Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell–mediated elimination of tumor cells

Joseph S. Palumbo, Kathryn E. Talmage, Jessica V. Massari, Christine M. La Jeunesse, Matthew J. Flick, Keith W. Kombrinck, Markéta Jirousková, and Jay L. Degen

To test the hypothesis that platelet activation contributes to tumor dissemination, we studied metastasis in mice lacking Gαq, a G protein critical for platelet activation. Loss of platelet activation resulted in a profound diminution in both experimental and spontaneous metastases. Analyses of the distribution of radiolabeled tumor cells demonstrated that platelet function, like fibrinogen, significantly improved the survival of circulating tumor cells in the pulmonary vasculature. More detailed studies showed that the increase in metastatic success conferred by either platelets or fibrinogen was linked to natural killer cell function. Specifically, the pronounced reduction in tumor cell survival observed in fibrinogen- and Gαq-deficient mice relative to control animals was eliminated by the immunologic or genetic depletion of natural killer cells. These studies establish an important link between hemostatic factors and innate immunity and indicate that one mechanism by which the platelet-fibrin(ogen) axis contributes to metastatic potential is by impeding natural killer cell elimination of tumor cells. (Blood. 2005;105:178-185)

Introduction

A persuasive body of evidence has accumulated associating hemostatic factors with tumor growth, stroma formation, and tumor dissemination.[1-3] Clinical studies have shown that expression of procoagulants by cancer cells is prognostic of poor outcome.[4-6] Furthermore, the expression of tissue factor by tumor cells has been shown to promote metastatic disease in experimental animals,[7] whereas inhibitors of thrombin and other coagulation factors diminished metastatic potential.[1,8] The available data support the general hypothesis that local thrombin generation enhances tumor dissemination. However, it is presently not clear which specific thrombin substrates are important mediators of the metastatic process. Recent studies revealed that fibrinogen deficiency significantly diminishes metastatic potential, suggesting that fibrinogen is at least one thrombin substrate important in metastasis.[9,10] However, it is likely that other thrombin substrates also contribute to tumor cell metastasis based on the finding that pharmacologic inhibition of thrombin resulted in decreased metastatic potential even in the *absence* of fibrinogen.[10]

Several studies support the view that thrombin-mediated platelet activation may play a role in tumor biology. The elimination of circulating platelets with antiplatelet antibodies was shown to result in a significant diminution in metastases using several transplantable murine tumor models.[11,12] Competitive inhibition of the key platelet integrin, $\alpha_{IIb}\beta_3$, either pharmacologically or with antibodies to β_3, also diminished metastatic potential.[13,14] Similarly, pharmacologic inhibitors of platelet activation have been shown to decrease the metastatic potential of circulating tumor cells.[15,16] More recently, the genetic loss of the integrin β_3 subunit in mice was shown to diminish metastasis.[17]

Platelets could influence metastatic potential via several mechanisms. Platelet granules contain a variety of cellular growth factors (eg, platelet-derived growth factor [PDGF], vascular endothelial growth factor [VEGF]), matrix proteins (eg, vitronectin, fibronectin), and inflammatory mediators (eg, platelet factor-4, interleukin-8, macrophage inflammatory protein 1α [MIP-1α], RANTES [regulated on activation, normal T expressed, and secreted], CCL17, CCXL1, CXCL5) that might influence tumor cell behavior and stroma formation.[18-20] Platelets may also contribute to the physical interaction between circulating tumor cells and vascular endothelial cells by supporting the stable adhesion to endothelium and/or transmigration of tumor cells out of the vasculature. Local platelet activation could promote the migration of inflammatory cells, enhancing tumor stroma formation. Alternatively, tumor cell–associated platelets could facilitate tumor cell metastasis by *preventing* interactions between tumor cells and innate immune cells. This latter concept has been suggested by immunodepletion studies in vivo and studies showing that platelets can limit the ability of natural killer (NK) cells to lyse tumor cells in vitro.[21] This finding is consistent with the fact that NK cell–mediated elimination of target cells requires direct cell-cell contact. Despite substantial data pointing to a role for the fibrinogen-platelet axis in tumor progression, a mechanistic understanding of the interplay between hemostatic system components and tumor progression has remained elusive.

From the Divisions of Hematology/Oncology and Developmental Biology, Children's Hospital Research Foundation and University of Cincinnati College of Medicine, Cincinnati, OH; and The Rockefeller University, New York, NY.

Submitted June 16, 2004; accepted August 13, 2004. Prepublished online as *Blood* First Edition Paper, September 14, 2004; DOI 10.1182/blood-2004-06-2272.

Supported in part by grants HL074363 (J.S.P.) and HL71555 (J.L.D.) from the National Institutes of Health.

An Inside *Blood* analysis of this article appears in the front of this issue.

Reprints: J. L. Degen, Children's Hospital Research Foundation, Children's Hospital Medical Center, CHRF Room 2042, 3333 Burnet Ave, Cincinnati, OH 45229-3039; e-mail: degenjl@cchmc.org.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

The recent development of gene-targeted mice with qualitative and quantitative platelet defects (eg, Gαq-, nuclear factor–erythroid 2 [NF-E2]–, and protease-activated receptor 4 [PAR-4]–deficient mice) has provided an opportunity to better define the role of platelets in disease processes.[22-24] In order to examine the role of platelets in tumor growth and dissemination, we focused on mice lacking Gαq, a G protein critical for platelet activation. $G\alpha q^{-/-}$ mice have normal platelet numbers, thus retaining normal rheology within the circulation. However, Gαq-deficient platelets do not respond in vitro to physiologically relevant agonists, including adenosine diphosphate (ADP), thrombin, collagen, and thromboxane.[23] We report that metastatic potential is dramatically diminished in mice with defective platelet activation, and the platelet-fibrin(ogen) axis supports tumor cell dissemination via a mechanism related to natural killer cell function.

Material and methods

Transgenic mice

Gαq- and fibrinogen-deficient mice have been previously described.[23,25] Transgenic mice expressing an Ly49A cDNA under the control of a granzyme A promoter resulting in a severe defect in natural killer cells were described by Kim et al[26] (here referred to as NK^- mice). Genotyping of fibrinogen-deficient and NK^- mice was accomplished as previously described.[25,26] Gαq-deficient mice were genotyped by a multiplex polymerase chain reaction (PCR) strategy using 3 primers. The wild-type Gαq allele was detected using primer 1 (5′TTC CCA AGA TAG CAC CAC CAA CCG AG3′) and primer 2 (5′CCA ACA GCA TTT CAA CAA CAA GAG GCA C 3′) yielding a 385–base pair (bp) PCR product. The mutant Gαq allele was detected with primer 2 (see above) and primer 3 (5′GAT TCG CAG CGC ATC GCC TTC TAT3′) yielding a 264-bp PCR product. Fibrinogen-deficient and NK^- mice were inbred into the C57Bl/6 background for 7 generations. Gαq-deficient mice were inbred into the C57Bl/6 background for 4 generations. Age- and sex-matched cohorts of wild-type $(G\alpha q^{+/+})$ and Gαq-deficient $(G\alpha^{-/-})$ animals were enrolled in all experiments. Studies of fibrinogen-deficient $(Fib^{-/-})$ mice were done with cohorts of age- and sex-matched $Fib^{+/-}$ controls. The study protocols were approved by the Cincinnati Children's Hospital Research Foundation Institutional Animal Care and Use Committee in accordance with the guidelines of the National Institutes of Health (NIH).

Carotid artery thrombosis

Thrombus formation in the carotid artery of anesthetized mice was induced using $FeCl_3$ as previously described.[27] Blood flow through the carotid artery was monitored using a Doppler flow probe (no. 0.5VB307; Transonic Systems, Ithaca, NY) connected to a flow meter (T106; Transonic Systems), and thrombus formation was recorded continuously for up to 30 minutes using a miniature video camera (ProVideo CVC-514; CSI/SPECO, Amityville, NY). After the 30-minute recording period, anesthetized animals were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) and the injured carotid arteries were collected for analysis under a Hitachi S350 electron microscope (Hitachi, Tokyo, Japan) and under an Olympus BX60 microscope equipped with a Plan-Apo 2×/0.08 objective lens (Olympus, Melville, NY).

Tumor cell transplantation and histologic analysis

B16-BL6 melanoma cells and green fluorescent protein–expressing Lewis lung carcinoma (LLC^{GFP}) cells (both C57BL/6-derived tumor cells lines) were grown in vitro and injected into mice as previously described.[9,10] For experimental metastasis assays, mice were anesthetized with 2% isoflurane and 300 μL of a single-cell suspension was injected into the lateral tail vein. Lung tissue was collected 14 days after injection of LLC^{GFP} cells and 17 days after injection of B16-BL6 cells for quantitative analysis of metastatic foci. In studies of primary tumor growth and spontaneous metastasis, mice were anesthetized and 80 μL of single-cell suspension was injected intradermally into the dorsal skin. Primary tumor growth was followed by serial calipation.[28] Tumor tissues were fixed in 10% neutral-buffered formalin and processed for microscopic analysis as previously described.[9,10]

Bone marrow transplantation

Wild-type C57Bl/6 recipient mice were conditioned with 1175 cGy delivered in 2 doses of 700 cGy and 475 cGy at a rate of 63 cGy/minute administered 3 hours apart using a cesium-source gamma-irradiator (Mark 1-68 cesium irradiator; J. L. Shepherd, San Fernando, CA).[29] Donor $G\alpha q^{+/+}$ and $G\alpha q^{-/-}$ mice inbred into the C57Bl/6 background for 4 generations were killed and the pelvis, femora, and tibiae were sterilely harvested. Bone marrow was flushed from the bones with PBS and passed through a 40-μm mesh cell strainer. Mononuclear cells were separated by centrifugation in a Ficoll gradient. Irradiated recipient mice were intravenously injected with approximately 4×10^6 mononuclear bone marrow cells in 300 μL of PBS. Mice were enrolled in cancer studies 8 weeks after transplantation.

Spontaneous metastasis assays

LLC^{GFP} cells were transplanted into the dorsal subcutis overlying the lumbosacral spine as previously described.[9] Twelve days later, the mice were anesthetized and the head, chest, and upper abdomen were shielded with 6 cm of SuperFlab (Radiation Products Design, Albertville, MN) as previously described.[9] The tumors were then irradiated with 6 000 cGy using an electron beam irradiator (Siemens Mevatron, Munich, Germany). The mice were harvested 14 days later and organs were harvested for assessment.

Fate of tumor cells labeled with [^{125}I]-iododeoxyuridine

LLC^{GFP} cells were radiolabeled with 5-[^{125}I]iodo-2′-deoxyuridine (MP Biomedical, Irvine, CA) essentially as previously described.[10] Either 3×10^5 (Gαq-deficient mice) or 8×10^4 (fibrinogen-deficient mice) radiolabeled cells were injected intravenously. Organs were harvested at specified times points and washed for 3 days in 70% ethanol to remove free isotope. Radioisotope levels in the input inoculums and in each organ were determined with a Cobra gamma counter (Packard, Meridien, CT).

Immunologic elimination of NK cells in vivo and NK cytotoxicity assays

Rabbit antimouse asialo GM_1 polyclonal antibody (Cedarlane, Hornby, ON, Canada) was reconstituted per the manufacturer's recommendation. For tumor metastasis studies, mice were pretreated intravenously with either 100 μL anti–asialo GM_1 polyclonal antibody or carrier 48 hours prior to inoculation with tumor cells. The mice received a second dose of antibody/carrier on the day of tumor cell injection. For splenic NK assays, mice were intravenously injected with 100 μL anti–asialo GM_1 polyclonal antibody 48 hours prior to harvesting spleens. Twenty-four hours prior to being killed, mice were given an intraperitoneal injection of 150 μg poly I:C (Sigma-Aldrich, St Louis, MO). At the time they were killed, the mice were perfused with 5 mL of PBS via intracardiac infusion prior to harvesting spleens. Mononuclear splenocytes were separated by Ficoll gradient centrifugation and used for in vitro ^{51}Cr-release assays using YAC-1 target cells essentially as previously described.[30]

Results

In vivo thrombus formation is severely compromised in Gαq-deficient mice

As a prelude to detailed studies of tumor progression, we explored whether Gαq deficiency[23] resulted in a major defect in platelet thrombus formation in vivo. Thrombus formation within $FeCl_3$-injured carotid arteries was compared in control and Gαq-deficient mice using intravital videomicroscopy.[27] In $G\alpha q^{+/+}$ mice (n = 4), visible thrombus formation was evident immediately after the $FeCl_3$ challenge and resulted in occlusion of the carotid artery

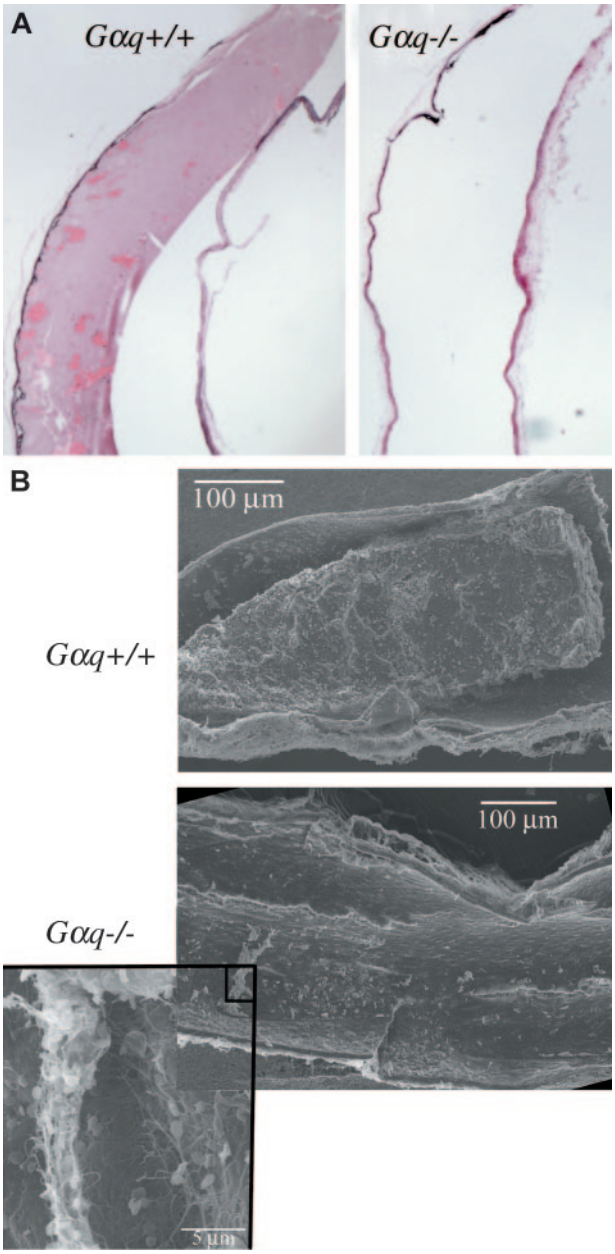

Figure 1. $G\alpha q^{-/-}$ platelets fail to support thrombus formation in vivo. Carotid vessels of wild-type and $G\alpha q^{-/-}$ mice were injured by the application of 20% $FeCl_3$ and thrombus formation analyzed by real-time intravital videomicroscopy (data not shown), histologic analysis (A), and scanning electron microscopy (B) 30 minutes after the initial challenge. Light microscopy of hematoxylin/eosin-stained sections revealed well-developed, occlusive thrombi within the carotids collected from wild-type mice, whereas vessels from $G\alpha q^{-/-}$ mice were effectively clear (representative views in panel A). Original magnification × 2. Electron micrographs of carotid arteries cut longitudinally to view the luminal contents (representative views in panel B) revealed densely packed thrombi in wild-type mice. In contrast, no occlusive thrombi were observed in the lumen of vessels from $G\alpha q^{-/-}$ mice. Rather, only trace amounts of fibrin-trapped platelets and red cells were observed (inset). Original magnifications × 100 and × 2000 (inset).

within 10.1 ± 0.9 minutes after $FeCl_3$ application. In contrast, $G\alpha q^{-/-}$ animals (n = 5) never developed visible platelet deposition, and blood flow through the carotid artery as measured using a Doppler flow probe remained unchanged for the entire 30-minute observation period (data not shown). Histologic analysis (Figure 1A) and scanning electron microscopic analysis (Figure 1B) of carotid arteries collected 30 minutes after $FeCl_3$ application confirmed the formation of large, dense thrombi in $G\alpha q^{+/+}$ mice. The artery walls in $G\alpha q^{-/-}$ mice, however, exhibited only a modest dusting of platelets (Figure 1) that could only be appreciated using high-magnification scanning electron microscopy (Figure 1B). These platelets appeared to be trapped within the small amounts of fibrin formed on the injured arterial surface, as opposed to being directly adherent to the vessel wall.

Gαq is an important determinant of the metastatic potential of circulating tumor cells

In order to examine the role of platelet activation in metastasis, we compared the formation of pulmonary metastatic foci within immunocompetent control and Gαq-deficient mice following the intravenous injection of either Lewis lung carcinoma cells (LLC^{GFP} cells; Figure 2A,C-D) or B16-BL6 melanoma cells (Figure 2B,E-F). Regardless of the tumor line employed, the number of lung metastases in $G\alpha q^{-/-}$ mice was 2 orders-of-magnitude less than that observed in control animals. Similar results were obtained in 3

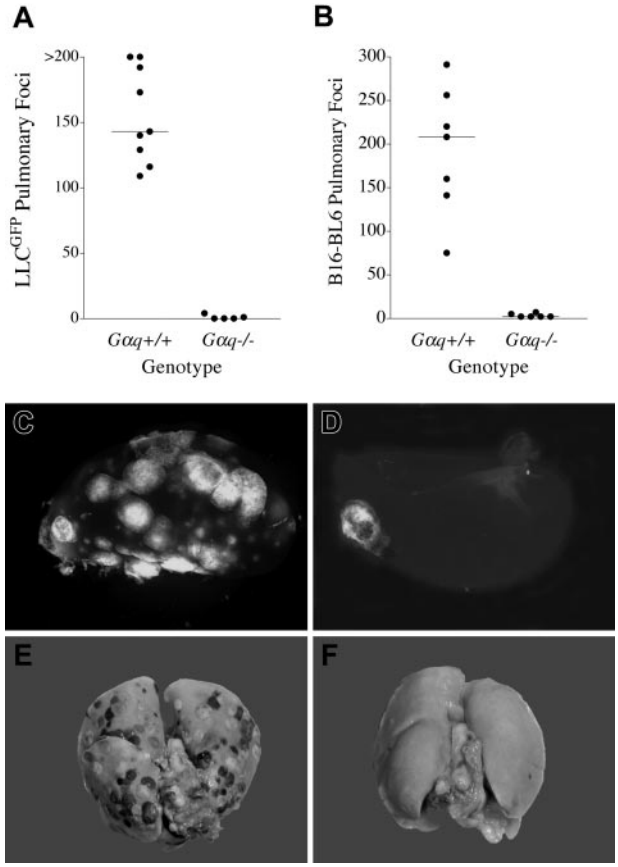

Figure 2. Gαq deficiency dramatically diminishes the metastatic potential of circulating tumor cells. Quantitative analysis of experimental pulmonary metastases in wild-type and $G\alpha q^{-/-}$ mice following intravenous injection of 4.5×10^5 LLC^{GFP} cells (A) or 8×10^4 B16-BL6 melanoma cells (B). The data shown represent the total number of surface pulmonary foci observed on all lung lobes for each mouse injected. The horizontal bars represent median values; $P < .005$ for each comparison using a Mann-Whitney *U* test. Gross appearance of pulmonary metastatic foci in animals injected with either LLC^{GFP} cells (representative views in panels C-D) or B16-BL6 (representative views in panels E-F) tumor cells. Note that the number of LLC^{GFP} tumor foci (detected based on GFP fluorescence) was dramatically higher in control mice (C) relative to $G\alpha q^{-/-}$ mice (D) but the size of individual lesions was similar. Images were obtained with a fluorescence-equipped Leica MZFL III stereomicroscope (Leica, Bannockburn, IL) equipped with an RTKE digital camera (RTKE Diagnostics, Sterling Heights, MI). SPOT RT software v. 3.5 was used to acquire images (RTKE Diagnostics). Similarly, high numbers of B16 melanoma lesions (detected based on melanin content) were seen in control mice (E) but not $G\alpha q^{-/-}$ mice (F).

independent experiments. While the number of metastases was strongly dependent on Gαq genotype, the size and overall appearance of individual pulmonary foci were similar in $G\alpha q^{+/+}$ and $G\alpha q^{-/-}$ mice. A microscopic analysis of lung tissues from control and $G\alpha q^{-/-}$ mice also did not reveal any difference in the qualitative features of individual metastatic lesions within these experimental animals (data not shown).

In order to establish that the diminution in metastases observed in $G\alpha q^{-/-}$ mice was due to the absence of Gαq within cells of hematopoietic origin, we limited the genetic deficit in Gαq to hematopoietic cells by bone marrow transplantation. Bone marrow mononuclear cells harvested separately from $G\alpha q^{+/+}$ and $G\alpha q^{-/-}$ mice were injected into lethally irradiated, wild-type C57Bl/6 mice. After a recovery period of 8 weeks to ensure the reconstitution of all blood cell lines, these mice were intravenously injected with LLC^{GFP} cells. Complete blood counts performed prior to tumor cell injection on 3 randomly selected mice of each genotype demonstrated no Gαq-dependent differences in white blood cell, red blood cell, or platelet reconstitution (data not shown). Similar to findings in mice with a constitutional deficit in Gαq, mice that received transplants of bone marrow from Gαq-deficient donors developed far fewer pulmonary metastases than mice that received transplants of bone marrow from wild-type donors (Figure 3). To establish the full recovery of hematopoiesis in transplant recipients used in tumor studies, cohorts of 4 randomly chosen tumor-bearing mice of each genotype were evaluated with complete blood counts at the time that they were killed. No significant difference was observed in any hematologic parameter, including white blood cell counts ($G\alpha q^{+/+}$ $3.7 \pm 0.47 \times 10^9$/L; $G\alpha q^{-/-}$ $3.54 \pm 0.54 \times 10^9$/L), hemoglobin concentration ($G\alpha q^{+/+}$ 112 ± 10.0 g/L; $G\alpha q^{-/-}$ 113 ± 8.0 g/L), and platelet counts ($G\alpha q^{+/+}$ $699 \pm 64 \times 10^9$/L; $G\alpha q^{-/-}$ $625 \pm 80 \times 10^9$/L; n = 4 for each genotype; P value not significant for each comparison using Mann-Whitney U test). Furthermore, these values were not significantly different from the hematologic parameters of wild-type and $G\alpha q^{-/-}$ mice that had not undergone the bone marrow transplantation procedure (data not shown). Microscopic analyses of blood smears also showed no differences in white cell subpopulations or red cell and platelet morphology. PCR analysis of both whole blood and bone marrow DNA demonstrated that at least 99% of the bone marrow cells were donor derived (data not shown). Bone marrow transplantation in itself was not a determinant of metastatic potential; no difference in pulmonary metastatic load was observed between nonirradiated wild-type mice and wild-type mice that received transplants of wild-type bone marrow (Figure 3). These data imply that Gαq-mediated signaling within hematopoietic cells is the dominant factor determining metastatic potential in Gαq-sufficient animals.

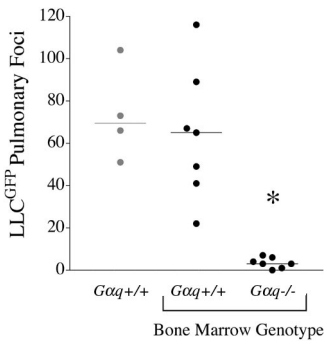

Figure 3. Gαq expression within hematopoietic-derived cells is a major determinant of metastatic potential. Lethally irradiated, wild-type, C57Bl/6 mice received transplants of bone marrow derived from either $G\alpha q^{+/+}$ or $G\alpha q^{-/-}$ mice. After recovery of hematopoietic function, the mice were intravenously injected with LLC^{GFP} cells. Shown is the total number of surface pulmonary foci observed within individual transplant recipients (●) as well as the number of foci observed in 4 wild-type mice that had not undergone the transplantation procedure (○) injected in parallel with LLC^{GFP} cells. The horizontal bars represent median values. *$P < .001$, Mann-Whitney U test.

Gαq-mediated platelet activation promotes spontaneous metastasis but does not affect established tumor growth

In order to explore the role of platelet activation in primary tumor growth, LLC^{GFP} cells were transplanted into the dorsal subcutis of $G\alpha q^{-/-}$ and $G\alpha q^{+/+}$ mice. Palpable tumors were present in mice of both genotypes within 4 days of inoculation. No genotype-dependent differences in tumor growth rate were observed based on serial calipation of tumor size[28] over a 12-day observation period (Figure 4A). Tumor weight measured at the time that the animal was killed was not influenced by Gαq deficiency. $G\alpha q^{+/+}$ mice had a median tumor mass of 387 mg (range, 60-785 mg; n = 9) compared with a median of 406 mg in $G\alpha q^{-/-}$ mice (range, 250-911 mg; n = 8; $P > .6$, Mann-Whitney U Test). Microscopic analysis of tissue sections prepared from LLC^{GFP} tumors did not reveal any genotype-dependent differences (Figure 4C-D). LLC^{GFP} tumors grew as dense masses of highly vascularized anaplastic cells with numerous mitoses. Small, focal areas of necrosis and numerous small blood vessels were evident in tumors from mice of both genotypes.

The fact that subcutaneous primary tumor growth was indistinguishable in control and $G\alpha q^{-/-}$ mice permitted a meaningful analysis of the platelet activation defect on spontaneous metastasis. To do this, we transplanted LLC^{GFP} cells into the dorsal subcutis overlying the lumbosacral spine. This caudal location was chosen so the primary tumor could later be eliminated by local radiotherapy.[9] Consistent with previous results, tumors grew similarly in $G\alpha q^{+/+}$ and $G\alpha q^{-/-}$ mice reaching approximately 300 mm^3 within 12 days. Radiotherapy (6000 cGy) was used to control the primary tumor in order to avoid the bleeding risk associated with surgical resection. During radiotherapy, the lungs and abdomen were shielded to minimize any general toxicity and to ensure the continued viability of any tumor cells within the lung.[9] Two weeks after elimination of the primary tumor, the mice were killed and metastatic foci on the lungs, liver, spleen, and kidneys enumerated. Like the experimental metastasis analyses, Gαq deficiency resulted in a significant decrease in spontaneous pulmonary metastases (Figure 4B). Spontaneous liver metastases also were less frequent in $G\alpha q^{-/-}$ mice relative to controls, but they were too few in number to make meaningful comparisons. Therefore, platelet activation appears to be a strong determinant of the more complex process of spontaneous metastasis.

Platelets enhance the survival of embolic tumor cells via a mechanism linked to natural killer cells

In order to examine the fate of circulating tumor cells in mice lacking platelet function, [^{125}I]-iododeoxyuridine–labeled LLC^{GFP} [8,10] cells were intravenously injected into 3 cohorts of $G\alpha q^{+/+}$ and $G\alpha q^{-/-}$ mice. Individual cohorts were killed 20 minutes, 5 hours, and 24 hours after tumor cell inoculation and the relative distribution of radiolabel in blood and organs was measured. Consistent with previous reports,[8,10] a significant fraction of tumor cells rapidly became localized within the lungs of control animals. Approximately half of the injected tumor cells were found in the lungs within 20 minutes of injection (Figure 5A). The residual tumor cells were widely dispersed with small numbers in blood

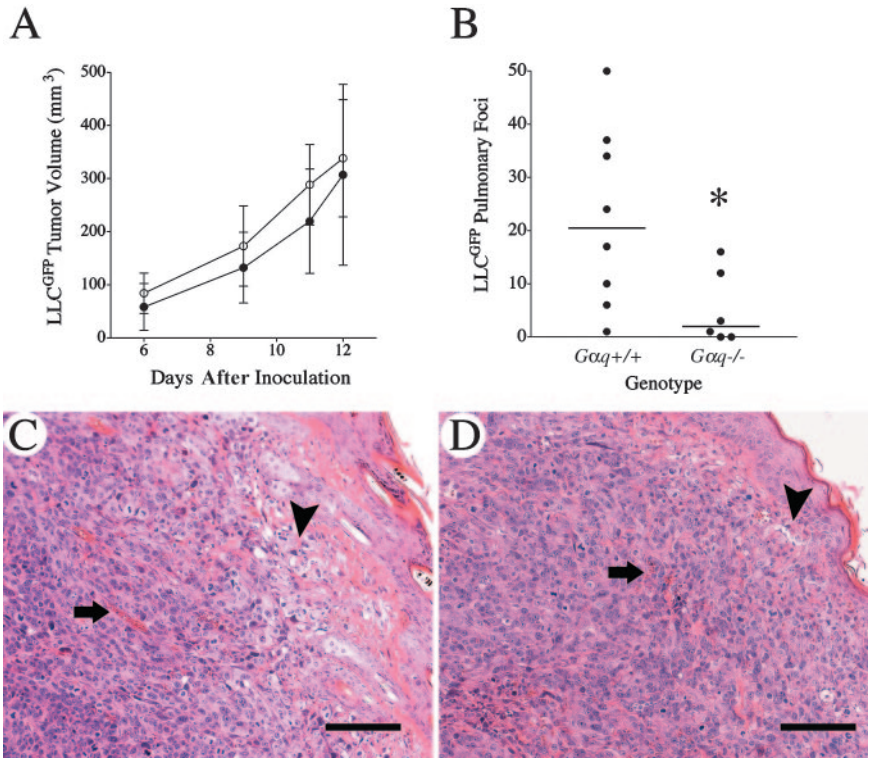

Figure 4. Platelet activation is a significant determinant of spontaneous metastasis but not tumor growth. (A) Serial measurements of tumor volume after transplantation of LLC^{GFP} cells into the dorsal subcutis of control (●, n = 9) and $G\alpha q^{-/-}$ mice (○, n = 8). There was no significant difference in tumor volumes between wild-type and mutant mice at any time point. Data shown are means with standard deviations. (B) Quantitative analysis of spontaneous pulmonary metastases. Metastatic foci were enumerated 14 days after primary tumor irradiation. Note that spontaneous metastasis, like experimental metastasis, was significantly diminished in Gαq-deficient mice. Horizontal bars indicate median values; $^*P < .03$, Mann-Whitney *U* test. No genotype-dependent difference was observed in overall primary tumor architecture based on histologic analyses of subcutaneous LLC^{GFP} tumors from control (representative data in panel C) and $G\alpha q^{-/-}$ (representative data in panel D) mice. Note that tumor cell invasion into overlying dermal structures (arrowheads) and numerous blood vessels (arrows) were observed in tumors harvested from mice of both genotypes. The horizontal bars denote 100 μm. Photomicrographs were obtained using a Zeiss Axioplan 2 microscope equipped with a 20×/0.75 objective lens and an Axiovision digital camera (Zeiss, Oberkochen, Germany). Axiovision software was used to acquire images (Zeiss).

(< 5%), liver (< 10%), spleen (< 1%), and other tissues. Also consistent with previous reports, a time-dependent process of tumor cell elimination was evident within the lungs of control mice, with only 10% of the inoculum remaining at 24 hours. The initial localization of tumor cells within the lungs (Figure 5A) and other tissues (data not shown) was not significantly different between $G\alpha q^{+/+}$ and $G\alpha q^{-/-}$ mice. However, consistent with their reduced risk of forming advanced pulmonary metastases, the loss of tumor cells within the lung was far more precipitous in $G\alpha q^{-/-}$ mice than in control animals. Only approximately 1% of the tumor cell inoculum remained within the lungs of $G\alpha q^{-/-}$ mice 24 hours after injection (Figure 5A). This same pattern of precipitous tumor cell elimination was observed previously in mice either lacking fibrinogen or treated with thrombin inhibitor,[8,10] suggesting a mechanistic linkage between platelet activation and fibrin(ogen) in circulating tumor cell survival.

One potential mechanism by which platelet-fibrin(ogen) microthrombi might support the metastatic success of embolic tumor cells is by forming a physical barrier that partially protects the malignant cells from natural killer (NK) cell–mediated elimination within the pulmonary vasculature.[21] In order to test this theory, comparative studies of tumor cell survival in vivo were done in control and $G\alpha q^{-/-}$ mice in which NK cells were depleted using anti–asialo GM_1 polyclonal antibodies.[31] As expected, control studies showed the successful immunodepletion of NK cells by this established method; splenic effector cells collected from wild-type mice treated with anti–asialo GM_1 displayed no natural killer function in vitro using standard ^{51}Cr-release assay with YAC-1 target cells (Figure 5B). An additional control study showed that the loss of Gαq did not result in any inherent loss of NK function; splenic NK cells prepared from wild-type and Gαq-deficient mice were equally effective in killing YAC-1 target cells in vitro at all target-to–effector cell ratios tested (data not shown). Nevertheless, the large difference in tumor cell survival observed within the lungs of control and $G\alpha q^{-/-}$ mice in vivo was found to be dependent on

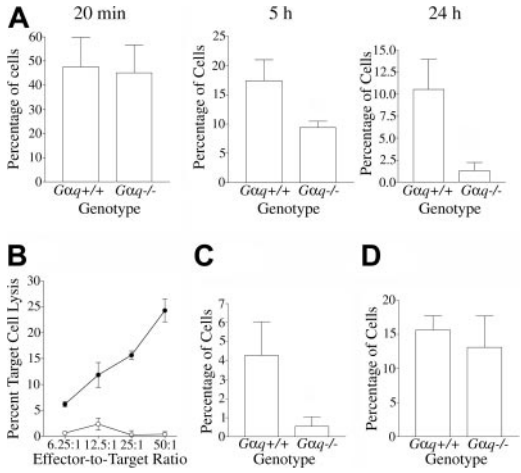

Figure 5. Platelet activation supports tumor cell survival in the lungs via a mechanism coupled to NK cell function. (A) Quantitative analysis of residual radiolabeled LLC^{GFP} cells within the lungs following intravenous injection. The values plotted are the percentage of the total tumor cells originally injected that were found within the lungs at the given time point (the data plotted are the mean values and standard deviations using cohorts of 5-6 mice). Note that the absence of Gαq had no effect on the initial localization of tumor cells in the lungs but resulted in a significant diminution in tumor cell survival when analyzed after either 5 ($P < .03$) or 24 hours ($P < .01$). (B) Loss of NK cell–mediated target cell lysis in vitro (standard chromium-release assays) by mononuclear splenocytes prepared from mice depleted of NK cells using an anti–asialo GM_1 antibody. Shown are representative results using splenocytes from carrier treated (●) and anti–asialo GM_1 antibody-treated mice (○). Note that anti–asialo GM_1 effectively eliminated NK cell lysis of YAC-1 target cells even at the highest effector-to-target cell ratio tested. (C-D) Quantitative analysis of residual radiolabeled LLC^{GFP} cells within the lung tissue 24 hours after intravenous injection into mice that were pretreated with anti–asialo GM_1 to deplete NK cells (D) or pretreated in parallel with antibody carrier (C). Note that in control (carrier-treated) mice (C), Gαq was affirmed to be an important determinant of tumor cell fate; $G\alpha q^{-/-}$ mice (n = 6) had significantly fewer pulmonary tumor cells than control animals (n = 6) 24 hours after tumor cell inoculation ($P < .01$). However, in mice depleted of NK cells (D), no significant diminution in lung-associated tumor cells was observed in $G\alpha q^{-/-}$ mice (n = 5) relative to control animals (n = 8). All *P* values were generated using a Mann-Whitney *U* test. Error bars represent standard deviation.

NK cell function in tumor cell fate studies. Here, ^{125}I-labeled LLC^{GFP} cells were injected into the circulation of cohorts of wild-type and $G\alpha q^{-/-}$ mice with and without NK cell immunodepletion and the residual tumor cells present within lung tissue were established 24 hours later using a gamma counter. Consistent with our earlier observations, in cohorts of carrier-treated mice (ie, no antibody), the number of residual pulmonary tumor cells was distinctly diminished in $G\alpha q^{-/-}$ mice relative to wild-type animals (Figure 5C). Furthermore, consistent with the known NK sensitivity of LLC tumor cells in metastasis assays, pretreatment with anti–asialo GM_1 antibodies resulted in a significant increase in the number of tumor cells in the lungs of wild-type mice at 24 hours (compare Figure 5C with 5D). However, a far more revealing finding was that while Gαq was clearly an important determinant of tumor cell survival in NK-sufficient mice, Gαq was no longer a determinant of tumor cell success in the context of NK cell depletion (Figure 5D). This experiment was repeated twice with similar results.

Fibrinogen supports the survival of embolic tumor cells via a mechanism linked to natural killer cells

In order to determine if the role of fibrin(ogen) in metastasis is also mechanistically linked to natural killer cell function, we took advantage of established transgenic mice shown to lack NK cells as a consequence of the inappropriate expression of Ly49A (kindly provided by Wayne Yokoyama, Washington University, St Louis, MO).[26] These mice (referred to here as NK^-) have a specific defect in NK cell function.[26] NK^- mice were crossed with fibrinogen-deficient mice in order to generate animals with single and combined deficits in fibrinogen and natural killer cells. Mice of the following 4 genotypes were challenged with an intravenous injection of LLC^{GFP} cells: Fib^+/NK^+, Fib^-/NK^+, Fib^+/NK^-, Fib^-/NK^-. Consistent with earlier reports,[26] the loss of NK function in NK^- mice resulted in a major increase ($>$ 20-fold) in the number of metastatic pulmonary foci that developed over a 2-week period relative to control animals (data not shown). In order to avoid a situation where NK$^-$ mice would have more metastatic foci than could reasonably be enumerated, cohorts of NK^+ mice were injected with 4×10^5 LLC^{GFP} cells, whereas cohorts of NK^- animals were injected with 8×10^4 cells obtained from the same cell suspension. The mice were killed 14 days later and pulmonary metastatic foci counted. In mice with intact NK function, the genetic elimination of fibrinogen resulted in a significant decrease in pulmonary metastasis (Figure 6A), consistent with previous results.[10] However, in mice lacking functional NK cells, fibrinogen deficiency was no longer a significant determinant of metastatic potential (Figure 6B). This experiment was performed 3 times with similar results.

To examine the interplay between fibrin(ogen) and NK cells in defining the early survival of circulating tumor cells, we examined tumor cell fate following the injection of 8×10^4 ^{125}I-labeled LLC^{GFP} cells into mice with single and combined genetic deficits in fibrinogen and NK cells. The mice were killed 24 hours after tumor cell injection and radiolabel in lungs and other tissues measured. Similar to our observations in Gαq-deficient mice and previously published results,[10] $Fib^{-/-}$ mice with intact NK function exhibited a more precipitous elimination of pulmonary tumor cells relative to fibrinogen-sufficient mice (Figure 6A). Predictably, the level of residual pulmonary tumor cells was significantly higher in NK^- mice relative to mice retaining NK function (compare Figure 6A with 6B). More importantly, in mice lacking NK cells, fibrinogen was no longer a significant determinant of early tumor cell survival within the lungs (Figure 6B). Thus, the diminution in metastatic lesions observed in the absence of either platelet activation or fibrinogen appears to result in part from differences in NK-mediated tumor cell elimination early after arrival in the circulation.

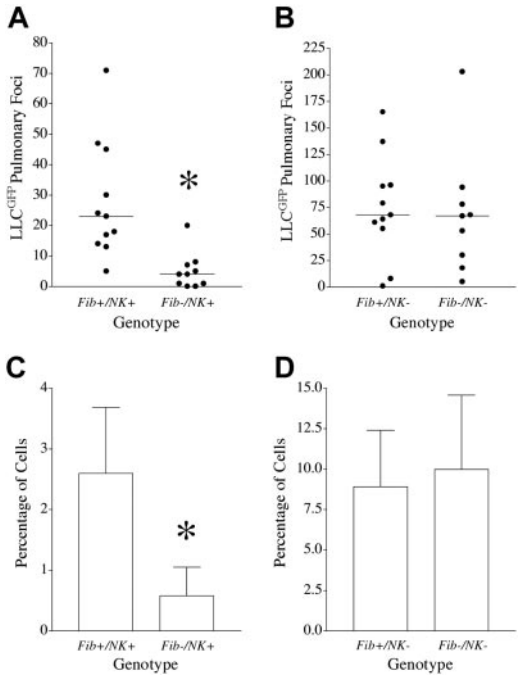

Figure 6. Fibrin(ogen) supports tumor cell survival in the lungs by a mechanism coupled to NK cell function. (A-B) Quantitative analysis of metastatic foci within the lungs of mice with single and combined genetic deficits in fibrinogen and NK cells 14 days after intravenous injection of LLC^{GFP} cells. Note that with natural killer cell function intact (A), $Fib^{-/-}$ mice developed significantly fewer metastatic foci than control animals challenged in parallel with 4×10^5 LLC^{GFP} ($^*P < .01$). In contrast, in transgenic mice with a life-long genetic deficit in NK cells (B), fibrinogen deficiency did not significantly alter metastatic potential (8×10^4 cells injected/mouse; $P > .5$). (C-D) Quantitative analysis of residual radiolabeled LLC^{GFP} cells within the lung tissue 24 hours after intravenous injection of 8×10^4 cells into mice with and without NK cells. Note that in mice with intact natural killer function (NK$^+$), fibrinogen deficiency (n = 15) resulted in a significant diminution in lung-associated tumor cells relative to control animals (n = 18; C). ($^*P < .008$). However, in transgenic mice with a life-long genetic deficit in NK cells (D), no significant diminution in lung-associated tumor cells was observed in $Fib^{-/-}$ mice (n = 12) relative to control animals (n = 19). All P values were generated using a Mann-Whitney U test.

Discussion

We demonstrate that both platelet activation and fibrin(ogen) increase the metastatic success of embolic tumor cells. Given that fibrinogen is actively engaged by activated platelets, these factors probably act in concert in altering tumor cell metastatic potential. Nevertheless, neither platelet activation (this report) nor fibrin(ogen)[9] is essential for the growth of established tumors. Rather, tumor cell fate analyses showed that platelet activation, like fibrinogen,[10] enhances the early survival of embolic tumor cells within the pulmonary vasculature. Furthermore, our studies demonstrate that at least one mechanism by which hemostatic factors support metastasis in vivo is by impeding tumor cell clearance by natural killer cells.

The loss of Gαq results in a severe defect in platelet activation in vitro[23] and here we extended this observation to show that

platelet thrombus formation is profoundly impaired in $G\alpha q^{-/-}$ mice in vivo. This defect also confers a dramatic decrease in the metastatic potential of tumor cells in both experimental and spontaneous metastasis assays. The finding that metastatic potential is similarly diminished in cohorts of mice constitutionally deficient in Gαq and cohorts of mice in which the loss of Gαq was restricted to hematopoietic cells (through bone marrow transplantation) further supports the notion that cells of hematopoietic origin constitute the predominant determinant of metastatic success in $G\alpha q^{-/-}$ mice. Platelet function appears to be central to metastatic potential (see "Discussion"), but a lingering question is whether the loss of Gαq in leukocytes also contributes to the diminution in experimental metastasis. While a contribution of leukocytes has not been formally excluded, loss of Gαq does not result in quantitative defects in leukocytes or the ability of hematopoietic stem cells to reconstitute bone marrow. Furthermore, we have found that the inherent capacity of NK cells to kill target cells in vitro is comparable using effector cells prepared from control and $G\alpha q^{-/-}$ mice. The current working view that the loss of platelet function constitutes the major determinant of metastasis in Gαq-deficient mice is consistent with earlier reports showing that pharmacologic or immunologic agents capable of diminishing platelet function markedly reduce metastasis.[11,12,32,33] This conclusion is further supported by recent reports of dramatically reduced metastatic potential in mice with either a quantitative platelet defect (eg, NF-E2–deficient mice) or a defect in thrombin-mediated platelet activation (eg, PAR-4–deficient mice).[34] Finally, mice lacking either fibrinogen or platelet fibrinogen receptor $\alpha_{IIb}\beta_3$ also exhibit reduced metastatic potential.[17] Taken together, the low metastatic potential observed in $G\alpha q^{-/-}$ mice is likely to be largely, if not solely, a consequence of the profound defect in platelet function.

Platelets and fibrin(ogen) could influence tumor biology through several possible mechanisms. Given the varied challenges confronting metastatic tumor cells, including transendothelial cell migration, safe transit within the circulation, stabilization within distant vascular beds, growth, and the establishment of a supportive vasculature, local platelet activation might offer some significant advantages at certain steps of this process and be a significant liability at other steps. Potential benefits include the following: (i) the local release of platelet-derived growth factors and other effectors (eg, inflammatory mediators) could promote tumor cell growth and stroma formation[18,20]; (ii) local platelet-fibrin deposition could sustain tumor cell immobilization within the circulation and provide a supportive matrix for cell proliferation; and (iii) platelets associated with embolic tumor cells could limit anti–tumor cell immune surveillance mechanisms. On the other hand, possible deleterious consequences of tumor-associated platelet-fibrin deposition include impeding local nutrient delivery and gas exchange or restricting tumor cell migration. It follows that any setting where persistent platelet thrombi were present within tumor neovasculature would tend to slow tumor growth.[35] While platelets may strongly influence tumor growth in specific contexts, the present studies indicate that platelet activation is neither strictly required nor uniformly deleterious for either tumor growth or the establishment of a supportive vasculature. Finally, whatever the benefits and liabilities of platelets for tumor progression, the data presented here show that in the balance, a functional defect in platelet activation strongly diminishes metastatic success.

The available data indicate that neither platelets (these studies) nor fibrin(ogen)[10] is important in the initial adhesion/localization of tumor cells within the pulmonary vasculature. Nevertheless, the adhesive properties of activated platelets and fibrin(ogen) may still be of significant importance in this process by supporting the *sustained adhesion* of tumor cells within high shear stress or turbulent vascular environments. However, perhaps the most intriguing facet of the present study is the finding that the benefits to tumor cell emboli that stem from platelet-fibrin deposition are not limited to the mechanical property of adhesion. Rather, the data presented here indicate that tumor cell–associated platelet-fibrin deposition provides a means for tumor cells to evade NK cell–mediated elimination, a finding that further ties hemostatic factors to immune surveillance. Given that NK-mediated killing requires direct contact with target cells, one attractive theory consistent with the available data is that platelet-fibrin microthrombi act as a *physical barrier* preventing contact of NK cells with target tumor cells. This general hypothesis is consistent with earlier studies showing that activated platelets can impede NK cell–mediated cell lysis in vitro.[21] Under this scenario, the contribution of fibrin(ogen) to tumor cell metastasis might be primarily the stabilization of tumor-associated platelets that effectively stand in the way of NK cell access. However, an alternative model is that NK cells are effectively *pacified* by immunomodulatory receptor engagement of tumor cell–associated microthrombi. In this regard, it should be noted that NK cells are known to express receptors (eg, $\alpha_M\beta_2$)[36] capable of binding immobilized fibrin(ogen) and platelet surface components. Similarly, the local release of cytokines from platelets associated with embolic tumor cells might regulate NK function. A final model consistent with the finding that Gαq ceases to be a determinant of metastatic potential in the absence of NK cells is that Gαq is a critical regulator of NK function. While still viable, this later hypothesis has the liability that in order to explain the experimental findings, the *loss* of any Gαq signaling in NK cells would have to markedly *improve* the ability of NK cells to kill tumor cells. The fact that NK cell lysis of target cells in vitro was not altered by Gαq deficiency would seem to argue against this concept. Furthermore, the parallel nature of our findings with fibrinogen- and Gαq-deficient mice points more toward microthrombus formation as being a central determinant of NK-mediated tumor cell elimination rather than a putative role of Gαq in NK cell function. Since platelets would be restricted to the vascular compartment, we would predict that whatever the interplay between activated platelets and NK cell–mediated tumor cell elimination, the interchange is likely to influence events while the tumor cells are still within the vasculature. While our studies highlight that tumor cells may capitalize on hemostatic system components to evade innate immune surveillance in certain contexts, it is important to emphasize that settings will almost certainly be found where hemostatic factors contribute to tumor biology, regardless of the presence or absence of NK function, such as tumors established within areas prone to recurrent trauma.[35]

In summary, these studies demonstrate that platelets and fibrin(ogen) enhance metastatic potential in part by impeding intravascular tumor cell clearance by NK cells. These observations are particularly noteworthy in light of clinical observations, suggesting that agents targeting thrombin generation and platelet function can improve outcome for certain types of cancer.[37-39] This would suggest that therapies designed to inhibit the association of tumor cells with platelets/fibrin(ogen), in combination with therapeutic strategies that augment the clearance of tumor cells via the innate immune system, could be effective for the treatment of certain malignancies. Agents that target specific signal transduction proteins involved in platelet activation or limit platelet aggregation

could be particularly attractive candidates since the remainder of the clotting cascade would be left intact.

Acknowledgments

The authors would like to thank Drs Christopher Karp and Jonathan Katz for their many helpful suggestions. We also thank Carol Moore for her expert assistance with the NK cell function assays, as well as Drs Ronald Gordon and Norman Katz (Mount Sinai School of Medicine, NY) for their help with electron microscopy, and Igor Chershnev and Heikki Vaanen (Mount Sinai School of Medicine) for their help with the thrombus model. We gratefully acknowledge Jeffrey Bailey and Victoria Seummey-Harmer for their assistance with the bone marrow transplant protocol and Dr Beth A. Myers for critically reviewing the manuscript.

References

1. Palumbo JS, Degen JL. Hemostatic factors in tumor biology. J Pediatr Hematol Oncol. 2000;22: 281-287.
2. Dvorak HF, Harvey VS, Estrella P, Brown LF, McDonagh J, Dvorak AM. Fibrin containing gels induce angiogenesis: implications for tumor stroma generation and wound healing. Lab Invest. 1987; 57:673-686.
3. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315: 1650-1659.
4. Akashi T, Furuya Y, Ohta S, Fuse H. Tissue factor expression and prognosis in patients with metastatic prostate cancer. Urology. 2003;62:1078-1082.
5. Seto S, Onodera H, Kaido T, et al. Tissue factor expression in human colorectal carcinoma: correlation with hepatic metastasis and impact on prognosis. Cancer. 2000;88:295-301.
6. Ueno T, Toi M, Koike M, Nakamura S, Tominaga T. Tissue factor expression in breast cancer tissues: its correlation with prognosis and plasma concentration. Br J Cancer. 2000;83:164-170.
7. Mueller BM, Ruf W. Requirement for binding of catalytically active factor VIIa in tissue factor-dependent experimental metastasis. J Clin Invest. 1998;101:1372-1378.
8. Esumi N, Fan D, Fidler IJ. Inhibition of murine melanoma experimental metastasis by recombinant desulfatohirudin, a highly specific thrombin inhibitor. Cancer Res. 1991;51:4549-4556.
9. Palumbo JS, Potter JM, Kaplan LS, Talmage K, Jackson DG, Degen JL. Spontaneous hematogenous and lymphatic metastasis, but not primary tumor growth or angiogenesis, is diminished in fibrinogen-deficient mice. Cancer Res. 2002;62: 6966-6972.
10. Palumbo JS, Kombrinck KW, Drew AF, et al. Fibrinogen is an important determinant of the metastatic potential of circulating tumor cells. Blood. 2000;96:3302-3309.
11. Kato Y, Fujita N, Yano H, Tsuruo T. Suppression of experimental lung colonization of mouse colon adenocarcinoma 26 in vivo by an anti-idiotype monoclonal antibody recognizing a platelet surface molecule. Cancer Res. 1997;57:3040-3045.
12. Pearlstein E, Ambrogio C, Karpatkin S. Effect of antiplatelet antibody on the development of pulmonary metastases following injection of CT26 colon adenocarcinoma, Lewis lung carcinoma, and B16 amelanotic melanoma tumor cells into mice. Cancer Res. 1984;44:3884-3887.
13. Amirkhosravi A, Mousa SA, Amaya M, et al. Inhibition of tumor cell-induced platelet aggregation and lung metastasis by the oral GpIIb/IIIa antagonist XV454. Thromb Haemost. 2003;90:549-554.
14. Trikha M, Zhou Z, Timar J, et al. Multiple roles for platelet GPIIb/IIIa and $\alpha_v\beta_3$ integrins in tumor growth, angiogenesis, and metastasis. Cancer Res. 2002;62:2824-2833.
15. Tzanakakis GN, Agarwal KC, Veronikis DK, Vezeridis MP. Effects of antiplatelet agents alone or in combinations on platelet aggregation and on liver metastases from a human pancreatic adenocarcinoma in the nude mouse. J Surg Oncol. 1991;48:45-50.
16. Honn KV, Cicone B, Skoff A. Prostacyclin: a potent antimetastatic agent. Science. 1981;212: 1270-1272.
17. Bakewell SJ, Nestor P, Prasad S, et al. Platelet and osteoclast β_3 integrins are critical for bone metastasis. Proc Natl Acad Sci U S A. 2003;100: 14205-14210.
18. Gear AR, Camerini D. Platelet chemokines and chemokine receptors: linking hemostasis, inflammation, and host defense. Microcirculation. 2003; 10:335-350.
19. Holt JC, Niewiarowski S. Biochemistry of α granule proteins. Semin Hematol. 1985;22:151-163.
20. Wagner DD, Burger PC. Platelets in inflammation and thrombosis. Arterioscler Thromb Vasc Biol. 2003;23:2131-2137.
21. Nieswandt B, Hafner M, Echtenacher B, Mannel DN. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. Cancer Res. 1999; 59:1295-1300.
22. Connolly AJ, Ishihara H, Kahn ML, Farese RV Jr, Coughlin SR. Role of the thrombin receptor in development and evidence for a second receptor. Nature. 1996;381:516-519.
23. Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G αq-deficient mice. Nature. 1997;389:183-186.
24. Shivdasani RA, Rosenblatt MF, Zucker-Franklin D, et al. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. Cell. 1995;81:695-704.
25. Suh TT, Holmback K, Jensen NJ, et al. Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev. 1995;9:2020-2033.
26. Kim S, Iizuka K, Aguila HL, Weissman IL, Yokoyama WM. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. Proc Natl Acad Sci U S A. 2000;97:2731-2736.
27. Jirouskova M, Chereshnev I, Vaananen H, Degen JL, Coller BS. Antibody blockade or mutation of the fibrinogen γ-chain C-terminus is more effective in inhibiting murine arterial thrombus formation than complete absence of fibrinogen. Blood. 2004;103:1995-2002.
28. Geran H, Greenberg N, MacDonald M, Schumacher A, Abbot B. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother Rep. 1972;3:1-16.
29. Puig T, Kadar E, Limon A, et al. Myeloablation enhances engraftment of transduced murine hematopoietic cells, but does not influence long-term expression of the transgene. Gene Ther. 2002;9:1472-1479.
30. Hackett J Jr, Bennett M, Kumar V. Origin and differentiation of natural killer cells, I: characteristics of a transplantable NK cell precursor. J Immunol. 1985;134:3731-3738.
31. Gorelik E. Augmentation of the antimetastatic effect of anticoagulant drugs by immunostimulation in mice. Cancer Res. 1987;47:809-815.
32. Honn KV, Tang DG, Crissman JD. Platelets and cancer metastasis: a causal relationship? Cancer Metastasis Rev. 1992;11:325-351.
33. Nierodzik ML, Klepfish A, Karpatkin S. Role of platelets, thrombin, integrin IIb-IIIa, fibronectin and von Willebrand factor on tumor adhesion in vitro and metastasis in vivo. Thromb Haemost. 1995;74:282-290.
34. Camerer E, Qazi AA, Duong DN, Cornelissen I, Advincula R, Coughlin SR. Platelets, protease-activated receptors and fibrinogen in hematogenous metastasis. Blood. 2004;104:397-401.
35. Palumbo JS, Talmage KE, Liu H, La Jeunesse CM, Witte DP, Degen JL. Plasminogen supports tumor growth through a fibrinogen-dependent mechanism linked to vascular patency. Blood. 2003;102:2819-2827.
36. Vetvicka V, Thornton BP, Wieman TJ, Ross GD. Targeting of natural killer cells to mammary carcinoma via naturally occurring tumor cell-bound iC3b and β-glucan-primed CR3 (CD11b/CD18). J Immunol. 1997;159:599-605.
37. Moysich KB, Menezes RJ, Ronsani A, et al. Regular aspirin use and lung cancer risk. BMC Cancer. 2002;2:31.
38. Thun MJ, Namboodiri MM, Heath CW Jr. Aspirin use and reduced risk of fatal colon cancer. N Engl J Med. 1991;325:1593-1596.
39. Kakkar AK, Levine MN, Kadziola Z, et al. Low molecular weight heparin, therapy with dalteparin, and survival in advanced cancer: The Fragmin Advanced Malignancy Outcome Study (FAMOUS). J Clin Oncol. 2004;22:1944-1948.