin. *A,* comparison of VEGF levels in LLC tumor-bearing control and platelet-depleted mice ($n = 5$). *B,* comparison of angiopoietin-1 levels between control and platelet-depleted mice ($n = 5$). *Inset,* Western blot detection of angiopoietin-1. *Lane 1,* PPP from control mouse; *lane 2,* plasma from platelet-depleted mouse; *lane 3,* serum from control mouse; *lane 4,* serum from platelet-depleted mouse. *Arrow,* angiopoietin-1. *C,* comparison of serotonin levels between control and platelet-depleted mice ($n = 4$–6). Platelet depletion led to disappearance of serotonin and angiopoietin-1 from serum without affecting VEGF levels.

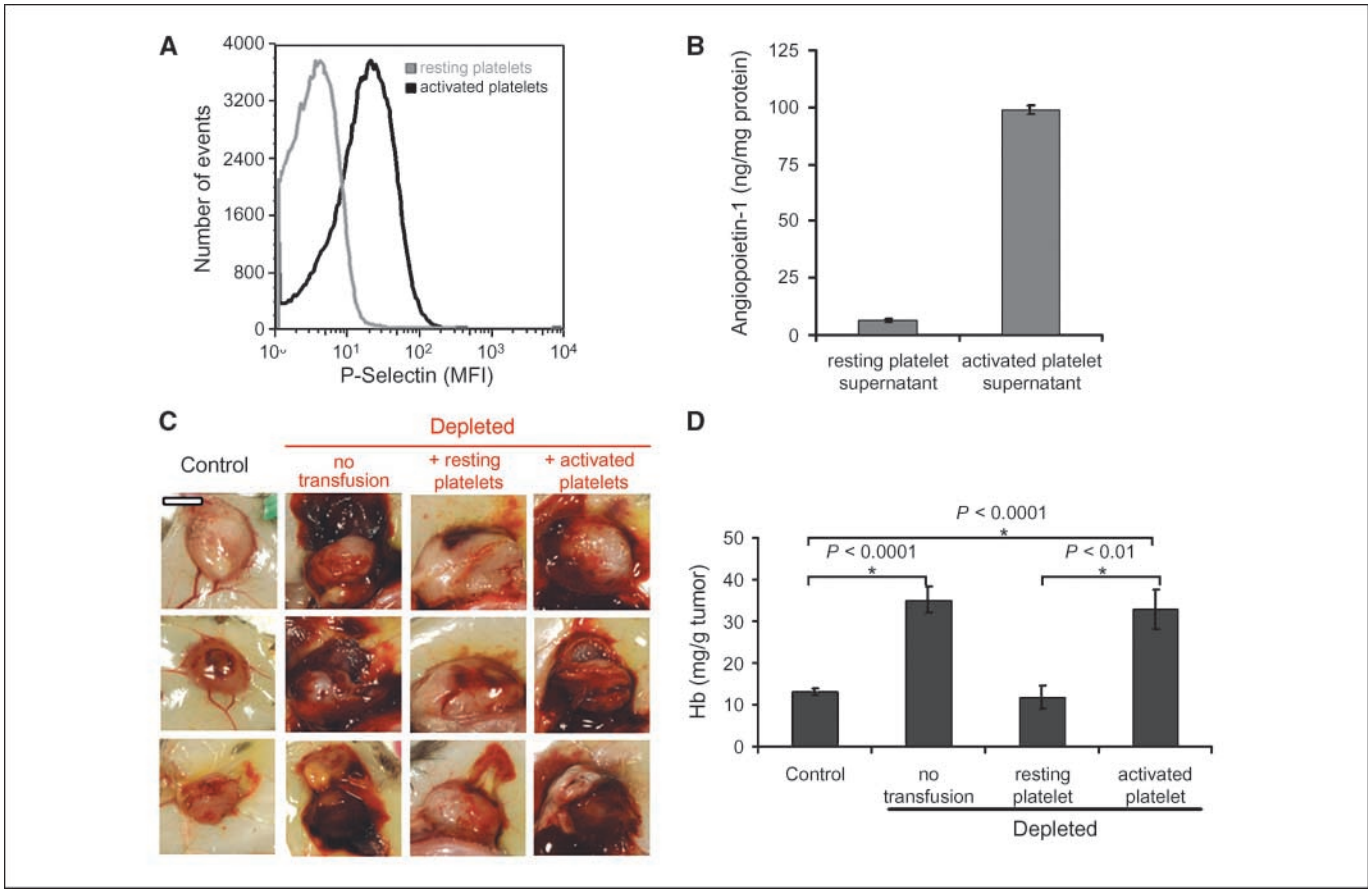

Figure 6. Degranulated platelets are unable to prevent thrombocytopenia-induced tumor bleeding. Degranulation of thrombin-stimulated platelets was assessed by FACS analysis of P-selectin surface expression (*A*) and by quantitation of angiopoietin-1 in platelet supernatants by ELISA (*B*). *MFI,* mean fluorescence intensity. *C,* at day 8 after tumor cell implantation, mice were injected with either the control IgG (*Control*) or the platelet-depleting IgG (*Depleted*). A subset of mice was transfused 30 min before the induction of thrombocytopenia with tyrode buffer (*no transfusion*) or with 7×10^8 of either resting or activated platelets and s.c. LLCs were photographed 18 h later. *Bar,* 5 mm. *D,* comparison of the hemoglobin content of control tumors and platelet-depleted tumors from mice transfused with either tyrode buffer, resting platelets, or activated platelets (n = 17–20).

vascular permeability in adult mice (37). We therefore investigated whether the absence of platelets could substantially affect the concentrations of these factors in PPP, serum, and tumor, thus resulting in a modification of the balance between propermeability and antipermeability factors. VEGF was found most abundantly in tumors and its levels in PPP, serum, and tumor were not significantly affected by thrombocytopenia (Fig. 5*A*). Angiopoietin-1 and serotonin were mostly found in serum in tumor-bearing mice (Fig. 5*B* and *C*) and were found in similar levels in serum of non–tumor-bearing animals (data not shown). In contrast to VEGF (Fig. 5*A*), angiopoietin-1 and serotonin levels in serum were dramatically decreased in tumor-bearing thrombocytopenic mice compared with tumor-bearing control mice (Fig. 5*B* and *C*). Similarly, thrombocytopenia led to the disappearance of serum angiopoietin-1 and serotonin in mice without tumors (data not shown). This indicates that the serotonin and angiopoietin-1 load in platelets accounts for the majority of the circulating levels of these two key regulators of vascular permeability. Thus, whereas the potent propermeability factor VEGF is consistently produced by the LLC tumor and relatively little is found in plasma or serum, the two antipermeability factors angiopoietin-1 and serotonin are primarily found in platelets and not in tumors. Our results document that severe acute thrombocytopenia leads to an altered balance between the platelet-derived pool of antipermeability factors and the tumor-derived propermeability factor VEGF, also known as vascular permeability factor.

Degranulated platelets are unable to prevent thrombocytopenia-induced tumor bleeding. To investigate the role of the content of platelet granules in preventing tumor hemorrhage, we compared the ability of transfused resting platelets and thrombin-stimulated platelets (with released granules) to rescue tumor bleeding in thrombocytopenic tumors. Thrombin-activated platelets have been previously shown to rapidly lose surface P-selectin when transfused but to continue to circulate and function (38, 39). To avoid clearance of the transfused platelets by the depleting anti-GPIbα antibody, platelets lacking the extracellular domain of GPIbα were used. The removal of the extracellular domain of GPIbα was performed with *O*-sialoglycoprotein endopeptidase before transfusion (32). Degranulation of thrombin-stimulated platelets was verified by fluorescence-activated cell sorting (FACS) analysis of P-selectin surface expression and by the presence of angiopoietin-1 in platelet supernatants (Fig. 6*A* and *B*). Whereas transfusion of resting platelets could prevent tumor bleeding in thrombocytopenic mice, transfusion of degranulated (P-selectin positive) platelets could not (Fig. 6*C* and *D*), indicating that prevention of tumor vessel bleeding by platelets likely relies on

the local release of a soluble factor from platelet granules rather than on the formation of platelet plugs.

Discussion

In the present report, we addressed the contribution of platelets to tumor vascular homeostasis. We show that platelets are crucial regulators of tumor vessel stability that are continuously needed to prevent severe tumor hemorrhage. In fact, absence of platelets leads to an immediate destabilization of tumor vessels with intratumor hemorrhage starting within the first hour following the induction of thrombocytopenia. This thrombocytopenia-induced tumor bleeding seems to be independent of the tumor type and location because it could be seen in s.c. LLC tumors, B16F10 melanoma, and in lung metastasis (Fig. 1). We recently reported that absence of platelets leads to high susceptibility to hemorrhage in blood vessels during inflammation (40). In this previous study, we showed that in thrombocytopenic mice inflammation induces hemorrhage at the inflamed site, indicating that platelets prevent tissue-damaging hemorrhage in inflamed organs (40). Solid tumors often show signs of chronic inflammation. These include the presence of leukocyte infiltration, the expression of cytokines such as tumor necrosis factor-α or interleukin-1, chemokines, and active tissue remodeling (41, 42). We hypothesize that the requirement of platelets for the prevention of tumor hemorrhage might include not only their supportive role in angiogenesis (29) but also their ability to prevent inflammation-induced vascular injury (40).

Platelets are known to carry biologically active agents, such as angiopoietin-1 and serotonin, which have been shown to promote endothelial integrity and barrier function *in vitro* and *in vivo* (36, 37, 43–46). Angiopoietin-1 is known to stabilize blood vessels, to inhibit vascular permeability, and to have anti-inflammatory properties (47–49). Serotonin was shown to prevent RBC extravasation in thrombocytopenic hamsters (46). In contrast, tumors release destabilizing factors, the most prominent being VEGF, and activated endothelium, as would be found in tumors, secretes angiopoietin-2, another potent destabilizer of vasculature (50). Angiopoeitin-2 action is through competitive inhibition of angiopoietin-1 (50). We hypothesize that the balance between the tumor and platelet-derived agents is regulating tumor vessel stability. Indeed, our study reveals the crucial contribution of the platelet-derived products in the prevention of tumor hemorrhage. We show that platelet depletion leads to a dramatic decrease in both angiopoietin-1 and serotonin levels in serum, whereas VEGF levels remain unaffected. This illustrates that severe acute thrombocytopenia leads to an impaired balance between available propermeability and antipermeability factors that may contribute to tumor vessel destabilization. It is of note, however, that systemic i.v. infusion of platelet releasate did not prevent tumor bleeding in thrombocytopenic mice (data not shown). We speculate that the platelet-derived factors responsible for tumor vessel stabilization have to be delivered by platelets to the tumor site for optimal activity.

Surprisingly, prevention of severe intratumor hemorrhage by platelets does not seem to require platelet plug formation, which relies on platelet adhesion receptors GPIbα/VWF and activation of the integrin αIIbβ3. Although CalDAG-GEFI$^{-/-}$, VWF$^{-/-}$, or GPIbα inhibitor-treated mice all have a severe bleeding phenotype, manifesting a highly prolonged bleeding time on injury, severe intratumor hemorrhage did not occur in any of these animals. Thus, similar to the prevention of hemorrhage in inflamed tissues (40), tumor vessel stabilization by platelets might require neither platelet adhesion to the vessel wall nor platelet aggregation. These observations agree with those of Manegold and colleagues (51) who could not detect an increase in platelet adhesion in tumor vessels. The fact that genetic inhibition of platelet adhesion and aggregation did not affect their capacity to stabilize tumor vessels raises questions about how platelets deliver vasoactive compounds to tumor vessels. As previously hypothesized by Pinedo and colleagues (52), platelet/tumor interactions might be facilitated by the impaired blood flow (51, 53–55) and the localized granular release by the procoagulant environment of the tumor (56–58).

The identification of the tumor vessel–stabilizing factor(s) delivered by platelets to solid tumors could lead to new therapeutic strategies. Inhibition of these platelet-derived factors may allow selective induction of tumor bleeding and thus decrease tumor viability and/or growth (Fig. 3) without affecting the immediate function of blood vessels in other tissues. Induction of tumor hemorrhage may also facilitate the selective delivery of chemotherapeutic agents to tumors and enhance antitumor immunity through better exposure of tumor antigens to circulating immune cells. Alternatively, mimicking the stabilizing effect of platelets on tumor vessels might help to normalize the tumor vasculature and its function, a strategy that was shown to have a synergistic effect when combined with cytotoxic therapy (55).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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