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

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To test the hypothesis that platelet activation contributes to tumor dissemination, we studied metastasis in mice lacking G\textsubscript{q}α, 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 G\textsubscript{q}α-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-fibrinogen 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.\textsuperscript{1-3} Clinical studies have shown that expression of procoagulants by cancer cells is prognostic of poor outcome.\textsuperscript{4-6} Furthermore, the expression of tissue factor by tumor cells has been shown to promote metastatic disease in experimental animals,\textsuperscript{7} whereas inhibitors of thrombin and other coagulation factors diminished metastatic potential.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{11,12} Competitive inhibition of the key platelet integrin, α\textsubscript{IIb}β\textsubscript{3}, either pharmacologically or with antibodies to β\textsubscript{3}, also diminished metastatic potential.\textsuperscript{13,14} Similarly, pharmacologic inhibitors of platelet activation have been shown to decrease the metastatic potential of circulating tumor cells.\textsuperscript{15,16} More recently, the genetic loss of the integrin β\textsubscript{3} subunit in mice was shown to diminish metastasis.\textsuperscript{17}

Platelets could influence metastatic potential via several mechanisms. Platelet granules contain a variety of cellular growth factors (e.g., platelet-derived growth factor [PDGF], vascular endothelial growth factor [VEGF]), matrix proteins (e.g., vitronectin, fibronectin), and inflammatory mediators (e.g., 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.\textsuperscript{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.\textsuperscript{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.
The recent development of gene-targeted mice with qualitative and quantitative platelet defects (eg, Gaq-, 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. In order to examine the role of platelets in tumor growth and dissemination, we focused on mice lacking Gaq, a G protein critical for platelet activation. Gaq−/− mice have normal platelet numbers, thus retaining normal rheology within the circulation. However, Gaq-deficient platelets do not respond in vitro to physiologically relevant agonists, including adenosine diphosphate (ADP), thrombin, collagen, and thromboxane. We report that metastatic potential is dramatically diminished in mice with defective platelet activation, and the platelet-fibrinogen axis supports tumor cell dissemination via a mechanism related to natural killer cell function.

Material and methods

Transgenic mice

Gaq- and fibrinogen-deficient mice have been previously described. Transgenic mice expressing a Ly49A CDNA under the control of a granzyne A promoter resulting in a severe defect in natural killer cells were described by Kim et al (here referred to as NK−/− mice). Genotyping of fibrinogen-deficient and NK−/− mice was accomplished as previously described. Gaq-deficient mice were genotyped by a multiplex polymerase chain reaction (PCR) strategy using 3 primers. The wild-type Gaq allele was detected using primer 1 (5′-TTT CCA AGA TAG CAC CAA CCG AG3′) and primer 2 (5′-CCA ACA GCA TTT CAA CAA AAG GAG GCA C 3′) yielding a 385–base pair (bp) PCR product. The mutant Gaq allele was detected with primer 2 (see above) and primer 3 (5′-GAT TCG CAG CGC ACF GCC TTC TAT3′) yielding a 264-bp PCR product. Fibrinogen-deficient and NK−/− mice were inbred into the C57Bl/6 background for 7 generations. Gaq-deficient mice were inbred into the C57Bl/6 background for 4 generations. Age- and sex-matched cohorts of wild-type (Gaq+/+) and Gaq-deficient (Gaq−/−) 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 FeCl3 as previously described. 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). 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 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 (LLCSP) cells (both C57BL/6-derived tumor cells lines) were grown in vitro and injected into mice as previously described. As a prelude to detailed studies of tumor progression, we explored whether Gaq deficiency resulted in a major defect in platelet thrombus formation in vivo. Thrombus formation within FeCl3-injured carotid arteries was compared in control and Gaq-deficient mice using intravital videomicroscopy. In Gaq−/− mice (n = 4), visible thrombus formation was evident immediately after the FeCl3 challenge and resulted in occlusion of the carotid artery.
within 10.1 ± 0.9 minutes after FeCl₃ application. In contrast, Gaq⁻/⁻ 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 FeCl₃ application confirmed the formation of large, dense thrombi in Gaq⁺/+ mice.

The artery walls in Gaq⁻/⁻ 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.

**Gaq 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 Gaq-deficient mice following the intravenous injection of either Lewis lung carcinoma cells (LLC<sub>GFP</sub> 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 Gaq⁻/⁻ mice was 2 orders-of-magnitude less than that observed in control animals. Similar results were obtained in 3
independent experiments. While the number of metastases was strongly dependent on Gaq genotype, the size and overall appearance of individual pulmonary foci were similar in Gaq+/+ and Gaq−/− mice. A microscopic analysis of lung tissues from control and Gaq−/− 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 Gaq−/− mice was due to the absence of Gaq within cells of hematopoietic origin, we limited the genetic deficit in Gaq to hematopoietic cells by bone marrow transplantation. Bone marrow mononuclear cells harvested separately from Gaq+/+ and Gaq−/− 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<sup>GFP</sup> cells. Complete blood counts performed prior to tumor cell injection on 3 randomly selected mice of each genotype demonstrated no Gaq-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 Gaq, mice that received transplants of bone marrow from Gaq-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 (Gaq+/+ 3.7 ± 0.47 × 10<sup>9</sup>/L; Gaq−/− 3.54 ± 0.54 × 10<sup>9</sup>/L), hemoglobin concentration (Gaq+/+ 112 ± 10.0 g/L; Gaq−/− 113 ± 8.0 g/L), and platelet counts (Gaq+/+ 699 ± 64 × 10<sup>9</sup>/L; Gaq−/− 625 ± 80 × 10<sup>9</sup>/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 Gaq−/− 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 Gaq-mediated signaling within hematopoietic cells is the dominant factor determining metastatic potential in Gaq-sufficient animals.

**Gaq-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<sup>GFP</sup> cells were transplanted into the dorsal subcutis of Gaq−/− and Gaq+/+ 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 calibration of tumor size<sup>28</sup> over a 12-day observation period (Figure 4A). Tumor weight measured at the time that the animal was killed was not influenced by Gaq deficiency. Gaq+/+ mice had a median tumor mass of 387 mg (range, 60-785 mg; n = 9) compared with a median of 406 mg in Gaq−/− mice (range, 250-911 mg; n = 8; P > .6, Mann-Whitney U Test).

Microscopic analysis of tissue sections prepared from LLC<sup>GFP</sup> tumors did not reveal any genotype-dependent differences (Figure 4C-D). LLC<sup>GFP</sup> 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 Gaq−/− mice permitted a meaningful analysis of the platelet activation defect on spontaneous metastasis. To do this, we transplanted LLC<sup>GFP</sup> cells into the dorsal subcutis overlying the lumbarosacral spine. This caudal location was chosen so the primary tumor could later be eliminated by local radiotherapy. Consistent with previous results, tumors grew similarly in Gaq+/+ and Gaq−/− mice reaching approximately 300 mm<sup>3</sup> 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.<sup>29</sup> 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, Gaq deficiency resulted in a significant decrease in spontaneous pulmonary metastases (Figure 4B). Spontaneous liver metastases also were less frequent in Gaq−/− 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, [125I]-iododeoxyuridine–labeled LLC<sup>GFP</sup> cells were intravenously injected into 3 cohorts of Gaq−/− and Gaq+/+ 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,<sup>8,10</sup> 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
(<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 Gaq+/- and Gaq-/- 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 Gaq-/- mice than in control animals. Only approximately 1% of the tumor cell inoculum remained within the lungs of Gaq-/- 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, 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. In order to test this theory, malignant cells from natural killer (NK) cell–mediated elimination were observed previously in mice either lacking Gαq-deficient mice. Horizontal bars denote 100 μm. Photomicrographs were obtained using a Zeiss Axioplan 2 microscope equipped with a 20×0.75 objective lens and an Axiosvision digital camera (Zeiss, Oberkochen, Germany). Axiovision software was used to acquire images (Zeiss).

Figure 4. Platelet activation is a significant determinate 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 Gaq-/- 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 metastases, like experimental metastases, was significantly diminished in Gaq-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 D) and Gaq-/- (representative data in panel E) 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 Axiosvision digital camera (Zeiss, Oberkochen, Germany). Axiovision software was used to acquire images (Zeiss).

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 Gaq 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). However, in mice depleted of Gaq cells using an anti-asialo GM1 antibody, shown are representative results using splenocytes from carrier-treated (●) and anti-asialo GM1 antibody-treated mice (▲). Note that anti-asialo GM1 effectively eliminated NK cell lysis of YAC-1 target cells. (B,C) Quantitative analysis of residual radiolabeled LLC-GFP cells within the lungs 24 hours after intravenous injection into mice that were pretreated with anti-asialo GM1 to deplete NK cells (D) or pretreated in parallel with antibody carrier (C). Note that in control (carrier-treated) mice (▲), Gaq was affirmed to be an important determinant of tumor cell fate; Gaq-/- mice (●) had significantly fewer pulmonary tumor cells than control animals (○) 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 Gaq-/- mice (▲) relative to control animals (○). 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, ¹²⁵I-labeled LLC<sup>GFP</sup> cells were injected into the circulation of cohorts of wild-type and Gaq<sup>−/−</sup> 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 (i.e., no antibody), the number of residual pulmonary tumor cells was distinctly diminished in Gaq<sup>−/−</sup> 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<sub>1</sub> 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 Gaq was clearly an important determinant of tumor cell survival in NK-sufficient mice, Gaq 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 fibrinogen 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).<sup>26</sup> These mice (referred to here as NK<sup>−</sup>) have a specific defect in NK cell function.<sup>26</sup> NK<sup>−</sup> 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<sup>GFP</sup> cells: Fib<sup>−/−</sup>/NK<sup>−</sup>, Fib<sup>−/−</sup>/NK<sup>+</sup>, Fib<sup>+/−</sup>/NK<sup>−</sup>, and Fib<sup>+/−</sup>/NK<sup>+</sup>. Consistent with earlier reports,<sup>26</sup> the loss of NK function in NK<sup>−</sup> 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<sup>−</sup> mice would have more metastatic foci than could reasonably be enumerated, cohorts of NK<sup>+</sup> mice were injected with 4 × 10<sup>4</sup> LLC<sup>GFP</sup> cells, whereas cohorts of NK<sup>−</sup> animals were injected with 8 × 10<sup>3</sup> 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.<sup>10</sup> 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 fibrinogen and NK cells in defining the early survival of circulating tumor cells, we examined tumor cell fate following the injection of 8 × 10<sup>4</sup> ¹²⁵I-labeled LLC<sup>GFP</sup> 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 Gaq-deficient mice and previously published results,<sup>10</sup> Fib<sup>−/−</sup> 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<sup>−</sup> 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.

**Discussion**

We demonstrate that both platelet activation and fibrinogen 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 fibrinogen<sup>9</sup> is essential for the growth of established tumors. Rather, tumor cell fate analyses showed that platelet activation, like fibrinogen,<sup>10</sup> 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 Gaq results in a severe defect in platelet activation in vitro<sup>23</sup> and here we extended this observation to show that
Platelet thrombus formation is profoundly impaired in \( \text{Ga}q^{-/-} \) 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 \( \text{Ga}q \) and cohorts of mice in which the loss of \( \text{Ga}q \) 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 \( \text{Ga}q^{-/-} \) mice. Platelet function appears to be central to metastatic potential (see “Discussion”), but a lingering question is whether the loss of \( \text{Ga}q \) in leukocytes also contributes to the diminution in experimental metastasis. While a contribution of leukocytes has not been formally excluded, loss of \( \text{Ga}q \) 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 \( \text{Ga}q^{-/-} \) mice. The current working view that the loss of platelet function constitutes the major determinant of metastasis in \( \text{Ga}q \)-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 \( \alpha_M\beta_3 \) also exhibit reduced metastatic potential.\(^{17}\) Taken together, the low metastatic potential observed in \( \text{Ga}q^{-/-} \) 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 neovascularization 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, \( \alpha_M\beta_2 \)) 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 \( \text{Ga}q \) ceases to be a determinant of metastatic potential in the absence of NK cells is that \( \text{Ga}q \) 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 \( \text{Ga}q \) 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 \( \text{Ga}q \) deficiency would seem to argue against this concept. Furthermore, the parallel nature of our findings with fibrinogen- and \( \text{Ga}q \)-deficient mice points more toward microthrombus formation as being a central determinant of NK-mediated tumor cell elimination rather than a putative role of \( \text{Ga}q \) 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.\(^{36}\)

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...
References

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