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PRINCIPAL INVESTIGATOR: Dr. Jerry Ware

CONTRACTING ORGANIZATION: University of Arkansas for Medical Sciences
Little Rock, AR 77205

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Dr. Jerry Ware

E-Mail: jware@uams.edu

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14. ABSTRACT

This report describes studies that document the use of mouse models of platelet dysfunction in the progression of cancer to metastatic disease. The work used genetically modified mouse strains with dysfunctional platelet membrane receptors. The studies examined the relevance of platelet receptors in models of spontaneous metastasis and models of experimental metastasis. A transgenic C57BL/6J mouse colony expressing MMTV-PyV middle antigen in mammary epithelium and dysfunctional platelets was developed. Mammary gland tumor and lung metastases develop in these mice spontaneously so they will be used in spontaneous metastasis experiments. Breeding with congenic colonies with defective platelet GP receptors will determine the relevance of platelet adhesion and activation to spontaneous tumor metastasis. The importance of metastasis in the prognosis for recovery from breast cancer cannot be under emphasized. Indeed, the spread of metastatic disease represents a fundamental change in significantly shortening the life span of patients with breast cancer. Thus, understanding the molecules that regulate metastasis identifies potential targets for therapeutic intervention that could significantly improve the prognosis for the breast cancer patient.

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Blood platelet, Tumor, Metastasis, Collagen

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Table of Contents

Page

Introduction

Our experiments were designed to determine the molecular basis for the participation of platelet adhesion and activation receptors in tumor metastasis. Two major platelet membrane receptors, glycoprotein Ib-IX and glycoprotein VI, are key to the normal platelet response of adhesion and activation, respectively. Murine models of deficiency of each have been developed in our lab and bred as congenic strains which provided a unique opportunity to examine tumor cell viability in animals with an intact immune system. Both receptors have been studied in great detail for their role in the more typical platelet response of hemostasis and thrombosis. However, we are learning that the platelet's role in blood clotting extends to other disease processes, as well, including cancer and inflammation. [1] From the blood coagulation perspective, an absence of GP Ib-IX correlates with a severe, and often life-threatening, bleeding disorder. The basis of our funded project was founded in preliminary data demonstrating the functional paradigms defining the role of GP Ib-IX in hemostasis and thrombosis extend beyond blood coagulation and are relevant to cancer biology. [2]

We proposed **the hypothesis that platelet receptor functions that support hemostasis and thrombosis also support tumorigenesis.** We have completed studies that characterize in greater depth the relevance of GPVI and GP Ib-IX even testing GP Ib-IX in an extremely aggressive tumor model of spontaneous breast cancer development, a mouse over-expressing the highly oncogenic Middle T antigen of the polyoma virus (PyMT). As we will show this model was refractory to GP Ib-IX. However, the relevance of GP Ib-IX in less robust models has garnered interest from a Massocheusetts-based startup company, Vasculogics, who has proprietary small molecule inhibitors of GP Ib-IX. As a result in our funded studies we have initiated a collaborative arrangement with Vasculogics to perform more pre-clinical testing of inhibiting the GP Ib-IX pathway. Below we outline the series of experiments that have been completed, to date. While many questions still exist in applying the hemostasis paradigm to cancer, we believe we have gained a better understanding of how platelets can contribute to cancer progression.

Body

Overall Research Strategy: Our original experimental plan involved 3 Specific Aims (SA). These aims were chosen to address at different levels the involvement of platelet GP Ib-IX in the spread of metastatic disease. **SA1** asks the questions at what step in the temporal sequence of events is platelet GP Ib-IX participating in tumor metastasis? **SA2** draws upon a large amount of literature linking coagulation, thrombin, platelets, and cancer to ask "is thrombin binding to GP Ib-IX relevant to tumor development"? **SA3** avoids the problems associated with experimental metastasis and asks the biological relevance of GP Ib-IX in spontaneous tumor metastasis. Each of these questions was based upon existing hypotheses for the role of platelet GP Ib-IX in hemostasis and thrombosis and addressing these questions bridges the relevance of platelet paradigms in the progression of metastatic disease, a critical event in the prognosis for breast cancer patients. Not listed in our original Statement of Work was investigating the relevance of platelet GP VI in tumor development. We chose to include studies on GP VI during the funded period for a couple of reasons. First, we had developed data in our ongoing hemostasis studies that demonstrated inhibition of GP VI did not result in significant bleeding. Indeed, any therapeutic targeting would like to have bleeding risks kept to a minimum. Thus, we performed a series of experimental metastasis studies that demonstrated GP VI is also a viable anti-tumor target. Our publication describing these results was well received with a corresponding Commentary highlighting the importance of the model and work [3,4]. Thus, we will report final data relevant to GP VI in tumor development.

Specific Aim 1. To define the temporal sequence of events linking platelet GP Ib-IX and tumorigenesis. *This aim was achieved with an integrated approach of in vivo imaging using reporter tagged cell lines and histological analyses.*

For our initial analysis we chose the highly metastatic mouse cell line, B16F10.1 (B16), representing a commonly used melanoma cell line [5]. B16 cells were administered to animals via tail vein injections (1×10^5 cells) and 14 days following injection, the number of visible tumor foci was determined on the surface of each lung. As shown in Figure 1, a dramatic decrease in the number B16 cells able to colonize the lungs was associated with the functional absence of GP Ib-IX. Specifically, wild-type lungs had 150 foci per lung (median value) while GP1b-/- and IL4-R/Ibα^{Cyto} animals both had

median values of 8 and 12, respectively (Fig. 1). The reduction in surface tumors was statistically significant with similar *p*-values for WT versus GP1b-/- or IL4-R/Ibα^{Cyto} of 0.0001. These results are consistent with the reported platelet role in

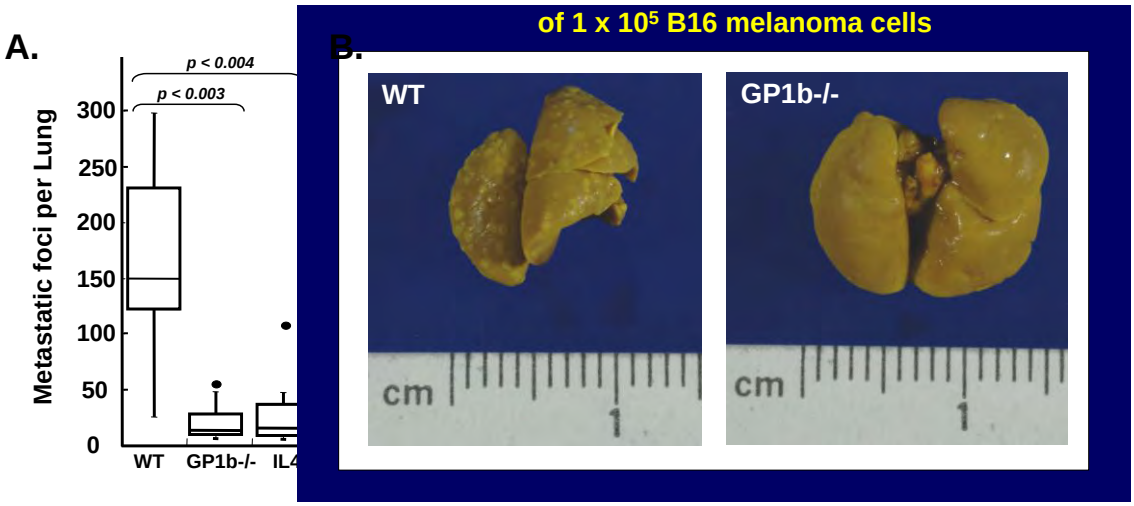
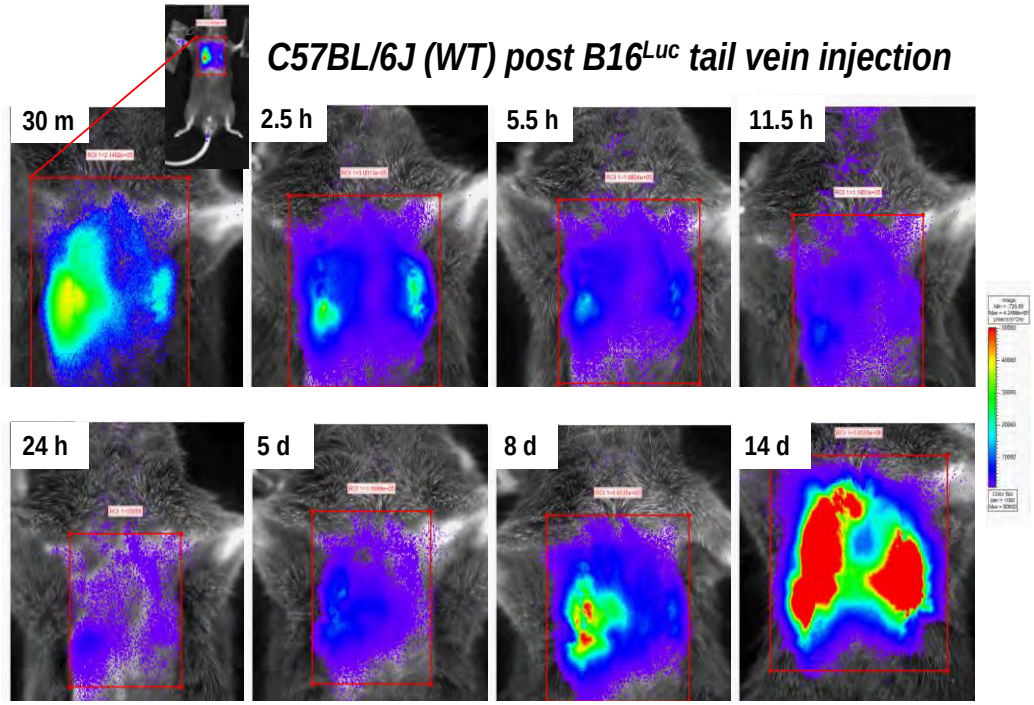


Fig. 1. *B16-F10.1 melanoma cells (10^5) were injected via a mouse tail vein. 14 days later, the lungs were removed and surface-visible tumors were counted in normal (WT), BSS mice (GP1b-/-) and mice with an absent extracytoplasmic domain of platelet GP Ibα (IL4-R/Ibα^{Cyto}). **(A)** Each box plot represents the number of surface foci from counting both lungs of individual mice. Median values are represented by the horizontal line. **(B)** Representative mouse lungs are shown with visible surface tumors.*



g. 2. *B16-F10.1 cells*
xpressing a luciferase
porter protein were injected
to the tail vein of normal
57BL/6J (WT) mice. Using an
vivo imaging system (IVIS,
enogen Corp) the fate of the
jected cells was followed
ith time. Mice were
nesthetized with 2.5%
olflurane via a nose cone
hile being imaged in the
upine position.
epresentative pictures were
ptured of the mouse thorax.
s shown, within 30 min of a
ost-tail vein injection B16^{Luc}
lls are present in the mouse
ng and reach a maximum
vel within a 2.5 hr time
indicative of the
maging an intraperitoneal
luciferin is available as a

e role of GP Ib-IX in this
ility to B16 cells to colonize

the lungs in this model of hematogenous metastasis. Is the GP Ib-IX dependent mechanism related to the role of platelets in protecting tumor cells from natural killer cells [6,7] and/or do the adhesive properties of GP Ib-IX influence the homing ability of B16 cells to the lung? Drs. Shaun Coughlin and Eric Camerer (UCSF) provided us with a stably-transfected B16F10 cell line ($B16^{Luc}$) expressing the firefly reporter protein – luciferase [8]. Using these cells allowed us to follow the fate of circulating $B16^{Luc}$ cells owing to their bioluminescent property. Briefly, the IVIS system is comprised of a CCD (charge-coupled device) camera equipped with software to track bioluminescent or fluorescent cells in the living organism. The bioluminescent feature differs from traditional fluorescence in that an excitation light is not required. If the animal is given a substrate, luciferin for luciferase-expressing cells, the location of the cells can be followed in an anesthetized animal. Figure 2 shows a representative IVIS scan from a single WT mouse following tail vein injection 5 x 10^5 $B16^{Luc}$ cells. The observed pattern represents a defined temporal sequence of events in which ***1)*** B16 cells accumulate in the lungs peaking within 2-3 hours, ***2)*** the majority of cells are cleared reaching the lowest level
elopment of luminescent tumors visible 4-5 days post-
us is an ability to home to lung is not influenced by GP Ib-
P Ib-IX, leads us to assume the platelet is influencing the
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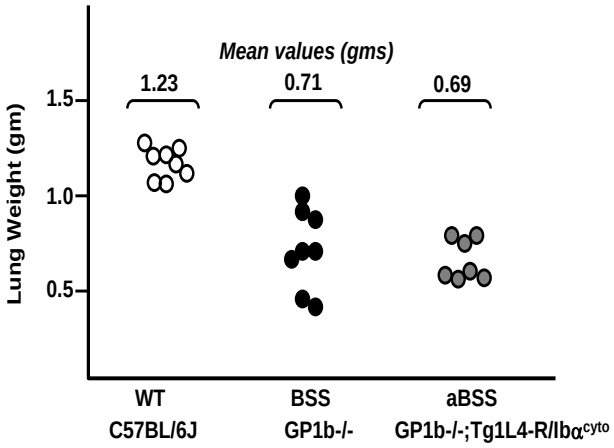


3. *Similar to experiments described in Fig. 1, tumor burden e lung following tail vein injection of Lewis Lung carcinoma is presented.*

e have suggested the molecular steps required for
astasis are similar for all solid tumors [9,10]. Is a role for GP
in lung colonization of B16 melanoma cells unique to B16
or would it apply to other malignant cell types? To address
question we performed tail vein injections using a Lewis
on, lungs were examined from WT, GP1b-/- and IL4-R/Ibα^{Cyto} animals. The animals were injected with 2.5 x 10^5 D121 cells and the resultant lung tumor burden was dramatic (Fig. 3). A determination of lung mass illustrated a significant decrease in tumor burden coinciding with the absence of GP Ib-IX. In this experiment we were unable to determine individual tumor foci as the carcinoma completely engulfed all surface visible lung tissue in the WT lung.

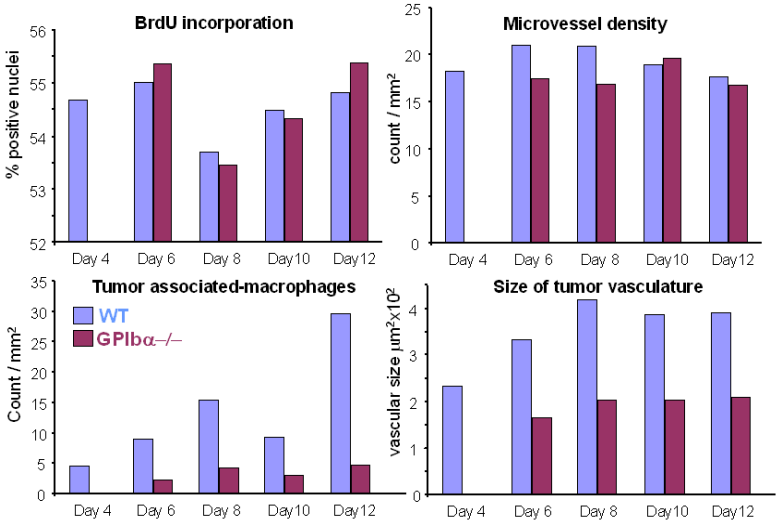


Fig 4. Quantitation of primary tumor growth (BrdU incorporation), microvessel density, tumor associated-macrophages, and size of the tumor vasculature is shown. Significant reduction in tumor associated macrophages was observed highlighting a platelet-inflammation component.

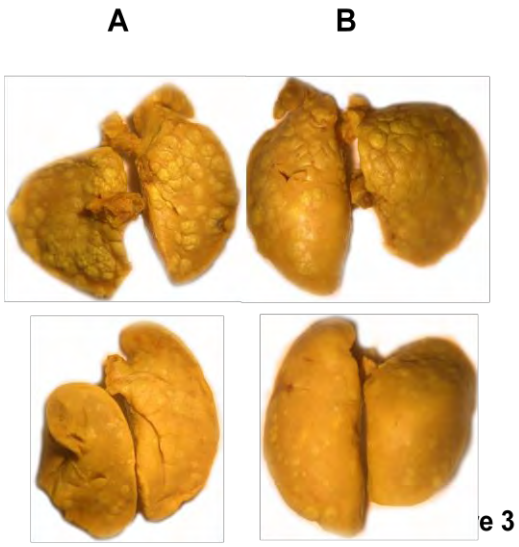
Immunohistochemical studies of the resultant tumors also revealed some striking differences dependent upon the presence of absence of GP Ib-IX (Fig. 4). Specifically, macrophage involvement in the tumor was significantly reduced as a

consequence of GP Ib-IX absence. Other tumor parameters, such as cell growth (BrdU incorporation), micro vessel density were unremarkable. Large vessel density was marked reduced consistent with Folkman's original hypothesis linking platelets and angiogenesis. [11]

Additional work relevant to Aim 1. To define the relevance of platelet glycoprotein VI in experimental metastasis. *This aim was achieved with tumor cell lines and monitoring of lung colonization.*

Platelet glycoprotein (GP)VI is a member of immunoglobulin super family specifically expressed on the surface of platelets [12,13]. GPVI is complexed on the platelet surface with FcR □-chain dimers to trigger platelet activation as a consequence of GPVI/collagen interactions [14]. Genetically altered mice devoid of GPVI show a slightly prolonged bleeding time and impaired thrombus formation [15,16], although strain variation appears to impact the severity of bleeding in individual mice [17]. The few patients described with a GPVI deficiency have a reported mild clinical bleeding phenotype [18,19], but a more recent analysis of more patients suggests the bleeding may be more severe than originally reported [20].

As describe above, mice deficient in a major platelet adhesion receptor, GPIb-IX, have an approximate 90% reduction in the mean number of lung tumors developing as a consequence of experimental metastasis [2]. These experiments utilized N10 congenic C57BL/6J strains deficient in GPIb-IX and tumor cells originally derived from the C57BL/6J strain of mice. As such, the animals were immunocompetent and represented a syngenic model of platelet dysfunction and tumor development. The effect of GPVI on experimental metastasis was examined by injecting B16F10.1 (B16) melanoma cells (10^5) into the lateral tail vein of the sex and age-matched mice. After 14 days we observed an approximate 50% reduction in the mean number of surface visible tumor foci on the lungs of GPVI^{null} mice (n = 7) as compared to wild type control C57Bl/6J mice (n = 6) (Fig. 1). The mean number of surface visible tumor foci on the lungs of GPVI^{null} mice was 134 as compared to 270 for wild type control C57BL/6J mice (Fig. 5). The *p*-value (Student's *t* test) was 0.013 indicating the reduction in the number of tumor foci on the lungs of GPVI^{null} mice was significant.



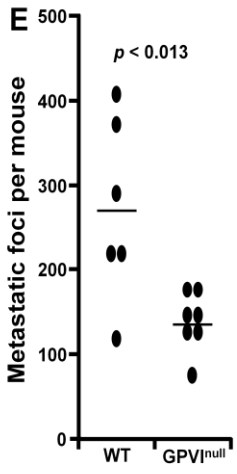


Fig 5. *B16F10.1 mouse melanoma cells (10^5) were injected via a mouse tail vein. Fourteen days later, lungs were removed and surface visible metastatic tumors on lungs were counted in wild type and GPVI^{null} mice.* ***(A, B)*** *Dorsal and ventral images of representative lungs are shown from a wild type mouse.* ***(C, D)*** *Dorsal and ventral images of representative lungs are shown from a GPVI^{null} mouse.* ***(E)*** *Individual animal data number for the number of tumor foci is shown for WT (n = 6) and GPVI^{null} (n = 7) mice. Horizontal bars are the mean values of 270 and 134 for WT and GPVI^{null} lungs, respectively.*

Similar experiments were performed using a highly metastatic cell line D121 (Lewis lung carcinoma). Tail vein injection of 4 X 10^5 cells produced surface visible tumor foci on lungs in 10 days (see Appendix). The mean number of metastatic foci on the lungs of GPVI^{null} mice (n = 12) was 69 as compared to 132 for control C57BL/6J (n = 15) mice with the *p*-value of 0.0003. Similar observations were made in 3 independent experiments suggesting Lewis lung carcinoma induced experimental metastasis is significantly reduced in the platelet GPVI deficient animals. Together these findings demonstrate platelet GPVI facilitates experimental metastasis in syngenic mouse models.

Specific Aim 2. To examine the relevance of thrombin binding to GP Ib-IX in tumorigenesis. Thrombin is a central molecule in hemostasis and a common thread for cancer as many tumor cells express thrombin. We have a

mouse model with normal human GP Ib-IX/vWF binding but blocked binding between GP Ib-IX and thrombin. Experiments are proposed to determine if a GP Ib-IX/thrombin axis is critical in tumor development.

The mouse model of with a defective GPIb-IX/thrombin binding axis was being bred during this funded period to generate a congenic strain of animals. The generation of a congenic strain requires a minimum of 10 generation backcrosses and then establishment of a colony that allows experimentation. For reasons that have never been defined husbandry to develop the congenic strain progressed at a very slow rate with low fecundity rates and high lethality of pups born. We do not believe this due to the specific mutation as breeding continued to not be a problem with the same mutation in a mixed genetic background. This husbandry issue impeded our ability to complete the proposed experiments during the funded period. However, we now have these animals and are going to get the experiments done with some internal funding. They will be completed as we still believe these experiments are critical for linking the central molecule of coagulation, thrombin, to platelet function in the progression of metastatic disease.

Specific Aim 3. To examine the relevance of platelet GP Ib-IX in a model of spontaneous metastasis. *Using a mammary tumor specific promoter overexpressing the oncogene middle T antigen (PyMT), we generated double transgenic animals devoid of platelet receptors in the presence of PyMT and characterized the tumor burden.*

Experimental metastasis (the primary approach for SA1 and SA2) is one of the most commonly used laboratory tools to study the role of various proteins in metastasis [21]. In xenograft models, various immortalized human cancer cells (such as MDA MB 231, MCF-7, etc.) are injected into the tail vein, portal vein, or left cardiac ventricle and monitored for their ability to establish tumor burden in mice. Xenograft models are useful to study the behavior of human cancer cell lines but the absence of a fully functional immune system in the host completely ignores the contribution of the immune system in tumor cell survival. In syngenic models there is a genetic match between the host mouse and the tumor cell allowing tumor cell survival to be monitored in the presence of a functional immune system. In addition, injections of immortalized cancer cell lines have been widely criticized for their indefinite *in vitro* culture which may have altered cellular properties. As such, experimental metastasis presents a rather artificial model which simplifies the process of metastasis to a single step of dissemination as opposed to an ongoing or continuous dissemination of cells from primary tumors in natural settings of metastasis [21]. However, the large bolus of tumor cells administered in experimental metastasis may reflect events occurring during the late stages of metastasis when a large number of tumor cells are shed from the primary tumor [22]. An alternative to experimental metastasis models are models of spontaneous tumor formation and metastasis. In these models, transgenic mice express an oncogene producing spontaneous primary tumors and metastases recapitulating some aspects of human cancer pathology [23].

In 1992, a mouse model of spontaneous breast cancer, MMTV-PyMT, was reported [24]. A transgenic insertion of the gene encoding the polyoma virus middle T antigen (PyMT) generated rapid and spontaneous multifocal mammary adenocarcinoma as a consequence of PyMT expression. Middle T antigen is a viral oncogene which provides a scaffold on the plasma membrane for constitutive activation of various signaling proteins [25]. Mammary tissue-specific expression is supported by a mouse mammary tumor virus long terminal repeat promoter (MMTV-LTR). The developing primary foci are followed by the appearance of spontaneous metastatic tumors on the lungs in more than 85% of 4-5 month old animals. In PyMT mice, palpable tumors are apparent on mammary glands by 9-10 wks and lung metastasis is detectable by 12-20 wks. Short latency, high penetrance, and a high incidence of lung metastasis occurring independent of pregnancy makes this a useful model to study spontaneous metastasis [26].

To complete this aim, we obtained a mouse colony from Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ) over-expressing the PyMT antigen [27]. Mice from this colony spontaneously develop primary breast tumors that at a frequency of approximately 80% will metastasize to the lung. We generated compound mice expressing the PyMT antigen and also containing our platelet mutation in the receptor complex, GP Ib-IX. We also performed studies characterizing the frequency of metastasis in these compound heterozygous animals. The flow diagram (Fig. 6) illustrates our strategy for generating these animals and also the strategy for phenotyping these animals. The breeding strategy was approximately a 12 month mouse husbandry strategy of genotyping and crossing to generate sufficient animals.

Primary tumor growth in GP1b^{null};PyMT mice

A compound transgenic mouse colony GP1b^{null};PyMT was generated which is devoid of platelet GP Ibα and expresses the polyoma virus middle T-antigen (PyMT) under the control of a mouse mammary tumor virus promoter (MMTV) (Fig 6). The PyMT oncogene in mice initiates the spontaneous development of a mammary adenocarcinoma by the age of 8-10 weeks without pregnancy or any other stimuli. To examine if the absence of platelet GP Ibα affects the growth of primary tumors, the primary tumor growth was followed on mammary glands of control GP1b^{WT};PyMT and GP1b^{null};PyMT mice for 20 weeks. Detectable tumors were first apparent on mammary glands by the age of 9-10 weeks.

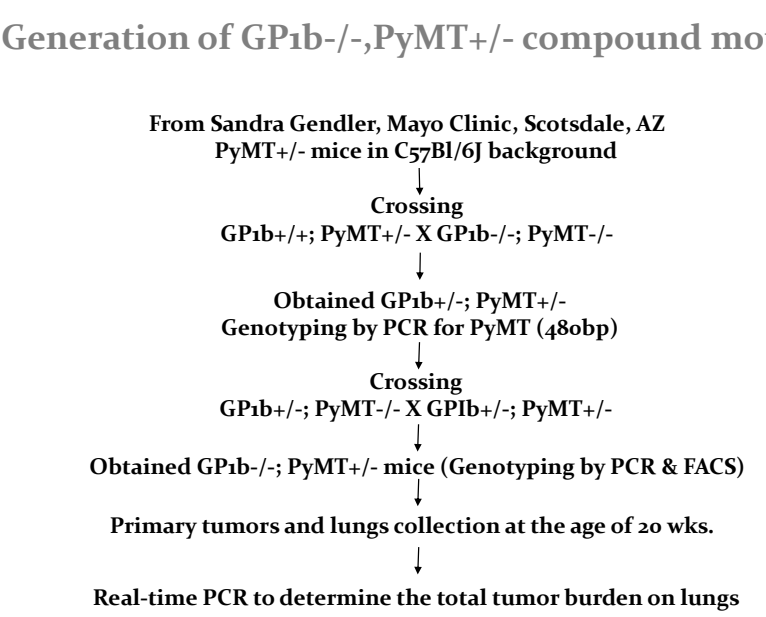


Fig. 6 *Breeding scheme for the generation of PyMT colonies.*

No difference in latency was found between control GP1b^{WT};PyMT and GP1b^{null};PyMT mouse colonies. The latency period on both the colonies varied from the age of 9-10 weeks to the age of 20 weeks. The expression of PyMT in both groups resulted in mammary adenocarcinomas by the age of 20 wks on single and sometimes multiple sites. The weight of primary tumors at 20 wks of age was more in the animals which developed primary tumors earlier than the animals in which primary tumor developed in later ages. At the age of 20 weeks animals were sacrificed (Fig 7).

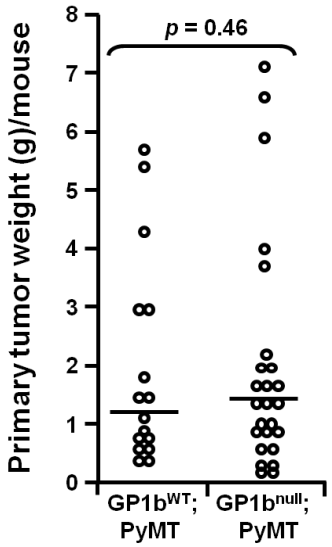


Fig. 7 Primary tumor weights. *The cumulative tumor weight at 20 weeks of age ranged from 0.3 g - 5.7 g in control GP1b^{WT};PyMT mice (n=16) and 0.13 g - 7.1 g in GP1b^{null};PyMT mice (n=25). The mean cumulative tumor weight of primary tumors in control GP1b^{WT};PyMT and GP1b^{null};PyMT mice was 1.9 g and 2 g respectively (p value = 0.46). These observations suggest growth of mammary adenocarcinoma is not affected by the absence of platelet GP Ibα in the MMTV-PyMT model.*

Spontaneous metastasis in GP1b^{null};PyMT mice

To quantitate total metastatic tumor burden within lungs, a real-time PCR assay was performed. The mRNA level for PyMT in lungs was indicative of spontaneous lung metastasis of the mammary adenocarcinoma since PyMT expression was restricted to mammary glands using the mouse mammary tumor virus promoter [28]. G3PDH (glyceraldehyde 3-

phosphate dehydrogenase) was used as a reference endogenous control transcript. A comparative C_T (threshold temperature) method was utilized for quantitation using the formula $2^{-\Delta\Delta CT}$. Before running the real-time PCR, efficiencies of primers for PyMT and G3PDH was checked by running validation experiments and found to be approximately equal. cDNA was prepared from PyMT induced primary mammary gland tumors and used as a positive control for the calculations by considering mRNA levels of PyMT as 1.

The extent of spontaneous lung metastasis was evaluated at the age of 20 wks in control GP1b^{WT};PyMT and GP1b^{null};PyMT animals. Mice were divided in two groups on the basis of cumulative primary tumor weight at the time of sacrifice; less than 1.5 g and greater than 1.5 g. In the mice which had cumulative primary tumor burden of less than 1.5 g; similar PyMT mRNA levels in lungs was found in the GP1b^{null};PyMT (n=14) mice as compared to PyMT mRNA in control GP1b^{WT};PyMT (n=9) mice with an insignificant *p* value of 0.38 (Fig 7A). In the other group of animals which had a cumulative primary tumor burden of more than 1.5 g the PyMT mRNA levels in lungs were similar between control GP1b^{WT};PyMT (n=7) and GP1b^{null};PyMT (n=9) (*p*=0.25) animals (Fig 7B). These data suggest no significant difference in spontaneous lung metastasis between control GP1b^{WT};PyMT and GP1b^{null};PyMT mouse colonies and the absence of GP Ibα from the platelet surface does not influence lung metastasis in the mouse model of MMTV-PyMT.

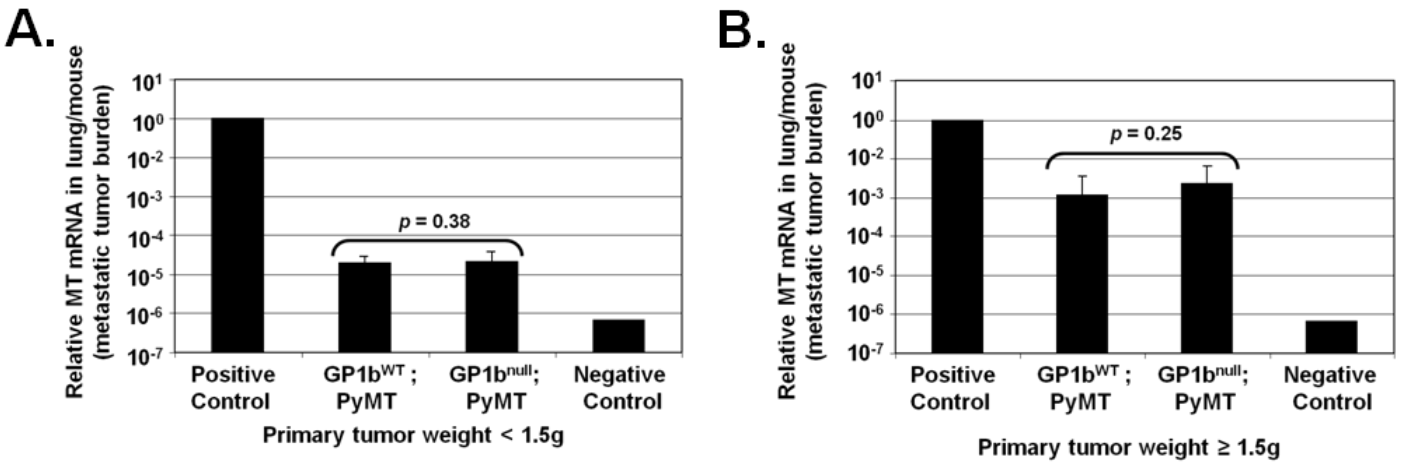


Fig 8. Lung metastatic burden in control GP1b^{WT};PyMT and GP1b^{null};PyMT mice. *Nulligravida control GP1b^{WT};PyMT and GP1b^{null};PyMT female mice were sacrificed at the age of 20 wks. Total metastatic burden on the lung of mice which had a cumulative primary tumor weight of less than 1.5 g (**panel A**) or greater than 1.5 g (**panel B**) was determined by measuring lung PyMT mRNA using real-time PCR assay. The positive control level is the mRNA from PyMT induced primary tumors. The negative control in each panel is the mRNA from the lung of a wild type C57BL/6J mouse. The graph illustrates the relative lung PyMT mRNA levels in control GP1b^{WT};PyMT (n = 9) and GP1b^{null};PyMT (n = 14) mice as compared to the positive control. Error bars represent the standard deviation. p value (Student's t test) is shown.*

Our first submission of this data for publication was met with questions on the hematologic parameters in the MMTV-PyMT model. Thus, we have in the past year generated addition PyMT expressing animals with normal platelets and evaluated platelet counts, bleeding times, and susceptibility to thrombosis. The data demonstrates these over expression of PyMT does not significantly impact normal hemostasis. Indeed, it could be speculated that mouse colonies expressing PyMT might elicit a Trousseau syndrome phenotype. However, we can now provide the data that shows their platelet function is not altered by a simple overexpression of PyMT. The revised data have now been resubmitted for publication.

Interpretation of Data

In the experimental metastasis assays, a 95% reduction in GP1b^{null} mice was observed, while in spontaneous lung metastasis no difference was found between control GP1b^{WT};PyMT and GP1b^{null};PyMT mouse colonies. Different cancer cells, immunologic response, routes of dissemination, and strain variation may be some of the reasons responsible for

the different outcomes in experimental and spontaneous metastasis assays. An immortalized cell line B16F10.1 mouse melanoma cells was used in the experimental metastasis assays. Although, these cells originated from the C57BL/6J strain, since they have been grown in a two dimensional tissue culture for an indefinite time, it is possible that they have lost some of their characteristic features and acquired some additional traits which may have changed their metastatic potential. In MMTV-PyMT mice metastatic tumors are formed by freshly dislodged tumor cells which might be assumed to retain all the cellular properties of a typical cell in a natural tumor environment. Nevertheless, discrepancy in conclusions on the relevance of GP Ib-IX for metastasis does exist. A similar discrepant conclusion was found when analyzing the protease activated receptor (PAR) 1 mouse knockout in the MMTV-PyMT model, PAR 1 knockout animals have reduced experimental metastasis but no difference in metastasis driven by MMTV-PyMT [29].
In experimental metastasis the direct injection of a large number of tumor cells into the venous circulation provides an excellent opportunity for tumor cells to interact with different vascular components, such as platelets, immediately after injection. In the model of MMTV-PyMT breast cancer metastasis, a smaller number of cells presumably first enter lymphatic vessels. Subsequently, they enter the blood circulation. As a result tumor cells have a longer and different vascular path to metastasize to the lungs as compared to experimental metastasis. This too, could alter the behavior of tumor cells leading to different outcomes in spontaneous PyMT metastasis versus experimental metastasis.

On the basis of observations obtained from control $GP1b^{WT}$;PyMT and $GP1b^{null}$;PyMT mouse colonies it can be concluded that platelet GP Ib-IXis dispensable for the growth of spontaneous primary mammary adenocarcinoma and associated spontaneous lung metastasis in the MMTV-PyMT model. However, while expression of PyMT may be a better model to study degradation of stroma surrounding the primary tumor and tumor cell migration; it has not proven to be the most reliable model for studying relevance of vascular components in hematogenous metastasis due to high variability in latency and the highly tumorigenic nature of PyMT generated tumor cells [30]. Future studies will investigate the contribution of platelet GP Ibα in a more controlled spontaneous tumorigenesis model where oncogenic activation can be induced at a precise time point.

Research Accomplishments

- In vivo imaging has demonstrated in models of experimental metastasis that platelet GPIb-IX does not facilitate tumor cell homing to the lung (SA1).

- Histological analysis has identified a platelet/macrophage axis that is significantly altered in animals devoid of platelet GPIb-IX (SA1-SA3).

- A congenic mouse colony expressing a variant of the GPIb-IX complex in which a Tyr 276 has been changed to Phe has been established after more than 10 generations of backcross mouse husbandry (SA2).

- A model of spontaneous breast tumor development and metastasis has been developed in C57BL/6J mice devoid of the platelet GPIb-IX receptor. Detailed studies of primary tumor growth and metastasis have been performed.

- Demonstrated reduced tumor metastasis in mouse models missing the platelet receptor GPVI

- Demonstrated the absence of platelet GPVI has no effect on primary tumor growth

- Established a mouse breeding colony with defective (absent) platelet glycoprotein receptors and a high incidence of primary breast tumors that can be analyzed to measure the metastatic potential in model of spontaneous tumor metastasis

Reportable Outcomes

- Oral symposia speaker at the American Society of Hematology meeting, Dec 2008, talk entitled "Platelets and Cancer"

- Oral symposia speaker at the International Society of Thrombosis and Hemostasis meeting, July 2009, talk entitled "Hemostasis and Cancer"

- Abstract: "Platelet Glycoprotein VI Contributes to Murine Experimental Metastasis" Shashank Jain, Susan Russell, Jerry Ware, 50th American Society of Hematology Annual Meeting and Exposition, San Francisco, CA, 2008.

- Abstract: "Platelet Glycoprotein GPVI Facilitates Murine Experimental Metastasis" Shashank Jain, Susan Russell, Jerry Ware, Oral presentation and Young Investigator Award to S Jain from International Society of Hemostasis and Thrombosis meeting, Boston, MA, 2009.

- Oral symposia speaker at the European Society of Hematology meeting, June 2010, talk entitled "The Platelet Paradigm in Thrombosis and Cancer"

- Abstract: "Platelet Glycoprotein Receptors in Metastasis" Jerry Ware, Department of Defense Breast Cancer Research Program, Orlando, Florida, Aug 2011

- Jain, S., Zuka, M., Liu, J., Russell, S., Dent, J.A., Guerrero, J.A., Forsyth, J., Maruszak, B., Gartner, T.K., Felding-Habermann, B. and **Ware, J.** 2007 Platelet glycoprotein Ibα supports experimental lung metastasis. *Proc Natl Acad Sci USA.,* 104:9024-9028.

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Conclusions

Our focus in the near future is to utilize our current understanding of the platelet GP Ib-IX / tumor cell axis and examine inhibitory molecules for their ability to decrease tumorigenesis. We know that antibody inhibition of platelet GP Ib-IX is not a viable option because antibody binding activates platelets and generates a severe thrombocytopenia. However, a novel inhibitory peptide has shown some biologic activity for inhibiting GP Ib-IX function in vitro and we plan to examine small molecule inhibition in models of metastasis (Fig 9). This becomes a translational initiative that is less hypothesis driven but is a direction that we believe we are able to pursue at this time with specific therapeutic possibilities.

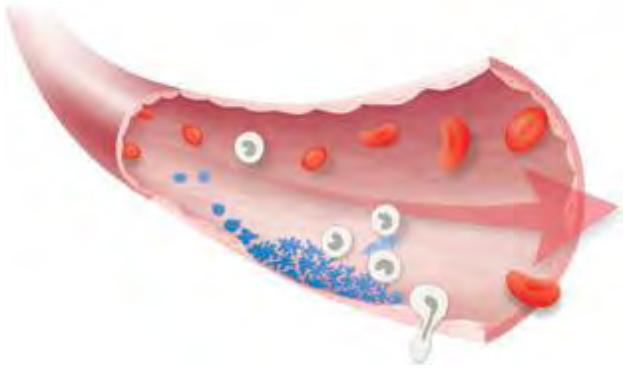

Fig 9. *A 15 amino acid peptide is being characterized for its ability to inhibit platelet GPIb-IX function. Abrogation of tumor cell extravasation from the bloodstream is a potential mechanistic target for cancer therapy.*

A "So What" Section

The main criticism of our work should be that we have defined a platelet / tumor cell axis that is important in an experimental setting but can this translate to improved health or treatment for a cancer patient. That is, the basic science approach is a "so what" if it cannot predict some improvement in medical care. Indeed, this is the crossroad for the current project and becomes our focus for the immediate future.

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Jerry Ware, Ph.D.
Judith Dent, Research Associate

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Appendices

Jain, S., Russell, S., and **Ware, J.** 2009 Platelet glycoprotein VI facilitates experimental lung metastasis in syngenic mouse models.

Farndale, R.W. 2009 Platelet glycoprotein VI as a mediator of metastasis. *J Thromb Haemost.* 7:1711-1712.

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IN FOCUS

Platelet glycoprotein VI facilitates experimental lung metastasis in syngenic mouse models

S. JAIN, S. RUSSELL and J. WARE
Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR, USA

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See also Farndale RW. Platelet glycoprotein VI as a mediator of metastasis. This issue, pp 1711–2.

Summary. *Background*: Glycoprotein (GP)VI is a key receptor for collagen on the platelet surface. It is a member of the immunoglobulin superfamily and is uniquely expressed on the surface of platelets, where it is assembled with the immunoreceptor tyrosine activation motif subunit, FcR-γ. We have previously reported the generation of a murine model of GPVI deficiency that revealed profound defects in collagen-induced platelet aggregation and in platelet activation following adhesion to collagen. Beyond the hemostasis/thrombosis paradigm, platelet receptors are emerging as significant participants in tumorigenesis and inflammation. *Objective*: In the current study, we have evaluated a role for platelet GPVI in primary tumor growth and experimental metastasis. *Methods*: Primary tumor induction and experimental metastasis assays were performed using syngenic immunocompetent animals and tumor cells derived from the C57BL/6J mouse strain in wild-type (C57BL/6J) and N10 C57BL/6J congenic GPVI-deficient mice. *Results*: Using either a Lewis lung carcinoma (D121) or melanoma (B16F10.1) cell line, we observed an approximately 50% reduction in the number of visible tumor foci in GPVI-deficient mice as compared with control C57BL/6J mice. Additional studies were performed to compare the size of subcutaneously implanted tumor cells, that is, primary tumor growth. Here, we observed no noticeable size difference when comparing the presence or absence of platelet GPVI. *Conclusions*: The results demonstrate that the presence of platelet GPVI facilitates experimental tumor metastasis but does not contribute to the growth of primary tumors.

Keywords: activation, adhesion, experimental metastasis, glycoprotein VI, hemostasis, primary tumor

Correspondence: Jerry Ware, Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, #505, 4301 West Markham Street, Little Rock, AR 72205-7199, USA.
Tel.: +1 501 526 6096; fax: +1 501 686 8167.
E-mail: jware@uams.edu

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Introduction

Platelet glycoprotein (GP)VI is a member of the immunoglobulin superfamily that is specifically expressed on the surface of platelets [1,2]. GPVI is complexed on the platelet surface with FcR γ-chain dimers to trigger platelet activation as a consequence of GPVI–collagen interactions [3]. Genetically altered mice devoid of GPVI show a slightly prolonged bleeding time and impaired thrombus formation [4,5], although strain variation appears to impact on the severity of bleeding in individual mice [6]. The few patients described with a GPVI deficiency have a reported mild clinical bleeding phenotype [7,8], but a more recent analysis of more patients suggests that the bleeding may be more severe than originally reported [9].

Beyond the well-characterized role for platelets in hemostasis and thrombosis, a role for platelets in hematogenous metastasis is well documented [10,11]. Recent studies have begun to define a molecular basis for the platelet's role in metastasis, ranging from prolonging the survival of tumor cells in the circulation [12] to facilitating the formation of tumor cell aggregates [13]. It has also been suggested that the tumor cell participates in platelet 'mimicry' to acquire the essential properties of a platelet that are so well characterized in hemostasis [14].

We recently reported that mice deficient in a major platelet adhesion receptor, GPIb–IX, have an approximate 90% reduction in the mean number of lung tumors developing as a consequence of experimental metastasis [15]. These experiments utilized N10 congenic C57BL/6J strains deficient in GPIb–IX and tumor cells originally derived from the C57BL/6J strain of mice. As such, the animals were immunocompetent and represented a syngenic model of platelet dysfunction and tumor development. We now report similar studies using animals lacking the receptor GPVI. The work was made possible after completing 10 generation backcrosses of $GPVI^{null}$ animals with C57BL/6J animals. Two different tumor cells were examined in a model of experimental metastasis and primary tumor growth. The results provide insights into the similarities between the platelet paradigm in hemostasis and

thrombosis and the developing platelet paradigm in tumorigenesis.

Materials and methods

Mice

Wild-type C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). GPVI^{null} mice have been described previously, and were generated by a gene-targeting strategy aimed at the mouse GPVI gene [4]. This GPVI^{null} mouse colony was backcrossed with C57BL/6J mice for 10 generations to produce N10 congenics. The breeding strategy involved stabilizing the mouse Y-chromosome in the first generation by using male C57BL/6J animals and choosing heterozygous male offspring for subsequent generations. The crossing of heterozygous GPVI$^{+/-}$ animals with normal C57BL/6J animals in the 10th generation led to GPVI$^{+/-}$ progeny; these were bred with each other, generating homozygous GPVI^{null} (B6.129S7-GP6^{tm1}) mice. All experimental protocols involving animals were IACUC-approved.

Tail bleeding time assay

Mouse tail bleeding times were determined by removing 3 mm of distal mouse tail and immediately immersing the tail in isotonic saline (37 °C). The bleeding time was defined as the period of time between the removal of the tail end and complete cessation of bleeding.

Flow cytometry

Whole blood was drawn from anesthetized mice via the retro-orbital plexus, using heparinized capillary tubes. One microliter of whole blood was added to 25 μL of modified Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 10 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 5 mM dextrose, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.4). One microliter of fluorescein isothiocyanate-conjugated rat anti-mouse GPVI stock antibody (Cat M010-1; Emfret, Eibelstadt, Germany) was added, and the reaction mixture was incubated on a rotary shaker for 30 min. The reaction was stopped by adding 250 μL of modified Tyrode's buffer. Samples were analyzed in a FACscan (Becton Dickinson, Franklin Lakes, NJ, USA) to confirm the absence of GPVI on the surface of platelets in the whole blood of N10 congenic GPVI^{null} mice. Similar procedures and dilutions were used with a phycoerythrin-labeled rat anti-mouse GPIbα monoclonal antibody (Cat M040-2; Emfret).

Cells

Lewis lung carcinoma cells (D121) were obtained from the ATCC (Manassas, VA, USA), and were grown according to the supplier's guidelines. B16F10.1 cells were obtained from B. Felding-Habermann (Scripps Research Institute, La Jolla, CA, USA), and grown in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 units mL^{-1} penicillin, 100 units mL^{-1} streptomycin, 2 mM glutamine and 1 mM sodium pyruvate in the presence of 5% CO_2.

Primary tumor induction

D121 cells were supplied with fresh medium 1 day prior to injection. Confluent cells (70–80%) were harvested by brief exposure to trypsin, and washed two times with serum-free medium prior to injection. Living cells were counted with the use of Trypan blue staining prior to injection to ensure that more than 95% of the cells were viable at the time of injection. Cells were kept on ice until injection. Mice were anesthetized, and 10^5 cells in 50 μL of serum-free medium were injected subcutaneously into the dorsal flank of sex-matched and age-matched control C57BL/6J and GPVI^{null} mice. After 17 days, the animals were euthanized, and primary tumors, lungs and liver were recovered from the animals. Primary tumors were weighed, and statistical analysis was performed using Student's *t*-test.

Experimental metastasis

Cells were supplied with fresh medium 1 day before tail vein injections. Confluent cells (70–80%) were harvested by brief exposure to trypsin, and washed two times with serum-free medium prior to injection. Living cells were counted with the use of Trypan blue staining prior to injection, to ensure that more than 95% of the cells were viable. Cells were kept on ice until injection. Cells were injected in volumes of less than 200 μL into the lateral tail vein of sex-matched and age-matched control C57BL/6J and GPVI^{null} mice. Mice were euthanized on day 10 for D121 cells and on day 14 for B16F10.1 cells. Liver, brain and lungs were recovered from mice, weighed, and stored in Bouine's fixative. After 24 h, surface-visible lung metastases were counted with the assistance of a stereo microscope (× 2 magnification; Tritech Research, Los Angeles, CA, USA). Briefly, lobes of the lung were separated to identify all surface areas on the lungs. Visible tumor foci were counted independently by two different laboratory personnel. Statistical analysis was performed by using Student's *t*-test.

Results

Generation of congenic GPVI^{null} mouse colony

We have previously reported a GPVI^{null} colony of mice generated by standard knockout technology [4]. The mice are viable, with no obvious deficiencies in fecundity, growth, or viability. To establish a congenic colony of GPVI^{null} mice, we backcrossed these animals with C57BL/6J animals for 10 generations. Expression of GPVI on the platelet surface was determined by flow cytometry using an anti-mouse GPVI antibody. No detectable GPVI expression was observed on the surface of platelets from N10 congenic GPVI^{null} mice (Fig. 1A). The expression levels of other platelet surface

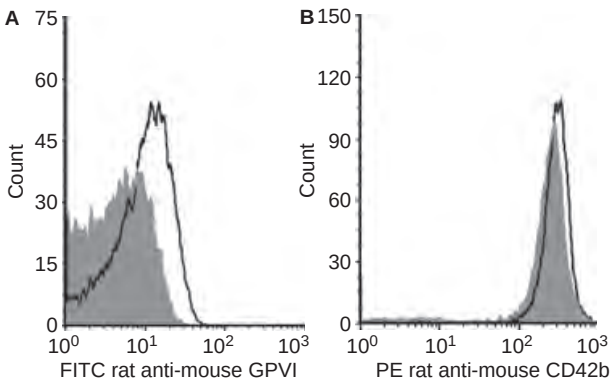

Fig. 1. Flow cytometry profiles of platelets of wild-type (C57BL/6J, black line) and glycoprotein (GP)VI^{null} (gray area) mice. (A) Profiles generated by a fluorescein isothiocyanate (FITC)-labeled rat anti-mouse GPVI monoclonal antibody. (B) Profiles generated by a phycoerythrin (PE)-labeled rat anti-mouse CD42b (GPIbα) monoclonal antibody.

receptors, such as the α-subunit of GPIb, were not significantly altered in the absence of platelet GPVI (Fig. 1B). Tail bleeding time assays on N10 congenic GPVI^{null} mice showed no prolonged bleeding time and, as originally reported, platelets from GPVI^{null} mice did not aggregate in the presence of type I fibrillar collagen (data not shown).

Primary tumor growth in GPVI^{null} mice

To investigate the role of GPVI in the growth of primary tumors, we induced primary tumors in control and N10 congenic GPVI^{null} mice. A highly tumorigenic Lewis lung carcinoma cell line (D121) was used to induce primary tumor by injecting 10^5 cells into the dorsal flank of sex-matched and age-matched control C57BL/6J ($n = 12$) and GPVI^{null} ($n = 12$) animals. Seventeen days later, primary tumors were removed along with lungs and livers (Fig. 2). The mean weight of primary tumors

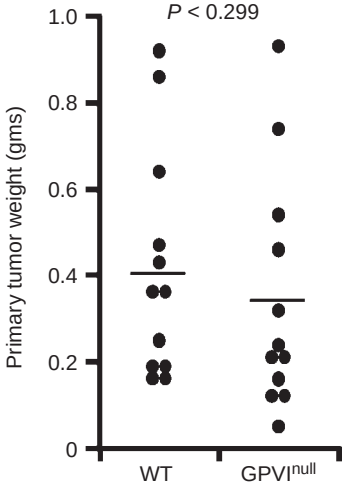

Fig. 2. D121 Lewis lung carcinoma cells (10^5) were subcutaneously injected into the dorsal flank of wild-type (WT) C57BL/6J ($n = 12$) and glycoprotein (GP)VI^{null} ($n = 12$) mice. Seventeen days later, primary tumors were excised and weighed. Primary tumor weights are presented for each animal. The horizontal bar is the mean value for each group.

from GPVI^{null} mice was 0.343 g, as compared with 0.417 g in control mice; the difference was statistically insignificant ($P = 0.299$). No surface-visible tumors were found on lungs or livers. We also counted PECAM-1-positive microvessels in the sections of primary tumors, and did not observe any significant differences between GPVI^{null} mice and control mice (data not shown) in the number of microvessels from tumors. Together, these results indicate that the absence of platelet GPVI does not affect the growth of primary tumors *in vivo*.

GPVI and experimental metastasis

The effect of GPVI on experimental metastasis was examined by injecting B16F10.1 (B16) melanoma cells (10^5) into the lateral tail vein of the sex-matched and age-matched mice. After 14 days, we observed an approximately 50% reduction in the mean number of surface-visible tumor foci on the lungs of GPVI^{null} mice ($n = 7$) as compared with wild-type control C57Bl/6J mice ($n = 6$) (Fig. 3). The mean number of surface-visible tumor foci on the lungs of GPVI^{null} mice was 134, as compared with 270 for wild-type control C57BL/6J mice (Fig. 3E). The *P*-value (Student's *t*-test) was 0.013, indicating that the reduction in the number of tumor foci on the lungs of GPVI^{null} mice was significant.

Similar experiments were performed using a highly metastatic cell line, D121 (Lewis lung carcinoma). Tail vein injection of 4×10^5 cells produced surface-visible tumor foci on lungs in 10 days (Fig. 4). The mean number of metastatic foci on the lungs of GPVI^{null} mice ($n = 12$) was 69, as compared with 132 for control C57BL/6J ($n = 15$) mice, with a *P*-value of 0.0003 (Fig. 4E). Similar observations were made in three independent experiments, suggesting that Lewis lung carcinoma-induced experimental metastasis is significantly reduced in the platelet GPVI-deficient animals. Together, these findings demonstrate that platelet GPVI facilitates experimental metastasis in syngenic mouse models.

Discussion

In recent years, platelet glycoproteins have been extensively studied for their role in normal hemostasis and thrombosis. What has been defined is a series of temporal events, including platelet adhesion, activation, aggregation, and thrombus formation [16]. A key receptor in these events, GPIb–IX, is a primary platelet adhesion receptor that binds to von Willebrand factor at sites of vasculature injury and has been well characterized as a key promoter of normal hemostasis [17]. Previously, we reported that GPIb–IX also contributes to experimental metastasis in murine models [15]. We found a significant decrease (90% reduction) in experimental metastasis in GPIb–IX-deficient mice as compared with control mice. GPIb–IX is important for normal platelet function, as its absence results in a severe and sometimes life-threatening bleeding disorder, Bernard–Soulier syndrome (BSS) [18]. Mouse models of BSS display a similar severe bleeding and antithrombotic phenotype that recapitulates the salient features of human BSS [5,19].

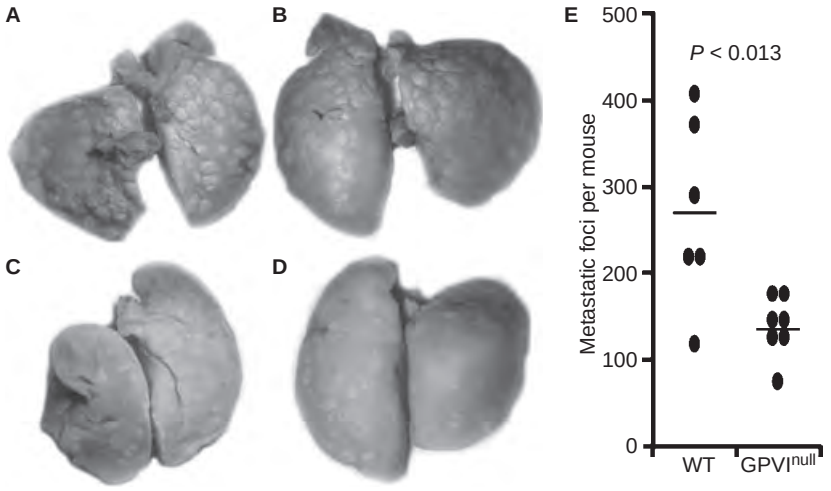

Fig. 3. B16F10.1 mouse melanoma cells (10^5) were injected via a mouse tail vein. Fourteen days later, lungs were removed, and surface-visible metastatic tumors on lungs were counted in wild-type (WT) and glycoprotein (GP)VI^{null} mice. (A, B) Dorsal and ventral images of representative lungs from a wild-type mouse. (C, D) Dorsal and ventral images of representative lungs from a GPVI^{null} mouse. (E) The number of metastatic foci per mouse for wild-type ($n = 6$) and GPVI^{null} ($n = 7$) mice. Horizontal bars are the mean values of 270 and 134 for wild-type and GPVI^{null} lungs, respectively.

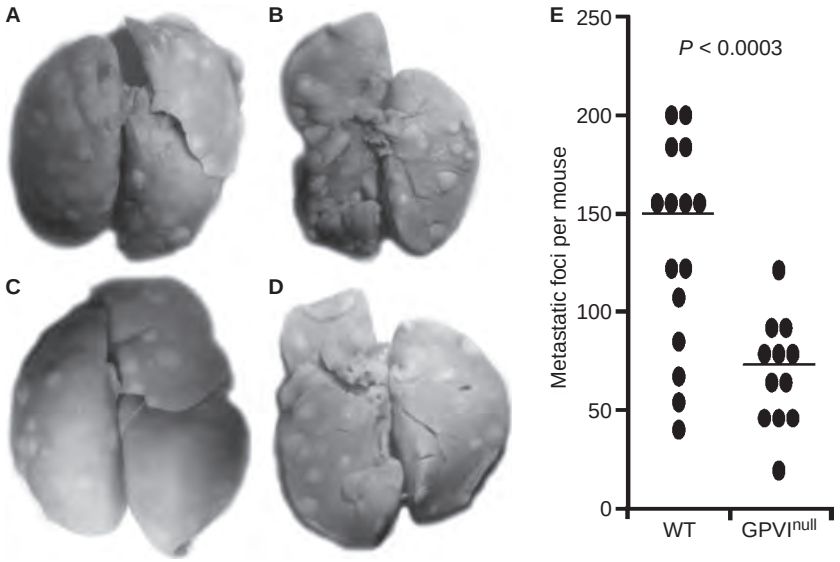

Fig. 4. D121 mouse Lewis lung carcinoma cells (4×10^5) were injected via a mouse tail vein. Ten days later, lungs were removed, and surface-visible metastatic tumors on lungs were counted in wild-type (WT) and glycoprotein (GP)VI^{null} mice. (A, B) Dorsal and ventral images of representative lungs from a wild-type mouse. (C, D) Dorsal and ventral images of representative lungs from a GPVI^{null} mouse. (E) The number of metastatic foci per mouse for wild-type ($n = 15$) and GPVI^{null} ($n = 12$) mice. Horizontal bars are the mean values of 132 and 69 for wild-type and GPVI^{null} lungs, respectively.

GPIb–IX is clearly important in primary hemostasis, but once bound to a damaged surface, the platelet undergoes activation, leading to a plethora of structural and biochemical changes that impact on the ability of the platelet to prevent blood loss. GPVI is involved in the activation step as one of the two main collagen receptors on the platelet surface [1,20]. The severity of the bleeding in GPVI-deficient humans seems to vary and, to date, has not been evaluated in the same detail as other genetic disorders, such as BSS. Nevertheless, the consensus seems to be that the congenital absence of GPVI does not lead to a severe bleeding phenotype, and this is certainly true for the GPVI-deficient mouse model that we have generated [4,5]. However, interpreting the mouse phenotype has been less than straightforward, as there appear to be strain-dependent influences on mouse GPVI function [6]. For the current study, we established N10 congenic C57BL/6J animals deficient in GPVI. In this strain of mice, we have observed minimal, at best, effects on hemostasis as assayed by tail bleeding times or time to occlusion following $FeCl_3$ damage to the mouse carotid artery (J. Ware, unpublished data). Thus, in contrast to mice mimicking human BSS, in the current study we have tested experimental metastasis in an animal with no functional GPVI and no significant bleeding phenotype. The results illustrate an approximately 50% reduction in experimental metastasis, as compared with the 90% reduction that we found when using mice with BSS. This suggests that the

severity of the bleeding phenotype might be an indicator of the significance of the platelet pathway to metastasis. Certainly, more models will need to be examined to determine whether such a hypothesis can be supported.

In this study, we have also evaluated the relevance for GPVI to the growth of primary tumors. We did not observe significant differences in primary tumor weight as a consequence of GPVI deficiency. We also did not observe any significant differences between GPVI-deficient mice and wild-type controls in the number of microvessels within the primary tumors of GPVI deficient mice. These results suggest that platelet GPVI, and/or GPVI-dependent platelet activation, does not have an effect on the neoangiogenesis for the growth of primary tumors. Although the GPVI-dependent activation pathway has been extensively studied [21], it is generally appreciated that platelet activation is highly redundant [22]. Thus, the present results do not completely address the relevance of platelet activation to primary tumor growth, but must be interpreted in relation to the presence or absence of platelet GPVI.

These studies underscore the well-established paradigm of platelet adhesion and activation in hemostasis and thrombosis and its application to mechanisms participating in the spread of cancer. Indeed, the spread of metastatic disease represents a fundamental change for the cancer patient that can negatively impact on the prognosis. Although the use of an experimental metastasis model can be criticized for being a less than perfect model of the events that result in the spread of human disease, it does represent a beginning in understanding the relevance of specific targets. To this end, testing the well-defined platelet paradigms of hemostasis and thrombosis for their relevance in other disease processes is a logical extension to allow the full utilization of animal models, reagents, and expertise. In cancer biology, GPIb–IX and GPVI represent potential targets for an antimetastatic effect. Experimental metastasis suggests that GPIb–IX might have a greater therapeutic benefit than GPVI, but the cost of excessive bleeding might be equally severe. As such, targeting GPVI, even if only a modest reduction in metastasis is achieved, might be of benefit if bleeding complications are not significant. Indeed, any reduction in tumor metastasis should be viewed with hope and optimism.

Addendum

S. Jain performed experiments and shared in the writing duties. S. Russell screened and maintained the colonies of mice. J. Ware designed experiments and wrote the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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COMMENTARY

Platelet glycoprotein VI as a mediator of metastasis

R. W. FARNDALE
Department of Biochemistry, University of Cambridge, Cambridge, UK

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See also Jain S, Russell S, Ware J. Platelet glycoprotein VI facilitates experimental lung metastasis in syngenic mouse models. This issue, pp 1713–7.

Platelets have previously been implicated in inflammatory processes, but there is a relatively short literature on their possible role in the progression of cancer. In this issue, Jain, Russell and Ware report that they injected metastatic cell lines into the tail vein of mice, and found that the development of metastases in the lung is impaired in platelet Glycoprotein VI knockout animals. Whilst the observed effect of ablation of the collagen receptor GPVI is partial, resulting in a halving of the number of metastases, this finding offers novel insight into the mechanisms underlying the recruitment of circulating tumor cells to susceptible tissues.

How can the platelet exert a pro-metastatic effect? Several possibilities present themselves, for some of which there is experimental evidence. First, the activated platelet may release or generate products that enhance tumor cell survival in the circulation or recruitment to sites of metastasis. One thinks especially of stored platelet alpha granule components that are released upon platelet activation, such as PDGF that might be chemotactic for tumor cells [1] or increase their rate of proliferation. VEGF is similarly released [2] and is a key mediator of tumor angiogenesis. In the present study, however, there was no effect of GPVI knockout on primary tumor mass or its complement of microcapillaries, suggesting that VEGF-mediated enhancement of tumor vascularity should be discounted. The activated platelet is also a rich source of newly-synthesized bioactive materials, including thromboxane A_2 and thrombin generated on the activated platelet surface. Some of these products could activate the vessel wall leading to endothelial retraction and increased opportunity for invasion, or may cause vasoconstriction that contributes to the entrapment of circulating tumor cells. Thrombin and other coagulation pathway components have been suggested as mediators of metastasis [3]. Such an indirect, soluble mediator, model would require circulating platelets to become activated, for example by cytokines released by primary tumors, but such mechanisms need not require any direct interaction of tumor cells with the platelet.

This is not the first such observation: a greater effect of complete platelet deficiency or PAR4 or fibrinogen knockout [3], or platelet GPIbα deletion [4], has been reported. Both GPIb and GPVI are adhesion receptors that support platelet interaction with collagen, indirectly in the former case via VWF, and directly in the latter. GPVI is a rapid and effective signaling receptor, whilst the VWF/GPIb axis is considered unable to elicit full activation of platelets under most conditions. The greater effect of ablation of GPIb, a weak binder of immobilized VWF at low shear but essential for platelet adhesion at high shear, raises the question of whether the shear rate at the site of platelet activation may be an important determinant of the mediation of metastasis, and suggests that in this system, the capture of platelets rather than their activation is the dominant process.

The possibility that platelets or platelet-derived microparticles may adhere to tumor cells and hitch a ride around the circulation until a suitably exposed subendothelial collagen is encountered, in the lung, supports the idea that platelet adhesion receptors play a role in targeting tumor cells to their metastatic niche [3].

Binding to tumor cells might result in platelet activation through GPVI, with consequent enhancement of metastasis through any or all of the mechanisms outlined here. Several direct and indirect ligand–receptor systems might mediate platelet–tumor cell interactions, utilizing, for example, GPIb or GPVI, the two platelet adhesion receptors studied by Jain in this and their previous study. GPIb-bound VWF can interact with RGD-binding integrins, for example αVβ3 that might be expressed on the tumor cell surface, whilst GPVI may bind laminin [5] and thence interact with apposed β1 integrins. Other direct interactions could occur, including that of the epithelial tumor-expressed podoplanin with platelet CLEC-2 [6], or tumor PSGL with P-selectin. The interaction of platelets with tumor cells has been found to perturb the ability of NK cells to target tumor cells [7], possibly by minimizing the scope for intercellular contact.

The therapeutic potential of GPVI in metastasis is of obvious interest. Because the bleeding defect in mouse associated with GPVI knockout is minor, and that of

Correspondence: Richard W. Farndale, Department of Biochemistry, University of Cambridge, Downing Site, Cambridge CB2 1QW, UK.
Tel.: +44 1223 766111; fax: +44 1223 333345.
E-mail: rwf10@cam.ac.uk

GPVI-deficient humans is believed to be modest, it seems quite plausible that the side effects of targeting GPVI would be more acceptable than the serious bleeding that would ensue from targeting either GPIb or fibrinogen. The balance between reduced metastasis and bleeding would need to be considered carefully, but it may be that antagonism of GPVI offers a novel, practical and specific means of minimizing the spread of metastatic tumors. This report [8] of Jain and colleagues needs careful extension if the underlying mechanism is to be identified, and then a balanced judgment on the potential of platelet GPVI as a metastatic target may be possible.

Disclosure of Conflict of Interests

The author states that he has no conflict of interest.

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Platelets: Linking Hemostasis and Cancer

Shashank Jain, John Harris and Jerry Ware

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Platelets

Linking Hemostasis and Cancer

Shashank Jain, John Harris, Jerry Ware

***Abstract*—**Platelets are the main cellular component in blood responsible for maintaining the integrity of the cardiovascular system via hemostasis. Platelet dysfunction contributes to a wide range of obvious pathological conditions, such as bleeding or thrombosis, but normal platelet function is also linked to diseases not immediately associated with hemostasis or thrombosis, such as cancer. Since the description of Trousseau syndrome in 1865, various experimental and clinical studies have detailed the interaction of platelets with primary tumors and circulating metastatic tumor cells. Observations have suggested that platelets not only augment the growth of primary tumors via angiogenesis but endow tumor cells physical and mechanical support to evade the immune system and extravasate to secondary organs, the basis of metastatic disease. Many laboratory and animal studies have identified specific targets for antiplatelet therapy that may be advantageous as adjuncts to existing cancer treatments. In this review, we summarize important platelet properties that influence tumorigenesis, including primary tumor growth and metastasis at the molecular level. The studies provide a link between the well-studied paradigms of platelet hemostasis and tumorigenesis. **(*Arterioscler Thromb Vasc Biol*. 2010;30:2362-2367.)**

Key Words: angiogenesis ■ experimental metastasis ■ hemostasis ■ primary tumor ■ spontaneous metastasis

Despite major advancements in the basic biology of cancer and new therapeutic interventions, cancer still remains among the deadliest diseases of the modern age. Over the last few decades, advances in the field of basic and clinical sciences have led to the recognition of hemostatic and coagulation systems in the growth and spread of different cancers in mouse models, as well as in human patients. Various distinct proteins originally described to participate in hemostasis are now found to be involved in different steps of cancer progression (Figure 1). The key mechanisms whereby hemostatic and coagulation systems cooperate are (1) platelets along with coagulation factors interacting with tumor cells to make platelet–tumor cell emboli aiding tumor cell extravasation to the metastatic niche; (2) a platelet cloak around tumor cells protecting them from natural killer (NK) cell cytotoxic activity; and (3) platelets storing various growth factors, proteases, and small molecules that help in tumor growth, invasion, and angiogenesis. In this review, we discuss the role of various platelets factors in tumorigenesis via these mechanisms. We have included thrombin and fibrinogen, given their importance to the platelet response but recognize many other coagulation factors not discussed are also important for cancer.

Platelet Involvement in Tumor Cell Dissemination

In 1865, Armand Trousseau described some patients with unusual migratory thromboses. These patients developed visceral malignancy later. Now, Trousseau syndrome is explained as a thrombotic event preceding the diagnosis of an occult visceral malignancy and diagnosed from an initial intravascular coagulopathy, platelet-rich microthrombi, microangiopathic hemolytic anemia, or thromboembolic problems.[1] For the homeostasis of the vasculature, it is crucial to maintain a normal platelet count in the blood. Experimental thrombocytopenia in mice induced by neuraminidase/antiplatelet serum resulted in a 50% reduction in experimental metastasis, and this could be reversed by transfusion of platelet-rich plasma transfusion.[2] Nf-E2 knockout mice (SCID background) with extreme thrombocytopenia have a significant reduction (94%) in metastatic burden in experimental metastasis models.[3] Others have shown that intravenous injection of some tumor cells may cause significant thrombocytopenia (50% to 70%) in mice.[4] Tumor cells that aggregate platelets in vitro produce more lung metastases than tumor cells lacking such ability, illustrating the platelet-activating potential of some tumor cells.[5,6] Taken together, these seminal observations suggest a robust interaction between circulating platelets and tumor cells.

After activation, platelets release small vesicles, called microparticles or microvesicles. Platelet microparticles are small in size (0.05 to 1 μm) with a defined plasma membrane and express selected platelet membrane and cellular proteins.[7] Lewis lung carcinoma cells treated with platelet-derived mi-

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From the Department of Physiology and Biophysics, University of Arkansas for Medical Science, Little Rock. Present address for S.J.: Department of Surgery, Duke Translational Research Institute, Duke University Medical Center, Durham, NC.

Correspondence to Jerry Ware, PhD, Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, #505, 4301 West Markham St, Little Rock, AR 72205. E-mail jware@uams.edu

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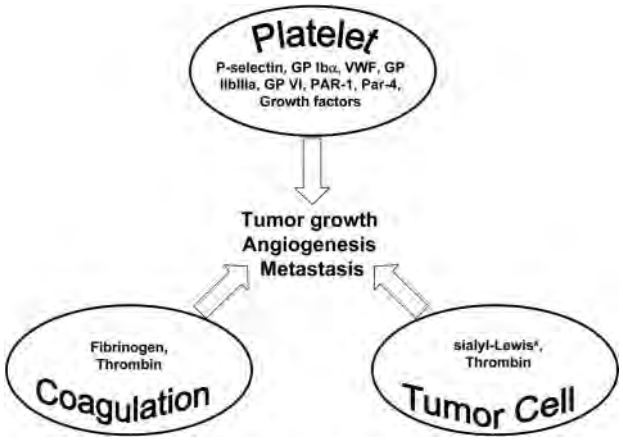

Figure 1. Interactions of platelets, coagulation, and tumor cells in tumorigenesis. Schematic diagram showing the interplay among various proteins: platelet receptors, coagulation proteins, and tumor cells interacting in the process of tumorigenesis.

croparticles have increased metastatic potential in syngenic mouse models.[8] Platelet microparticles increase invasive potential by increasing adhesion, proliferation, chemotaxis, and survival of breast cancer cell lines MDA-231 and BT-549 and the prostate cancer cell line CL-1. In the presence of microparticles, a number of cellular events have been documented, including upregulation of CXCR4, mitogen-activated protein kinase (MAPK) p42/44, matrix metalloproteinase (MMP)-2, and MMP-9, along with AKT phosphorylation.[9,10] Like the platelet, the platelet microparticle facilitates tumorigenesis (Table).

Platelet Receptors and Ligands Supporting Tumor Cell Growth and Survival

Tumor cells contain various membrane receptors that can bind directly to platelets and mediate tumor cell–platelet binding and activate platelets (Figure 2). Flow cytometry, fluorescence microscopy, and intravital microscopy have revealed the presence of platelet–tumor cell aggregates in vitro and in vivo.[11,12]

Table. Hemostasis/Cancer Associations

Factor	Origin	Primary Tumor	Metastasis
Platelet count	Platelet		↑
Platelet microparticles	Platelet		↑
P-selectin	Platelet	↑	↑
MAPK p38α	Platelet	No effect	↑
GPIbα	Platelet	No effect	↑
VWF	Platelet, EC	No effect	↓
GPVI	Platelet	No effect	↑
GPIIb-IIIa	Platelet		↑
Fibrinogen	Platelet		↑
PAR-1	Platelet, EC	↑	↑
PAR-2	EC	↑	↑
PAR-4	Platelet		↑
Thrombin	EC, TC	↑	↑
Chondroitin sulfate glycosaminoglycans	Tumor cells		↑
Sialyl Lewis X	Tumor cells		↑
Thrombospondin-1	Platelets	↓	

EC indicates endothelial cells; TC, tumor cells; ↑, stimulatory effect; ↓, inhibitory effect.

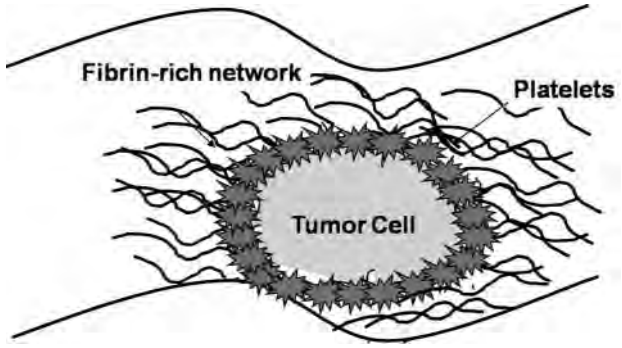

Figure 2. Interaction of platelets, coagulation, and tumor cells. Illustration showing some of the molecules implicated for tumor cells and platelets to promote interaction and influence tumor cell growth and survival.

P-selectin is an adhesion receptor found in the α-granules of platelets and Weibel–Palade bodies of endothelial cells.[13] After platelet activation, P-selectin appears on the platelet surface and aids the recruitment of other circulating platelets and leukocytes. Chondroitin sulfate glycosaminoglycans on the surface of human MDA-MET cells and murine 4T1 cells have been shown to bind selectively P-selectin.[14] It has also been suggested that platelet P-selectin recognizes sulfated galactosylceramide SM2, SM3, and SM4 on MC-38 cells, and sulfatide removal results in inhibition of in vitro platelet P-selectin binding to MC-38 cells and reduced syngenic experimental metastasis in vivo.[15] Experimental metastasis and subcutaneously implanted tumor growth was reported to be reduced in P-selectin–deficient mice and in an immunocompetent model with MC-38 colon carcinoma cells and B16 melanoma cells.[16,17] Not only is the rate of tumor cell homing to lungs diminished in P-selectin–deficient mice, but tumor cells fail to make aggregates with platelets, resulting in a decreased number of metastatic nodules in the lungs of P-selectin–deficient mice.[16]

A selectin ligand mimicry peptide, IELLQAR, has been found to have an inhibitory effect on B16-induced experimental metastasis.[18] An inhibitor of sialyl Lewis X, such as AcGnG-NM, not only reduces binding of tumor cells to selectin coated surfaces, activated platelets, and tumor necrosis factor-α–activated endothelial cells but also diminishes experimental metastasis in SCID mice.[19] Heparin inhibits the binding of P-selectin to its receptors and has been shown to inhibit experimental metastasis in syngenic mouse models.[20] Human platelets also express MAPK p38α, a serine threonine kinase, and the expression of MAPK p38α is directly linked to platelet P-selectin expression. Mice lacking MAPK p38α are not viable, but heterozygous p38$\alpha^{+/-}$ mice have reduced experimental metastasis, with no effect on primary tumor growth.[21] More recently, a role for P-selectin using models of spontaneous tumor metastasis has been presented.[22] Thus, through a wide array of studies, it can be concluded that P-selectin facilitates direct binding to tumor cells and augments tumor metastasis.

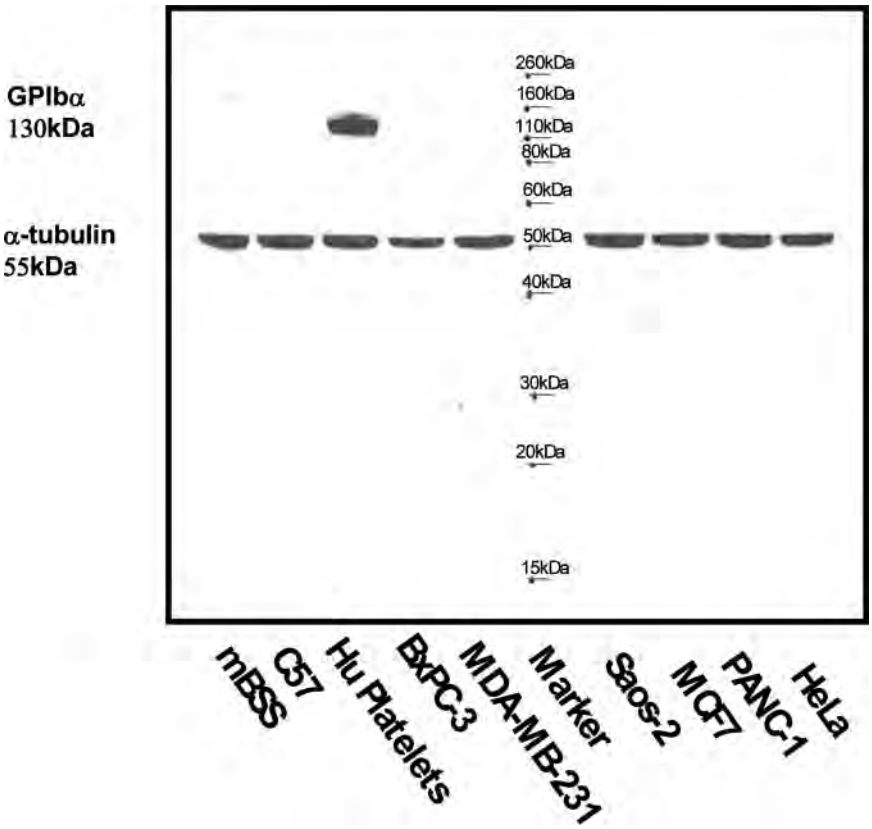

Figure 3. Western blot analysis of human tumor cell lines for the presence of human GP Ibα antigen. Also shown are normal mouse and human platelet lysates for representative signals. The blot was reacted with an anti–α-tubulin antibody as a positive control. Human GP Ibα antigen is observed in lysates from purified human platelets (Hu Platelets) and noticeably absent in all other samples (anti-human GP Ibα monoclonal antibody, LJ-Ib10, kindly provided by Zaverio Ruggeri, The Scripps Research Institute). mBSS indicates mouse platelet lysate missing GP Ibα; C57, normal mouse platelet lysate; Hu Platelets, human platelet lysate. All other lanes contain lysate from the indicated human tumor cell line.

The platelet receptor glycoprotein (GP) Ib-IX supports adhesion of platelets on a compromised vascular wall and, as such, is a key initiator of the platelet paradigm in hemostasis.[23] We reported that B16F10 mouse melanoma cell metastasis was reduced 15-fold in GP Ib-IX–deficient mouse colonies, suggesting an important role for adhesion in a syngenic mouse model.[24] However, overexpression of the polyoma middle T antigen in mouse mammary tissue and lung metastasis were not affected by the absence of platelet GP Ib-IX in a model of spontaneous tumor formation (S.J. and J.W., unpublished observation, 2010). Confounding results have been described with the administration of the anti–GP Ib-IX antibodies and the opposite effect, namely increased colonization of the lung.[25] The genetic absence of the platelet collagen receptor, GP VI, is also associated with a 50% reduction in experimental metastasis.[26] However, primary tumor growth and angiogenesis was not altered in GP VI–deficient mice.[26]

Although GP Ib-IX is widely considered to be a platelet-specific complex, several studies have suggested the expression of GP Ib-IX subunits by a variety of tumor cells.[27–29] In examining the expression of the major subunit of the GP Ib-IX complex, the α-subunit of GP Ib, in lysates of commonly used human tumor cell lines, we have been unable to document the presence of GP Ibα (Figure 3). At this time, we conclude that the expression of GP Ib-IX by cancer cells is not a common mechanism contributing to tumor formation or metastasis.

Von Willebrand factor (VWF) is a key major ligand for the platelet GP Ib-IX complex. Lewis lung carcinoma and B16-B6–mediated experimental metastasis was increased 2- and 5-fold, respectively, in VWF-deficient mice.[30] Lung colonization of tumor cells was increased 1 to 4 hours after injection in VWF-deficient mice, suggesting VWF may be responsible for tumor cell clearance in the circulation. VWF deficiency did not have any effect on the growth of primary tumors. However, it has also been reported that an anti-VWF antibody protects mice from experimental metastasis in mouse models.[31] It is possible that in the absence of VWF, platelet GP Ib-IX availability is increased, resulting in increased experimental metastasis. More definitive experimental proof is required to test this possibility.

Integrin αIIbβ3 (GP IIb-IIIa) is the most abundant receptor on the platelet surface. It participates in hemostasis by bridging platelet/platelet interactions via the ligand, fibrinogen.[32] GP IIb-IIIa inhibition by the monoclonal antibody 10E5 has been reported to diminish binding of CT26 and HCT28 cells to platelets in vitro.[31] Integrin $\beta3^{-/-}$ mice show a reduction in B16F10 melanoma-induced osteolytic experimental metastasis and reduced osteolytic bone invasion, both reversed by bone marrow transplantation of $\beta3^{+/+}$ marrow.[33] Antibody inhibition of GP IIb-IIIa diminishes tumor cell adhesion on extracellular matrix under flow conditions, suggesting a role for GP IIb-IIIa in platelet–tumor cell emboli extravasation.[34] c7E3 (ReoPro) a mouse–human chimeric antibody for GP IIb-IIIa has antiangiogenic and antitumor properties in mouse models.[35] A single treatment of this antibody in a xenograft model of SCID mice reduces experimental metastasis significantly. In addition, c7E3 also inhibits vascular endothelial growth factor (VEGF) secretion from

platelets in the presence of tumor cells.[36] Taken together, these studies suggest the major platelet integrin receptor plays a significant role in tumorigenesis at several different mechanistic levels.

The major ligand of GP IIb-IIIa, fibrinogen, is also implicated in metastasis. As a central ligand supporting platelet–platelet interactions and as a key cleavage substrate for thrombin in coagulation, fibrinogen is essential in the well-characterized paradigm of hemostasis and thrombosis.[37] In the realm of tumor biology, fibrinogen supports the formation of platelet–fibrinogen–tumor cell emboli as tumor cells intravasate.[38] Local deposition of fibrin and fibrin products have been found in solid tumors[39] and reported to support angiogenesis.[40] Experimental metastasis, spontaneous hematogenous metastasis, and lymphatic metastasis are significantly diminished in fibrinogen knockout mice.[3,41,42] Soluble fibrin monomer infusion facilitates platelet–tumor cell adhesion in vitro and experimental metastasis in vivo.[43] Thus, fibrinogen and fibrin participate in a variety of pathways contributing to tumor cell survival and growth.

Thrombin and Tumorigenesis

The role of thrombin in normal platelet function and in the pathways of blood coagulation highlight its importance as a central molecule linking the cellular (platelet) and biochemical (coagulation) paradigms of hemostasis.[44] Thrombin treatment of platelets facilitates platelet adhesion on tumor cells by 2- to 4-fold in various cancer cells (HM54, HCT8, CT26, and B16).[45] Thrombin-activated tumor cells (CT26 and B16F10) show a 10- to 156-fold increase in experimental metastasis.[46] Use of the thrombin agonist TRAP (thrombin receptor activation peptide) on CT26 or B16F10 cells also results in an increase in experimental metastasis.[47] Thrombin has been found to break endothelial junctions and aid in VE-cadherin– and β-catenin–mediated angiogenesis and tumor growth.[48,49] Thrombin also acts as a mitogenic agent for various mesenchymal tissues and cells by activating growth-stimulatory signals.[50] Hirudin, a thrombin antagonist, has been found to diminish 4T1 mouse primary tumor growth and spontaneous tumor metastasis in the mouse.[51]

Thrombin also upregulates the expression of various growth factors. It induces the secretion/expression of MMP-1, MMP-2, VEGF, angiopoietin-2, CD31, and receptors KDR and CXCR2 from human umbilical vein endothelial cells (HUVECs) and angiopoietin-1 from platelets.[10,52–56] Recently, it has been shown that thrombin upregulates secretion of GRO-α from MCF7 and HUVECs, and anti–GRO-α antibodies inhibit various angiogenic properties of MCF7 and HUVECs.[57] Thrombin was also found to upregulate expression of TWIST (an angiogenesis and tumor growth promoting transcription factor) in tumor cells and endothelial cells.[58] Interestingly, thrombin regulates proangiogenic and antiangiogenic factors differentially.[59] In platelets, the protease-activated receptor (PAR)-4 agonist (ATPGFK) inhibits VEGF-A secretion while increasing endostatin secretion. The PAR-1 agonist (TFLLR) increases VEGF-A secretion and inhibits endostatin secretion.

Thrombin cleaves the platelet receptors PAR-1, PAR-3, and PAR-4 at their N-terminal end, which in turn activates G protein–mediated intracellular signaling.[60] PAR1 expression has been directly correlated with the degree of invasiveness in primary breast tissue specimens.[61] Overexpression of PAR-1 in B16 cells results in a 5-fold increase in experimental metastasis.[62] It has also been reported that MMP-1 cleaves PAR-1 and enhances tumor growth and invasion of MDA-MB-231 cells in vivo.[63] PAR-4–deficient mouse colonies (SCID and C57 background) display a significant reduction in B16F10 cell–induced experimental pulmonary metastasis.[3] In a spontaneous tumor metastasis model, PAR-$2^{-/-}$ mouse colonies have reduced mammary adenocarcinoma growth and associated spontaneous metastasis.[64]

Platelets, NK Cells, and Tumorigenesis

As tumor cells intravasate to the circulation from a primary tumor, they interact with various components of the circulation system including platelets and immune cells. In mouse models of experimental metastasis, it has been found that most tumor cells entering the circulation do not survive, with $\approx$0.01% colonizing the lung.[12] NK cells are largely responsible for the elimination of cancer cells from the circulation.[65,66] Experimental and genetic depletion of NK cells in mice causes a 2- to 5-fold increase in experimental metastasis.[11,12] It has been proposed that platelets make a cloak around tumor cells and protect the tumor cell from NK cells.[11] It addition, platelets and fibrinogen are linked to a significant reduction in the cytolytic activity of NK cells in vitro.[11] NK cells express Mac-1 (integrin $\alpha_M\beta_2$), which has been shown to bind to platelet GP Ibα.[67] Whether a GP Ibα–Mac-1–mediated platelet–NK cell interaction plays a role in regulating NK cell cytolytic activity for cancer cells remains to be examined. Taken together, these observations highlight the platelet and coagulation interplay in the NK cell response to tumor cells.

Platelets, Angiogenesis, and Tumorigenesis

In 1971, Judah Folkman proposed that tumor growth is dependent on angiogenesis.[68,69] Platelets store various angiogenesis-regulating factors such as VEGF, platelet-derived growth factor, fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, insulin-like growth factor, angiopoietin, lysophosphatidic acid, sphingosine 1-phosphate, CD40 ligand, MMP-1, MMP-2, MMP-9, gelatinase A, and heparanase. Most of these angiogenic agents have been shown to participate in angiogenesis for tumor growth directly or indirectly. Platelets also contain antiangiogenic agents such as angiostatin, thrombospondin-1, platelet factor-4, endostatin, transforming growth factor β, and TIMP (tissue inhibitor of matrix metalloproteinases). Dissecting the relevance of pro- and antiangiogenic factors in the milieu of the platelet releasate represents a major challenge for the future.[70,71]

Recently, it has been found that expression of a negative regulator of angiogenesis, platelet-derived thrombospondin-1, is increased in tumor-bearing mice after tumor resection. Primary tumor growth of Lewis lung carcinoma cells was significantly increased in thrombospondin-1–deficient mice, suggesting a role for angiogenesis in tumor growth.[72] A chemically synthesized COOH-terminal peptide of platelet factor-4 (CXCL4L1) can inhibit angiogenesis and B16-induced melanoma growth in

vivo.[73] Together, these results suggest there is a role for platelet-derived antiangiogenic factors and may represent new directions for future studies.

Conclusions

The role of platelets in hemostasis and thrombosis has been studied for several decades, with remarkable molecular insights defining the hemostasis or thrombosis paradigm. Indeed, many of the well-studied platelet receptors and pathways can influence other diseases. Obvious connections to tumor growth, angiogenesis, and metastasis have been described here. Thus, the potential for insights from one discipline to rapidly contribute to new understandings in a different discipline is exciting. Future studies will hopefully contribute to both disciplines ultimately leading to better prevention, diagnosis, and treatment of disease.

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None.

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The hemostasis/thrombosis paradigm in cancer

S. Jain
J. Ware

Department of Physiology & Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA

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A B S T R A C T

Uncontrolled cell growth and spread in the human body leads to cancer and represents the leading worldwide cause of death, according to the American Cancer Society. Despite incredible advances in understanding the basic science of cancer and major advancements in therapeutics, cancer remains one of the deadliest diseases of the modern age. The metastatic conduit by which a tumor cell travels is the vascular system. Thus, the normal physiologic function of blood can influence spread and growth of a cancer cell. Indeed, mechanisms that control blood loss (hemostasis) and pathological clot formation (thrombosis) can have dramatic effects on the progression of cancer. The intersection of hemostasis, thrombosis, and cancer is being defined at the molecular level where known paradigms of hemostasis and thrombosis are seen to have significant relevance for tumor progression. We will summarize some of these key pathways focusing on the relevance of circulating blood platelets and the biochemical enzymes supporting blood coagulation. Many of the key platelet and coagulation elements have been studied for decades as targets for anti-thrombotic and anti-coagulation therapy. Future studies will better define the therapeutic potential of these targets for improving cancer patient prognosis while maintaining the critical hemostatic balance.

Platelets and tumorigenesis

The best studied function of platelets is in the formation of the hemostatic plug to prevent hemorrhage. Platelets circulate in a resting, or inactive, state and, following vascular injury, a variety of events can activate platelets, leading to irreversible adhesion at the damaged area, secretion, and sequestration of more activated platelets. Central to the formation of a platelet plug is platelet stabilization with insoluble fibrin, an end product of blood coagulation, which will be discussed later. A model has emerged bridging platelets, coagulation, and tumor cells to support survival of the tumor cell (Figure 1). The basis of such a model started from a seminal report in 1968 showing that experimental thrombocytopenia in mice induced by an anti-platelet serum resulted in a 50% reduction in experimental metastasis.[1] The conclusion was made that tumor cell attachment to the host vascular endothelium might be augmented by platelets.[1-3] The idea that platelets supported tumor progression was further elaborated in 1998 by Judah Folkman.[4] Later, a mouse genetic model with severe thrombocytopenia (Nf-E2 knockout) was studied and found to have a significant reduction (94%) in metastatic burden using a similar model of experimental metastasis.[5] Indeed, the interrelationship between tumor cells and platelets is highlighted by the intravenous injection of tumor cells, leading to a significant reduction in circulating platelet counts (50-70%) in mice.[6] Tumor cells which aggregate platelets in vitro produce more lung metastases than tumor cells lacking such ability.[7]

Both tumor cells and platelets contain various membrane receptors which support their bridging and may also lead to subsequent platelet activation (Figure 2). Flow cytometry, fluorescent microscopy, and intravital microscopy have revealed the presence of platelet/tumor cell aggregates both in vitro and in vivo.[8,9] A model of platelets "cloaking" the tumor cell has evolved, which suggests the platelet protects the tumor cell from natural killer cells or the body's innate immune response.[9] An immunoglobulin like molecule, Necl-5 has been found to be upregulated in various cancer cells and binds to platelet DNAM-1 (CD226).[10] Overexpression of Necl-5 in C26 colon adenocarcinoma cells results in increased experimental metastasis in BALB/c mice. Monoclonal antibody inhibition of Necl-5 on CT26 cells also causes reduction in metastatic burden on lungs but does not affect primary tumor growth.[11]

Contributing to the platelet/tumor cell axis on the platelet side, a wide range of proteins has been described, each with previously acknowledged roles in normal hemostasis. P-selectin (originally described as GMP-140 and PADGEM), has been extensively studied both on platelets and endothelial cells as a resident marker of the platelet α-granule and the endothelial cell Weibel-Palade body, respectively.[12] After platelet activation P-selectin appears on the platelet surface and helps in the recruitment of circulating platelets and leukocytes. Many tumor cells, such as small cell lung carcinoma cells and neuroblastoma cells contain sialyl-Lewis X, which binds to the P-selectin of platelets.[13] Chondroitin sulfate glycosaminoglycans on

the surface of MDA-MET breast tumor cells and 4T1 cells also bind platelet P-selectin.[14] Experimental metastasis and tumors produced by subcutaneously implanted tumor cells were both reduced in P-selectin deficient mice.[15] It was found that not only the rate of tumor cell homing to lungs was diminished in P-selectin deficient mice, but they also fail to make aggregates with platelets and resulting in a decreased number of metastatic lung nodules.[15] A selectin ligand mimicry peptide, IELLQAR, has been found to have an inhibitory effect on B16 (melanoma) induced experimental metastasis.[16] For P-selectin, a myriad of studies has suggested that platelet P-selectin participates in tumor growth by facilitating angiogenesis and binding directly to tumor cells to augment tumor metastasis.

Two major receptors on the platelet surface are the adhesion receptor for von Willebrand factor (VWF), glycoprotein (GP) Ib-IX, and the integrin receptor for fibrinogen, αIIbβ3.[17] We have reported a role for the α-subunit of GP Ib-IX in experimental metastasis[18] and others have demonstrated the ability of αIIbβ3 inhibitory antibodies to block platelet tumor cell interactions.[19] Genetic models of β3 deletion have also demonstrated β3 as essential to tumorigenesis but contribution of the platelet β3 subunit is not solely validated by this model since β3 gene expression occurs in a variety of cell types, including tumor cells.[20-22] Indeed, both GP Ib-IX and αIIbβ3 are key platelet receptors in the hemostasis and thrombosis paradigm, as shown by the experiments of nature, the Bernard-Soulier syndrome and Glanzmann's thrombasthenia, respectively.[23] The integrin receptor has been studied for a number of decades with a wide array of inhibiting therapeutic drugs for their antithrombotic properties and the utility of these agents for antitumor properties should be further examined. Similar therapies for the platelet GP Ib-IX receptor have not been widely tested, but will require close scrutiny for their adverse bleeding side effects.

The role of platelets in the hemostasis paradigm is to tether to damaged vascular surfaces but at some point platelets are irreversibly bound and there is inherent platelet activation. As a consequence there are dramatic changes in the biological properties of the platelet. Thus, the issue of platelet activation in tumorigenesis becomes relevant to understand if platelet activation contributes to the behavior of a tumor cell. One well characterized activation signaling molecule is Gαq in platelets. Mice deficient in Gαq have significant thrombotic impairment but they have also have dramatically reduced experimental metastasis.[9] Primary tumor growth is unaffected in Gαq deficient mice.[9]

Receptor mediated platelet activation can also occur via the platelet collagen receptor, GP VI. As a platelet specific receptor, GP VI assembles on the surface of platelets with the immunoreceptor tyrosine activation motif (ITAM) bearing subunit, FcR-γ.[24] Using models of experimental metastasis, both B16F10 (melanoma cells) and D121 (Lewis lung carcinoma cells) produce 50% fewer tumor lung foci in mice lacking GP VI.[25] Similar to the Gαq studies, no differences were seen in primary tumor growth as a consequence of GP VI absence.[25]

Aggrus/Podoplanin is a type-I transmembrane sialomucin-like glycoprotein found on tumor cells that can bind to the C-type lectin like receptor 2 (CLEC-2) on the

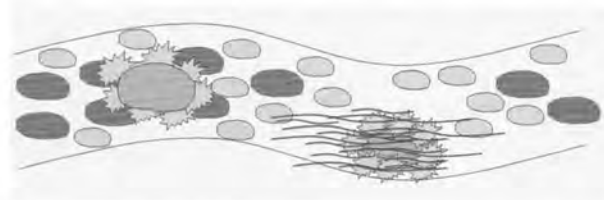

Figure 1. Tumor cell platelet/coagulation interactions. The tumor cell (brown) interacts in the circulation with platelets presumably leading to a "cloaked' tumor cell that subsequently supports platelet activation and coagulation. The end result is a tumor cell embedded in a fibrin/platelet mesh protecting the tumor cell from the host's innate immunity and providing a microenvironment that supports extravasation to a secondary site for tumor growth.

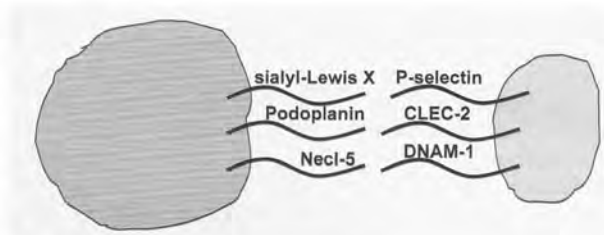

Figure 2. Platelet/tumor cell adhesion. A wide array of proteins have been described on the tumor cell and platelet that can support their interaction in the circulation. Shown are a few proteins on the tumor cell (left) and platelet (right) that have been extensively studied in metastasis and primary tumor growth.

platelet surface inducing activation and aggregation of platelets.[26] Podoplanin expression on CHO cells promotes experimental and spontaneous metastasis of CHO cells in BALB/c-nu/nu mice.[27] It has also been reported that ectopic CD9 expression on HT1080 cells that also express podoplanin results in reduced pulmonary metastasis suggesting that CD9 neutralizes podoplanin-mediated platelet aggregation by acting as a tumor suppressor.[28]

Thus, the opportunity for platelets, their receptors, and intracellular signaling molecules to promote or modulate tumorigenesis is quite extensive. The role for many of these molecules is a modulation via a direct means while for others a more indirect mechanism is responsible. From a simple standpoint, it would appear that platelet processes, important when the platelet interacts with a surface, albeit a damaged, vascular bed or a circulating tumor cell, also elicit a potential to influence a tumor cell in its immediate microenvironment. The tumor cell by simply being in this microenvironment may be changed with the outcome many times favoring the survival of the tumor cell.

Coagulation and tumorigenesis

The link between cancer and blood coagulation dates to 1865 with a description by Armand Trousseau of some cancer patients with thrombosis. Today, Trousseau's syndrome is explained as thrombotic events preceding the diagnosis of an occult visceral malignancy.[29] Often associated with intravascular coag-

ulopathy, platelet-rich microthrombi, microangiopathic hemolytic anemia, and thromboembolic problems, tumor cells have inherent procoagulant properties.[29,30] Tumor cells secrete many proteins that may initiate coagulation, resulting in the formation of a fibrin-rich tumor cell-platelet emboli (Figure 1). Many of these coagulation factor-tumor cell interactions have been studied in animal models and found to support angiogenesis, tumor invasion and experimental and spontaneous tumor metastasis.

Tissue factor (TF) is a transmembrane glycoprotein constitutively expressed by fibroblasts of the vessel wall.[31,32] TF is essential for the extrinsic pathway of coagulation. The binding to factor VII/VIIa to form a bimolecular complex which activates factor X leads to the generation of thrombin and fibrinogen [33]. Higher plasma concentrations of TF are found to be correlated with bigger tumor size in xenograft mouse models,[34] as opposed to TF inhibition, which suppresses tumor growth.[35] TF and TF positive microparticles also promote cancer associated venous thromboembolism (VTE).[36] In experimental metastasis assays TF inhibition by antibodies or TF pathway inhibitors significantly reduces lung metastasis.[37-39]

Thrombin, as a central regulator of platelet function and coagulation, activates platelets and tumor cells by increasing tumor cell-platelet adhesion in vitro and metastasis in vivo.[40-42] In addition, the thrombin receptors (PARs) influence tumor progression.[43] Thrombin also acts as a mitogenic agent for various mesenchymal cells by activating growth-stimulatory signals.[44] Hirudin, a thrombin antagonist has been found to diminish primary tumor growth of 4T1 mouse breast cancer cells and decrease spontaneous tumor metastasis in mice by 4-11 fold.[45] Clearly, thrombin sits in a middle of the platelet and coagulation axis influencing various aspects of tumor cell metastasis and primary tumor growth.

Following the generation of thrombin, the cleavage of fibrinogen into insoluble fibrin serves as the anchoring mortar that stabilizes the platelet plug and creates a blood impermeable barrier preventing blood loss. Thus, fibrinogen is a final key protein in the coagulation mechanism. It has been found that platelet-fibrinogen-tumor cell emboli form as tumor cells intravasate aiding in metastasis.[46] Local deposition of fibrin and fibrin products are associated with solid tumors.[47] Fibrin and fibrin degradation products (FDP's) have also been shown to support angiogenesis facilitating tumor growth.[48] In fibrinogen knockout mice multiple models of metastasis have shown significant reduction providing direct genetic proof for the crucial role of fibrinogen.[5,49,50] Beyond the direct relevance of the coagulation process, the interaction of fibrin with adhesion molecules, tumors cell integrins, inflammatory cells, stromal cells, and extracellular matrix can all potentially control cell proliferation and ultimately tumorigenic potential.

Roles for coagulation factors VII, VIII, and XIII, in addition to those proteins mentioned above, have been reported to facilitate tumorigenesis via direct and indirect mechanisms.[51-53] Coagulation as an intrinsic mechanism to stabilize blood flow lends itself to the tumor cell helping to evade innate immunity, stabilizing tumor microemboli in the metastatic nice, and providing a matrix to support extravasation.

Concluding remarks and future directions

It is now well accepted that hemostatic and coagulation proteins play a significant role in the growth of primary tumors as well as metastasis by various direct and indirect mechanisms. Various antiplatelet and anticoagulant drugs are widely used in clinics to manage a variety of vascular complications. Coumadin, heparin, aspirin, and LMWH are some of the anticoagulant drugs which have been used in clinical studies for their effects on cancer prognosis. Zacharski et al. reported that coumadin prolongs survival of patients from 23 to 50 weeks.[54] LMWH and heparin have been reported to prolong survival of cancer patients.[55,56] Anticoagulant drugs can be beneficial for the treatment of cancer patients but they come with the inherited risk of bleeding complications. The anticancer potential of some antiplatelet agents, such as Integrillin, Reopro, or Aggrastat targeting αIIbβ3 and clopidogrel targeting P2Y12 receptors have not been studied in clinical settings and warrant closer attention from basic and clinical scientists. Most likely, the use of any antiplatelet or anticoagulant agent must be carefully evaluated for therapeutic benefit.

Decades of work dissecting the role of platelets in hemostasis and thrombosis can now be quickly applied to other disease processes, the case being made here for cancer biology. That said, antithrombotic and anticoagulant therapies may have benefits far beyond that of clot inhibition. Armed with information derived from decades of basic research on the mechanisms supporting blood coagulation and analysis of bleeding disorders, we have the potential to contribute to new platelet paradigms and hopefully aid in the advancement of treatment for the cancer patient.

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CHAPTER 38

Tumor Growth and Metastasis

Jerry Ware and Shashank Jain
Department of Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas

I. Introduction

The link between hemostasis, thrombosis and cancer dates to the 19th century with astute observations by the French internist, Armand Trousseau. His insights, referred to as the "Trousseau sign of malignancy" associated a hypercoagulable state and malignancy. (1) Indeed, the platelet/coagulation interface contributes to many aspects of tumor cell extravasation, migration, and growth. (2) The intrinsic properties of the platelet relevant for normal hemostasis are exploited in the pathological setting by tumor cells to assist in the growth and survival of the malignant cell. (3) Among the relevant roles for the platelet are platelet – tumor cell interactions that allow the tumor cell to evade the innate immune system, platelet storage granules loaded with pro-angiogenic and growth factors, and specific platelet receptor interactions that facilitate arrest in the bloodstream. Likely, most relevant in these processes are the platelet – tumor cell interactions occurring in the bloodstream and ultimately leading to metastatic disease and a worsening prognosis for the patient. Understanding the molecular details of these interactions may

rapidly translate into therapeutic options for the cancer patient given the decades of research defining mechanisms of platelet function in hemostasis and thrombosis.

II. The Platelet – Tumor Cell Axis

A common experimental strategy to investigate the platelet – tumor cell axis is experimental metastasis where a bolus of tumor cells is injected into the venous circulation and tumor burden is evaluated at a distant site. Most commonly, mouse models of experimental metastasis have been exploited where tumor cells are injected into the tail vein of mice. Under this experimental setting a variety of methods that reduce circulating platelet count have been shown to result in a reduced tumor burden in the lung. (4;5) The relevance of the lung in this model reflects the pulmonary circulation as the first capillary bed for which tumor cells pass after entering the circulation via the tail vein. Thus, tumor emboli become lodged in the pulmonary vasculature and provide a microenvironment whereby the tumor cells are able to extravasate from the vessel and mimic a metastatic lesion in the lung.

While experimental metastasis does not completely mimic all of the processes occurring during the progression of human metastatic disease, the model has been powerful for examining the molecular interactions between platelets and tumor cells in the vasculature. One drawback of experimental metastasis has been the focus on a limited number of cell lines. These models are typically performed in sygeneic mice where the host animal and tumor cell share a common strain origin. Most laboratory mouse strains where targeted gene disruptions exist have been backcrossed to create a

congenic mouse model in the C57BL/6J strain. Two mouse tumor cells originating in the C57BL/6J background are the melanoma cell, B16, and a Lewis lung carcinoma, D121. Thus, a wealth of literature exists using B16 and D121 cells in experimental metastasis. (2) Whether the results extrapolate to all tumor cells is unlikely given the wide range of heterogeneity that tumor cells present. Indeed, injection of some tumor cells can result in thrombocytopenia while others do not. (6)

The artificial nature of the experimental metastasis model has lead to some controversial findings related to studying anti-platelet aggregating agents. (7-11) Recently, more sophisticated in vivo imaging technologies have allowed the injected tumor cells to be followed in the circulation. Strikingly, in this model the majority of tumor cells can be found in the lung just minutes after injection into the lung (Figure 1). After 24 hours the majority of tumor cells are no longer detectable most likely reflecting removal by the innate immune system (discussed below). However, the presence of tumor cells 2 weeks later is apparent as grossly visible tumor foci on the surface of lungs that can be quantitated. Presumably, the relevance of platelets in this model is restricted to a very short time frame of less than 30 minutes, an idea first proposed to explain the controversial nature of early experiments. (9)

The bias to consider a platelet – tumor axis should also include considerations of blood coagulation pathways (Figure 2). The platelet providing a membrane surface for proteolytic pathways leading to fibrin generation are likely to represent a major mechanism stabilizing the platelet - tumor cell interaction. (5;12;13) The infusion of soluble fibrin monomer facilitates experimental metastasis in vivo and platelet – tumor

cell interactions in vitro. (14) The extrinsic pathway of coagulation triggered by factor VII (FVII) and tissue factor can be activated in cancer patients. (15) Expression of tissue factor by tumor cells leads to thrombin generation, fibrin formation and ultimately supports malignancy. (16-18) Additionally, thrombin activity exhibited by tumor cells likely affects the relevance of platelets, coagulation and tumor cell viability. (18) The thrombin agonist TRAP (thrombin receptor activation peptide) results in an increase in experimental metastasis. (19) Conversely, the thrombin antagonist, hirudin, diminishes primary tumor growth and spontaneous tumor metastasis in the mouse. (20)

III. Platelet - Tumor Cell Emboli and Ischemia

Key to the process of metastasis is the ability of circulating tumor cells to survive in the circulation and reside long enough at a particular site to extravasate from the vessel to create a secondary metastatic site. (21) (Figure 3) As discussed above, the relevance of platelets in the microenvironment of the bloodstream was originally established with platelet depletion and an observed reduced in experimental metastasis. (4) However, a more definitive role for platelets utilized the elegant genetic model of mouse NF-E2 deletion whereby platelet production is significantly impaired. (5;22) In this model which is essentially devoid of circulating platelets experimental metastasis is almost completely abolished. The relevance of the normal platelet physiologic response to this process was further validated using PAR-4 and fibrinogen knockout mice which also are severely impaired in metastasis. (5).

The platelet mechanism exploited by blood borne tumor cells is related to the platelet/coagulation interface that can "cloak" the tumor cell in a fibrin-rich web. (13;23;24) During this process, the platelet integrin receptor, aIIbβ3, serves as a receptor linking fibrin, platelets, and tumor cells into a fibrin rich clot normally associated with a thrombus. (25;25) Indeed, it can be speculated the fibrin-rich clot surrounding a tumor cells is one mechanism whereby the tumor cell can evade the host animal's innate immunity. (12)

IV. Platelets, Tumor Cells and Innate Immunity

The platelet - tumor cell axis is described above but additional cell interactions exist which do affect tumor cell viability. Specifically, an added level of complexity has been documented, an immune cell - platelet – tumor cell axis. It is estimated in the experimental metastasis model that less than 0.01% of injected tumor cells ever colonize the lung. (12) In many of these models an intact immune system exists raising the possibility that the animal's innate immune system can recognize the tumor cell. As seen in Figure 1 most of the injected tumor cells do quickly reside in the lung so if the animal's immune system is capable of recognizing the abnormal tumor cell, a killing of the tumor cell can take place.

Natural killer (NK) cells are the major immune components capable of eliminating tumor cells from the circulation. (26;27) Interestingly, platelets can inhibit lysis of tumor cells by NK cells in mice. (28) This is explained by what has been referred to as a "cloak" of platelets that coat the tumor cell and allow it to evade

the animal's immune system. (12) However, in the case of an incomplete coating of tumor cells there exists known receptor – counter receptor interactions that could support a close interaction between the platelet – tumor cell leading to a NK cell – platelet – tumor cell. NK cells express the integrin, αMβ2 (or Mac-1) which can interact with platelets via the platelet-specific adhesion receptor, glycoprotein (GP) Ib-IX. (29-31) How relevant to tumor cell survival in the circulation is the Mac-1 – GP Ib-IX interaction remains to be explored.

V. Platelets and Angiogensis

The relevance of platelets to angiogenesis was a concept proposed by the late Judah Folkman based on the wealth of growth factor potential present in platelet lysates. (32;33) Among the relevant stored proteins in platelet granules are vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (EGF), hepatocyte growth factor, and insulin-like growth factor (IGF). (34;35) The insatiable appetite of growing tumors has led to an explosion of studies defining the molecular mechanisms of angiogenesis. (36) As such, anti-angiogenic drugs have shown efficacy in certain clinical situations for cancer and eye diseases but resistance in some diseases remains a problem. (36)

Among these proteins experimental evidence supports a highly relevant role for VEGF in several aspects of tumorigenesis. Foremost is the platelet's capacity to store the majority of the VEGF available to the vasculature. (37) Indeed, the

relevance of VEGF to tumor growth is best exemplified by the ability to target VEGF for therapeutic efficacy. (38-40) Blockade of the major platelet integrin receptor, αIIBβ3, inhibits the release of VEGF from tumor-cell activated platelets and also reduces experimental metastasis. (41) VEGF is also known to increase vascular permeability with the potential to support extravasation from the vessel and ultimately metastasis. (42)

While the proangiogenic potential of platelets is clear there is a need to fully understand the relevance of anti-angiogenic proteins also present in stored platelet granules. Known anti-angiogenic proteins present in platelet α-granules include angiostatin, thrombospondin-1 (TSP-1), platelet factor-4 (PF-4), endostatin, transforming growth factor β (TGF-β), and tissue inhibitor of matrix metalloproteinases (TMPs). (43) The relevance of TSP-1 is based on an increased primary tumor mass of Lewis lung carcinoma cells as a consequence of TSP-1 deletion in knockout mice. (44) A COOH-terminal peptide of PF-4 can inhibit angiogenesis of B16 melanoma growth *in vivo*. (45) Thus, platelet derived anti-angiogenic factors do exist and represent areas for future research to evaluate their potential for anti-tumor therapies.

The balance of angiogenic and anti-angiogenic factors in the platelet raises a question as to how opposing effects are regulated from the platelet. (46) First, is the possibility that platelets selectively store pro- or anti-angiogenic factors in distinct subsets of platelet α-granules. (47) However, the identification of distinct subsets of α-granules is not without some controversy. (48) The presence of a single α-granule

population that has heterogeneous zones appears to be the more likely structure that has for decades been referred to as an α-granule. (49) However, heterogeneity within a single population of α-granules may be the explanation that leads to the identification of counter-regulatory activities of protease activated receptors (PAR) -1 and -4. Each PAR receptor has been reported to selectively release endostatin and VEGF from platelets. (50)

The release of stored angiogenic regulating molecules from the platelet coincides with platelet activation or the platelet release reaction. Upon activation, some platelet material can be found as plasma membrane bound particles of less than micron in diameter, termed platelet microparticles. (51) Via mechanisms that are still being defined platelet microparticles are involved in a variety of vascular events from coagulation to inflammation, and including angiogenesis and tumor progression. (52-54) High platelet microparticle levels are correlated with aggressive tumors and worsening prognosis. (54-59) One explanation of these findings is the microparticle represents a mini-activated platelet capable of transferring functional receptors to and from the microparticle with increased adhesion potential. Recent studies have demonstrated the platelet collagen receptor, GP VI, is key in one aspect of platelet microparticle generation and the genetic absence of GP VI coincides with a reduced potential for metastasis. (53;60) Thus, future studies of platelet microparticles as therapeutic targets and biomarkers of disease are warranted and likely to lead to the development of novel therapeutic strategies. (54)

VI. Platelet Activation and Metastasis

Platelets store lysophosphatidic acid (LPA) and release it after activation. LPA has been found to have a mitogenic effect on breast cancer cells. It has been suggested that tumor cells induce LPA release from activated platelets which stimulates tumor growth and cytokine-mediated bone destruction. (61). Bone metastasis was reduced 50% by Integrilin (a platelet GP IIbIIIa blocking peptide) in MDA-BO2 cells overexpressing LPA an effect that might related to a reduction of platelet number rather than a direct LPA effect. (61) CD43 is a transmembrane sialoglycoprotein of neutrophils, monocytes, T lymphocytes and platelets. In $CD43^{-/-}$ animals primary tumor growth was reduced. Interestingly metastasis was reduced transiently in KO mice but was reversed in the long term. (62)

Intracellularly, platelet $G\alpha q$, is critical for signaling that mediates platelet activation. It has been reported that D121 induced experimental and spontaneous tumor metastasis is significantly reduced in $G\alpha q$ deficient mouse colonies but not primary tumor growth (12). Aggrus/Podoplanin is a type-I transmembrane sialyomucin-like glycoprotein and found on tumor cells. It is shown to bind to the C-type lectin like receptor 2 (CLEC-2) on the platelet surface and induce activation and aggregation of platelets via Src, Syk, and PLCγ. (63) Podoplanin expression on CHO cells promoted experimental and spontaneous metastasis of CHO cells in BALB/c-nu/nu mice. (64) It has also been reported that ectopic CD9 expression on HT1080 cells which also express podoplanin results in reduced pulmonary

metastasis suggesting that the CD9 neutralizes podoplanin-mediated platelet aggregation and acts as a tumor suppressor. (65)

Integrin αIIbβ3 (GP IIbIIIa is the most abundant receptor on the platelet surface. It participates in hemostasis by activating platelets and is responsible for the formation of fibrin rich platelet aggregates. GP IIbIIIa inhibition by a monoclonal antibody 10E5 has been reported to inhibit binding of human CT26 and HCT28 cells to platelets *in vitro*. (10) β3 deficient mice show reduction in B16F10 melanoma cells induced osteolytic experimental metastasis and reduced osteolytic bone invasion which can be reversed by bone marrow transplantation of $\beta3^{+/+}$ marrow illustrating a role for integrin β3 in bone metastasis. (66) Antibody inhibition of GP IIbIIIa reduces tumor cell adhesion on extracellular matrix under flow conditions suggesting a role for GP IIbIIIa in platelet-tumor cell emboli extravasation. (67) c7E3 (ReoPro™) a mouse-human chimeric antibody for GP IIbIIIa presents anti-angiogenic and anti-tumor properties in mouse models. A single treatment of this antibody in SCID mouse reduces experimental metastasis significantly. (68) In addition, c7E3 also inhibits VEGF secretion from platelets in the presence of tumor cells. (69) Taken together these studies suggest the major platelet integrin receptor plays a significant role in tumor metastasis by supporting tumor cell-platelet emboli formation.

VII. Platelet Therapies and Cancer

To date, the administration of anti-platelet or anti-coagulant medications focuses on the management of the venous thromboembolism for the cancer patient. The most widely used anti-thrombotic agent, aspirin, targets platelet cycloxygenase (COX1). (70;71). However, clinical trials examining anti-metastatic or anti-cancer effects have only minimally been examined with primary focus on the progression of large bowel cancer. (70)

The current understanding of platelet function and cancer suggests the need for more rigorous evaluation of other anti-platelet medications, such as Clopidogrel. Indeed, established anti-platelet targets for activation all represent new directions for study that might provide some key insights into mechanisms of tumor progression. However, the use of animal models in these studies may generate some confounding data owing to some of the unique mouse/human species differences that exists between mice and humans. In particular, human platelets are activated through PAR1 , while mouse platelets lack PAR1 and are activated through a PAR4 mechanism. (72) In mouse models of experimental metastasis the blockade of the major platelet adhesion receptor, GPIb-IX, increases metastatic potential in contrast to the genetic absence of GPIb-IX which was associated with reduced experimental metastasis. (11;73)

Another major challenge in managing the platelet biology of the cancer patient is the thrombocytopenia that can be induced as a consequence of chemotherapy. Clearly, the bleeding associated with the severe thromobocytopenia that can occur must be balanced with the knowledge of the pro-metastatic potential of the platelet.

Again, a rigorous evaluation of this situation is warranted and suggests a better understanding of the molecular basis of platelet involvement might identify new clinical approaches.

VIII. Conclusion and Perspectives

The platelet – tumor cell axis is just another example of how intrinsic platelet function has been hijacked in a disease state. (74) The platelet properties that regulate platelet-platelet interactions, platelet-leukocyte interactions, and in this case, platelet-tumor cell interactions are revealing some unexpected and striking relevance in a variety of disease states. (74) While manipulating the platelet – tumor cell axis for the benefit of the patient might seem obvious, maintaining normal hemostasis or a balance of hemostatic pathways will be a formidable challenge. The dynamic nature of hemostasis, thrombosis, and malignancy is a formidable challenge. However, the decades of research devoted to hemostasis and thrombosis have defined solid paradigms which may give rise to new paradigms relevant to the cancer patient providing hope for improvement and management of the disease.

TABLE 1.

Knockout mouse models of major platelet proteins implicated in tumor metastasis

Protein	*Reference*
b3 Integrin	**(66)**
Gaq	**(12)**
GP Iba	**(73)**
GP VI	**(60)**
LPA	**(61)**
NF-E2	**(5)**
P-selectin	**(47;75)**
PAR4	**(5)**

Figure Legends

Figure 1. B16-F10.1 melanoma cells expressed a luciferase reporter protein were injected into the tail vein of normal C57BL/6J (WT) mice. Using an in vivo imaging system (IVIS, Xenogen Corp) the fate of the injected cells was followed with time. Mice were anesthetized with 2.5% isolflurane via a nose cone while being imaged in the supine position. Representative pictures were captured of the mouse thorax. As shown, within 30 min post-tail vein injection $B16^{Luc}$ cells are present in the mouse lung and reach a maximum level within a 2.5 hr time frame. Subsequently, the number of visible cells declines only to reappear after several days indicative of the establishment of individual tumor foci within the lung. Approximately 15 min prior to each imaging an intraperitoneal injection of luciferin (1.5 mg/10 gm of mouse) is provided as a luciferase substrate. *In vivo*, luciferin is available as a substrate for approximately 1 hr following injection (not shown).

Figure 2. Platelet – tumor cell emboli in the vasculature. Schematic diagram showing platelet – tumor cell microemboili in the vasculature embedded in a fibrin-rich mesh.

Figure 3. Platelets coordinate a number of cell-cell interactions occurring in the vasculature that regulate tumor survival and growth. A platelet – tumor cell axis that is influenced by coagulation pathways and an innate immunity – platelet – tumor cell axis

that affects the activity of natural killer cells are just two of the characterized bloodstream events that affect tumorigenesis.

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