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14. ABSTRACT
   This report describes studies during the previous 12 months that document the use of mouse models of platelet dysfunction in the progression of cancer to metastatic disease. During the next year we propose to examine the relevance of platelet receptors in models of spontaneous metastasis. A transgenic C57BL/6J mouse colony expressing MMTV-PyV middle antigen in mammary epithelium has been 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. Although the proposed studies are basic in their approach, the ability to target platelet receptors in anti-thrombotic therapy (inhibitor of blood clotting) is an ongoing avenue for product development within several large pharmaceutical companies.

15. SUBJECT TERMS
Blood platelet, Tumor, Metastasis, Collagen

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Table of Contents

Introduction

The platelet paradigm in hemostasis and thrombosis involves an initiation step dependent upon platelet membrane receptors binding to ligands on a damaged or inflamed vascular surface. Once bound to the surface, platelets provide a unique microenvironment supporting the accumulation of more platelets and the elaboration of a fibrin-rich network produced by coagulation factors. This paradigm has been established from decades of research. The platelet-specific receptor, glycoprotein (GP) Ib-IX, is critical in this process and can initiate the formation of a platelet-rich thrombus by tethering the platelet to a thrombogenic surface. Several ligands binding to GP Ib-IX have been identified, including von Willebrand factor (vWF) and thrombin, illustrating platelet GP Ib-IX as a major initiator of platelet thrombus formation in the arterial circulation. Newer, emerging data supports a role for platelets in pathological events beyond the prevention of blood loss. In our ongoing studies, **we are testing the hypothesis that platelet GP Ib-IX contributes to malignancy**. Knockout and transgenic mouse colonies have been generated in our laboratory and bred to C57BL/6J animals to generate several congenic strains (> 10 generation backcrosses) with dysfunctional platelet GP Ib-IX. Our outlined studies are providing results to test the hypothesis and will provide new information on the link between platelets and cancer. Metastasis is estimated to be the cause of nearly 90% of all human cancer deaths. Long term, the outlined studies will evaluate whether adjunct anti-GP Ib-IX therapy could benefit the breast cancer patient with malignant disease.
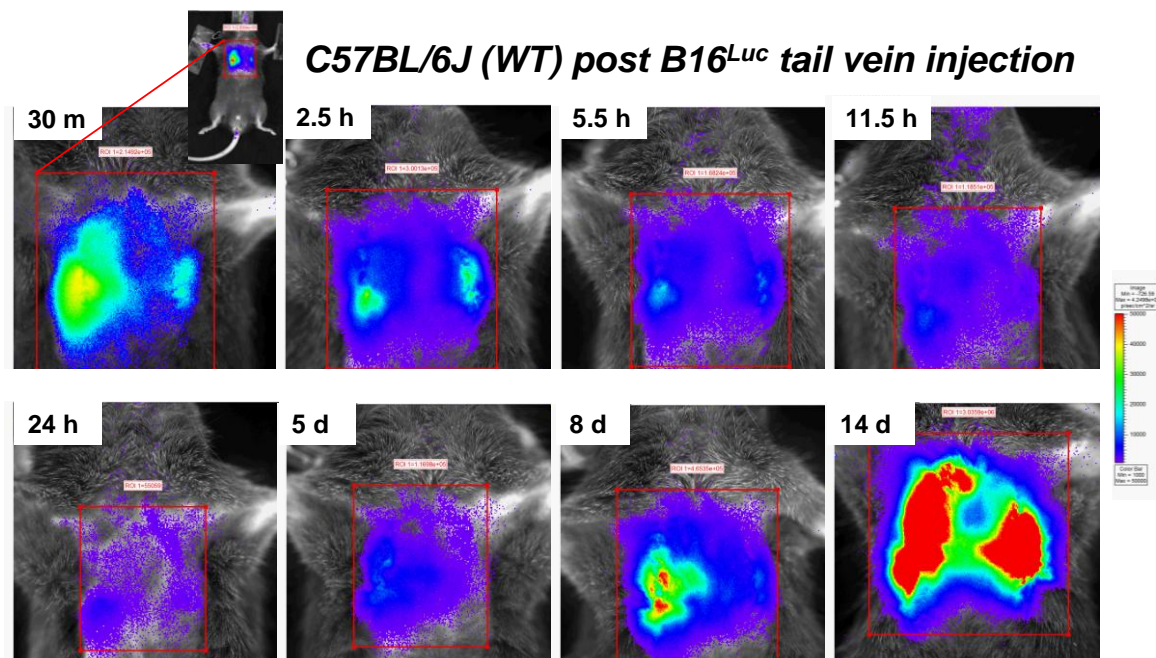
Body

Below we list the 3 Specific Aims from our original submission (blue font) followed by specific details (black font) for how each Aim has progressed during the covered date for this report (Sep 1, 2008 – Aug 31, 2009).

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

Our preliminary results supported the hypothesis that platelet GP Ib-IX effects the ability to B16 cells to colonize the lungs in this model of hematogenous metastasis [1]. Drs. Shaun Coughlin and Eric Camerer (UCSF) provided us with a stably-transfected B16F10 cell line (B16^{Luc}) expressing the firefly reporter protein – luciferase [2]. Using these cells we were able to follow the fate of B16^{Luc} cells owing to the bioluminescent properties of the tagged cells (Fig 1). The UAMS Center for Orthopedic Research has an IVIS 200 (In Vitro Imaging System, Xenogen, Inc.) which can be used to detect photons emanating from cells in the intact animal. The IVIS system is comprised 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. The observed pattern for cells injected in a control animal is similar to

4

experiments reported by others and represents a defined temporal sequence of events in which **1)** B16 cells accumulate in the lungs peaking within 2-3 hours, **2)** followed by a reduction in B16 cells that reaches the lowest level approximately 24 hours post-injection, and **3)** a gradual development tumors visible via the IVIS in approximately 4-5 days post-injection. Fig 2 illustrates an example comparing wild-type and mice missing the platelet GP Ib-IX receptor (Gp1b-/-). More than 10 animals of each genotype have been used confirming the reproducibility of the findings. The results illustrate that homing to the lung is not dependent upon the platelet receptor as the lung luciferase levels between control and knockout mice is indistinguishable. It is assumed an immune-based clearance of tumor cells has occurred and those cells that have evaded the immune system are able to colonize the lungs resulting in the formation of tumors that become visible by IVIS screening 4 days following injection (Fig. 2). Thus, the IVIS system provides a noninvasive method to follow the tumor cell's ability to colonize the lung.

In vivo Imaging of Lung Tumor Burden – IVIS 200

C57BL/6J (WT) post B16^{Luc} tail vein injection

Fig 1. Tumor cell imaging over a 2 week period is shown as an example of the ability to follow tumor cell homing to the lung and subsequent colonization of the lung. The same mouse is imaged at the various time points following the injection B16 tumor cells into the tail vein (experimental metastasis). The IVIV camera allows quantification of the tumor cell burden as graphically illustrated in Fig 2.

Histological analysis of the tumor has been performed and in this case we have made some key insights that are providing a fundamental linkage between platelets and tumor development.

While fibrin deposition has not been dramatically different between WT and GP1b-/- animals, other histological parameters have been. For example, shown in Fig 3 is a quantitative analysis
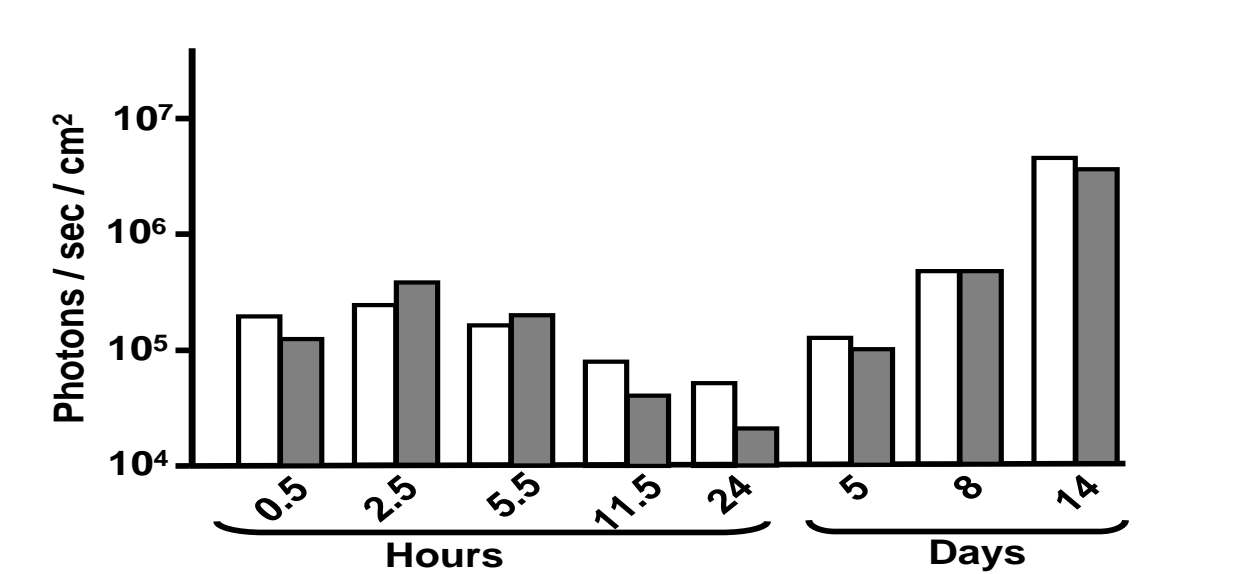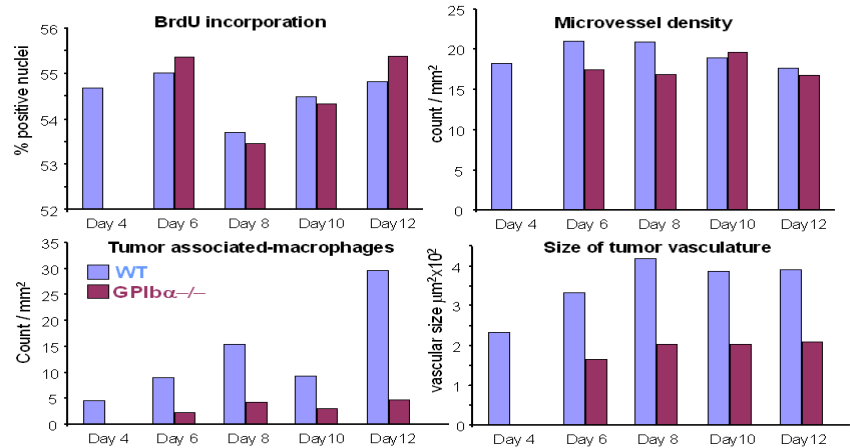

Fig. 2. Tumor cell burden in the lung is shown comparing a WT animal (open bar) and an animal genetically missing the platelet GPIb-IX receptor (gray bar). The analysis of multiple animals has confirmed the GPIb-IX receptor does not facilitate homing of the tumor cell to the lung in this model of experimental metastasis.

of tumor vascularity and the presence of macrophages, a key cell that complements mechanisms of angiogenesis (new blood vessel formation) [3]. Note the dramatic reduction in macrophage infiltration to the tumor as a consequence of platelet GP1b absence (Fig 3). Thus, a key mechanistic insight from this work is the possibility of a platelet/macrophage interaction that is critical to long term tumor development. Microvessel density is not changed, but large vessels size is impaired with dysfunctional platelets. We speculate this may be due to the platelet GPIb-IX receptor and the integrin receptor, Mac-1, present on the surface of macrophages but the definitive proof represents some of our ongoing work.

Fig 3. Histological analysis of tumors reveals a marked reduction in tumor-associated macrophages in animals missing platelet GPIb-IX.

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.

We developed the mouse Y276F model that represents a single tyrosine to phenylalanine mutation within the extracellular domain of platelet GP Ib. The thrombosis consequences of this mutation have been published but this work was peformed in a mixed genetic strain background comparing littermates from het/het crosses, a situation unsuitable syngenic model of metastasis [4]. To perform the experiments for SA2 required the generation of the Y276F mutation in a syngenic mouse background, i.e, a mouse model where the strain background (C57BL/6J) matches the source of mouse tumor cell lines. In order to achieve this we have been performing extensive mouse husbandry crossing the Y276F to wild-type C57BL/6J mice for 10 generations, the gold-standard in generating a congenic mouse strain. Such work had already been done with the straight knockouts utilized in SA1. We can now report we have the congenic Y276F strain in place and are expanding the congenic colony to perform the outlined studies. Thus, during the past year SA2 has been mouse husbandry and genetic screening to get us the necessary mouse colony to answer the questions posed for SA2. The utilization of the mouse colony will be the immediate focus for the upcoming reporting period.

Specific Aim 3. To examine the relevance of platelet GP Ib-IX in a model of spontaneous metastasis.

Relative to this aim we have made significant progress briefly outlined below. First, we obtained a mouse colony from Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ) over-expressing the PyMT antigen [5]. Mice from this colony spontaneously develop primary breast tumors that at a frequency of ~80% will metastasize to the lung. We have generated compound mice expressing the PyMT antigen and also containing our platelet mutation in the receptor complex, GP Ib-IX. We have also just initiated studies characterizing the frequency of metastasis in these compound heterozygous animals. The flow diagram (Fig 4) illustrates our strategy for generating these animals and also the strategy for phenotyping these animals. We point out the breeding strategy itself (just to generate sufficient animals for analysis) has taken ~18 months.

The breeding and generation of the compound mouse colony has progressed without major challenges. We show in Fig 5 some of our genotyping and phenotyping characterizations that identify the PyMT gene product and FACs analysis to identify platelets either containing (WT or PyMT+) or missing (knockout) the platelet GPIb-IX complex.

Generation of GP1b-/-,PyMT+/- compound mouse

From Sandra Gendler, Mayo Clinic, Scotsdale, AZ
PyMT+/- mice in C57Bl/6J background

↓

Crossing
GP1b+/+; PyMT+/- X GP1b-/-; PyMT-/-

↓

Obtained GP1b+/-; PyMT+/-
Genotyping by PCR for PyMT (480bp)

↓

Crossing
GP1b+/-; PyMT-/- X GPIb+/-; PyMT+/-

↓

Obtained GP1b-/-; PyMT+/- mice (Genotyping by PCR & FACS)

↓

Primary tumors and lungs collection at the age of 20 wks.

↓

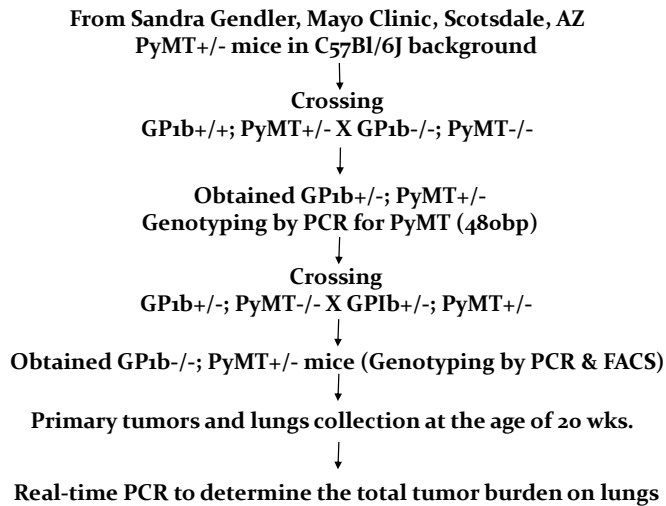**Real-time PCR to determine the total tumor burden on lungs**

Fig 4. We have performed the initial breeding required to obtain the compound mouse colony expressing the PyMT antigen but devoid of platelet adhesion receptor, GPIb-IX. Our current studies are focusing on the last 2 steps of the diagram, collecting tumor tissue and analyzing tumor burden in the lung. During the next 6 months this will be the major focus of our continued studies.

Generation of GP1b-/-,PyMT+/- compound mouse

1- Marker
2- 416
3- 417
4- 418
5- 419
6- 420
7- 421
8- 422
9- 423
10-424

WT

FL1- FITC anti-mouse GPIbα
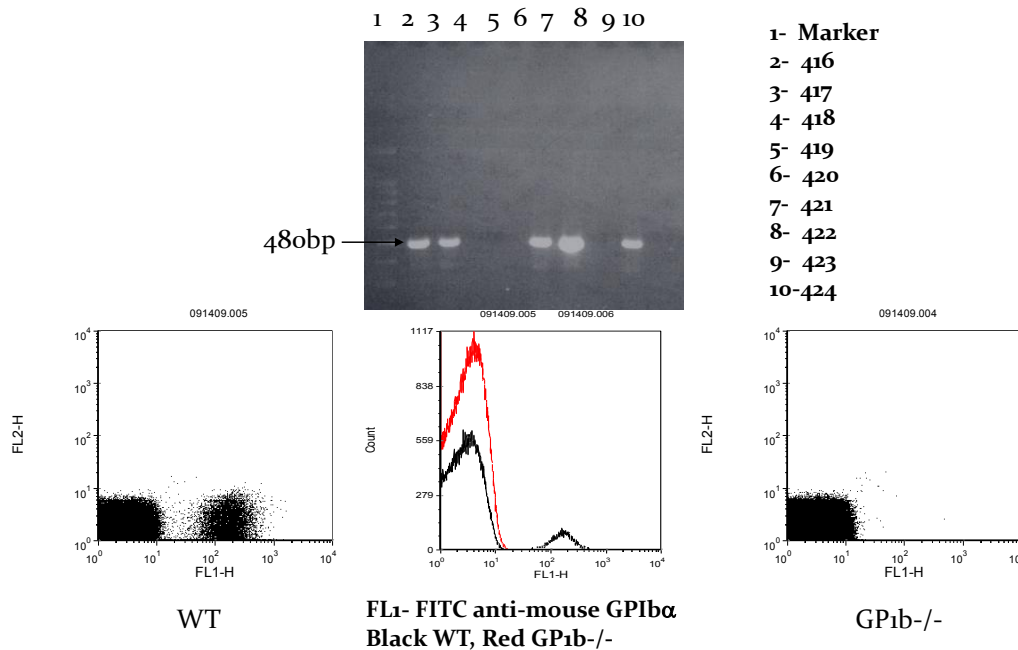Black WT, Red GP1b-/-

GP1b-/-

Fig. 5. A PCR product of 480 bp is visualized from mice containing the PyMT gene that drives formation of primary breast tumors. Mice that are positive for the PyMT transgene are subsequently screened for the presence or absence of the GPIb antigen by FACs.

8

We have just started collecting primary tissues from WT and compound mice. So far, the data has suggested no effect on the mass of the primary tumors (Fig 6). However, we are observing significant tumor variability with respect to cellularity. How this influences our analysis of tumor burden in the next months will have to be carefully examined.

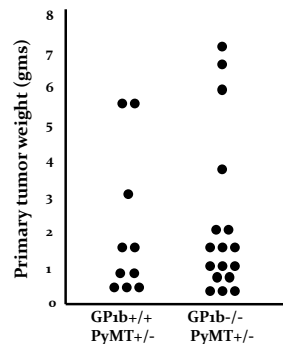**No inhibition of spontaneous mammary primary tumor growth in GP1b-/- mouse**

Fig. 6. Primary breast tumor size (gms) has been determined following the dissection from WT (PyMT+) or compound mice (PyMT+;GP1b-/-).

Key Research Accomplishments

1. In vivo imaging has demonstrated in models of experimental metastasis that platelet GPIb-IX does not facilitate tumor cell homing to the lung.
2. Histological analysis has identified a platelet/macrophage axis that is significantly altered in animals devoid of platelet GPIb-IX.
3. 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
4. A model of spontaneous breast tumor development and metastasis has been developed in C57BL/6J mice devoid of the platelet GPIb-IX receptor. Current studies are determining the influence of platelets on metastasis in this spontaneous tumor model.

Reportable Outcomes

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

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

Conclusion

Our project tests the hypothesis that circulating blood platelets contribute to tumorigenesis with a significant impact on molecular events leading to metastatic disease. We continue to use the mouse models we developed in our laboratory to address fundamental questions related to platelet adhesion and activation in the breast cancer disease process. We are excited to have new models to test in the coming year that spontaneously develop primary breast tumors owing to the presence of a transgene over-expressing the PyMT antigen driven by a gene promoter specific to breast tissue (murine mammary tumor virus). Thus, we believe interesting results using a model that more closely mimics the events that occur in vivo, in humans, will provide new data on the fundamental role of platelet receptor function and cancer.

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Supporting Data

Included in the Body.

Appendices

None