Effects of vascular endothelial growth factor receptor inhibitor SU5416 and prostacyclin on murine lung metastasis

Kyle C. Cuneo[a], Allie Fu[b], Katherine L. Osusky[b] and Ling Geng[b]

The majority of patients with a diagnosis of cancer die from metastatic disease. Targeting specific steps in the metastatic process has the potential to improve patient outcomes. In this study, a novel lung metastasis model was developed by injecting DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbo-cyanine perchlorate)-labeled Lewis lung carcinoma cells into the tail vein of mice. The temporal development of tumor metastases was studied in the lung, liver and spleen. Additionally, the effects of vascular endothelial growth factor receptor inhibitor SU5416 and platelet activation inhibitor prostacyclin were tested in this metastasis model. Systemically injected Lewis lung carcinoma cells present in the lung at 15 min slowly accumulated in the liver and spleen reaching a peak at 4 days. After 8 days, tumor development was only evident in the lung. Use of SU5416 or prostacyclin lowered the initial density of Lewis lung carcinoma-labeled cells in the lung by a factor 1.8 and 2.3, respectively ($P<0.05$). Furthermore, treatment with prostacyclin or SU5416 decreased lung weight by over 50% and the number of visible metastatic nodes by over 90% ($P<0.05$). Combined treatment resulted in grossly normal lung tissue. Additionally, systemic treatment with prostacyclin reduced harvested metastatic cell adherence to endothelial cells by a factor of 10 and treatment with SU5416 attenuated vascular formation ($P<0.001$). In conclusion, SU5416 and prostacyclin effectively attenuated metastasis formation in this model. DiI labeling is an effective technique to monitor the temporal and spatial distribution of metastatic cells. *Anti-Cancer Drugs* 18:349–355 © 2007 Lippincott Williams & Wilkins.

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[a]Department of Radiation Oncology, Vanderbilt University School of Medicine and [b]Department of Radiation Oncology, Vanderbilt University Medical Center, Nashville, Tennessee, USA.

Correspondence to Dr Ling Geng, MD, Vanderbilt University Medical Center, 1161 21st Avenue South, E1219 Medical Center North, Nashville, TN 37232, USA.
Tel: +1 615 343 3508;
e-mail: ling.geng@vanderbilt.edu

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Introduction

Over 90% of cancer-related deaths are due to metastatic disease. Our understanding of this process is limited and effective treatments are lacking. The development of metastasis involves a complex sequence of events [1]. Two of these key events are angiogenesis [2] and endothelial adherence [3]. Both these processes have been studied extensively and have been identified as potential therapeutic targets in antimetastatic therapy. In the current study, we have examined how molecular inhibitors of these processes affect the natural history of metastasis.

Angiogenesis plays a critical role in tumor metastasis. Tumor growth beyond 2 mm requires blood vessel formation to obtain nutrients and to remove waste products [4,5]. These newly created vessels provide access to the circulation for tumor cells. Furthermore, once cells travel to distant sites, angiogenesis is required for the newly formed tumor colonies to grow. A complex interaction exists between the endogenous angiogenesis promoters and inhibitors that control these processes [6,7]. Owing to its important role, inhibition of angiogenesis has been studied as a strategy to prevent tumor growth and metastasis [8–10]. SU5416 is a small-molecule tyrosine kinase inhibitor that selectively inhibits the kinase insert domain-containing receptor (KDR/Flk-1) for vascular endothelial growth factor (VEGF) [11]. Previous studies have shown VEGF receptor (VEGFR) inhibitors to be effective in preventing metastasis in lung and colon carcinoma [12,13]. Although blocking angiogenesis plays a major role in restricting metastasis growth, other factors including reduced vascular permeability may complement the antimetastatic properties of these drugs [13]. In the current study, the effect of SU5416 on the transient process of metastasis formation was studied.

Cellular adhesion molecules are necessary for tumor cell migration from the primary tumor to distant sites. It has been shown that platelets play a critical role in this process by increasing adherence, aggregating tumor cells, and protecting cells from the host immune system [14]. Prostacyclin (PGI_2) inhibits platelet adhesion and has been shown to attenuate the metastatic process [15–17]. Previous reports have revealed that aggregated platelets

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directly contact and surround tumor cells [18]. This action is believed to protect tumor cells from immune surveillance, enhance tumor cell adherence to endothelium and increase vascular permeability promoting extravasation [19,20].

To study the metastatic process at the microscopic level *in vivo*, tumor cells need to be detectable. Prior studies have used indicator genes such as the *Escherichia coli* lacZ gene [21] luciferase reporters [22], or green fluorescent protein (GFP)-transfected cell lines [23,24] to label tumor cells. Currently, GFP labeling is the preferred method for models used in metastasis research. Chishima *et al.* [24] first used this method to visualize single-cell micrometastases less than a decade ago. Labeling of individual tumor cells has been a valuable tool allowing for the elucidation of specific steps in the metastatic process [23]. Additionally, labeling and monitoring of tumor cells has been used to study many antimetastatic compounds [25,26]. The major drawbacks to using GFP are that tumor cells must be transfected and selected for. Furthermore, GFP has been shown to induce apoptosis in specific cell lines [27], generate reactive oxygen species [28,29] and induce an immune response [30].

In the present study, a model was developed to study the process and time course of lung metastasis. Using DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)-labeled Lewis lung carcinoma (LLC), the progression of metastasis formation was studied in the lung, liver and spleen. This labeling technique of tumor cells was shown to be effective in tracking individual metastatic cells as they travel through various organs. The known antimetastatic compounds SU5416 and PGI_2 were used to examine how VEGFR inhibition and platelet activation inhibition affect metastasis formation.

Methods

Cell culture and DiI labeling

The LLC mouse cell line was obtained from American Type Culture Collection (Manassas, Virginia, USA) and maintained in high-glucose (4.5 g) Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Human umbilical vein endothelial cells were obtained from Clonetics (East Rutherford, New Jersey, USA) and were maintained in EBM-2 medium supplemented with EGM-2 MV single-quots (BioWhittaker, East Rutherford, New Jersey, USA). All cells were incubated at 37°C in a 5% CO_2 incubator. DiI was obtained from Molecular Probes (Carlsbad, California, USA) and a 20-μg/μl stock solution was created in dimethylsulfoxide (DMSO). DiI was added to 80–90% confluent plates in media at a 1:1000 dilution. Cells were incubated with DiI at 37°C for 10 min, kept at 4°C for 15 min and then trypsinized.

Metastasis model

All animal studies were conducted following Institutional Animal Care and Use Committees approved protocols. A LLC metastasis model was used to test the effect of platelet activation inhibitor PGI_2 and VEGFR tyrosine kinase inhibitor SU5416 on tumor metastasis. In total, 5×10^5 LLC cells were injected into the tail vein of 6-week-old male C57BL6/J mice. Then 1.0 mg PGI_2 (Sigma, St Louis, Missouri, USA) was dissolved in 50 μl of 0.05 mol/l Tris buffer to create a stock solution. In the PGI_2 and PGI_2 + SU5416 treatment groups ($n = 5$), 100 μg of PGI_2 was injected with the LLC cells into the tail vein. In the SU5416 ($n = 5$) and the PGI_2 + SU5416 treatment groups, 25 mg/kg SU5416 was administered by intraperitoneal injection. The mice were treated for 5 consecutive days. Control groups received 50 μl of DMSO. Fifteen days after the initial injection, the animals were killed and lungs were removed. Lungs were individually weighed and tumor nodes were counted under a dissection microscope. Lungs were sectioned and stained with hematoxylin & eosin.

4,6-Diamidino-2-phenylindole staining

The above procedure was repeated using 5×10^5 LLC cells stained with DiI. Mice from each group (control, SU5416 and PGI_2) were killed at 15 min, 1 day, 4 days, 8 days, 13 days and 15 days. The lung, liver and spleen were sectioned and stained with 5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 5 min. Images were quantified using ImagePro software (Silver Spring, Maryland, USA).

In-vivo/in-vitro adhesion model

Passage 3 human umbilical vein endothelial cells (48 000 cells) were plated onto wells lined with 300 μl Matrigel and then incubated until capillary-like tubule structures formed. Mice were injected with 5×10^5 DiI-labeled LLC cells and half of them were simultaneously injected with PGI_2 ($n = 4$). Five minutes later, 100 μl of whole blood from the mice in both the PGI_2 and the control groups was harvested and added to the Matrigel-lined wells with the formed endothelial tubules. The wells were incubated with the blood sample for 30 min. The media was then aspirated, and the cells were fixed with cold absolute methanol, stained with DAPI and examined by microscopy.

Vascular window model

The window model was used in C57BL6/J mice to visualize tumor vascular formation as previously described [31]. The SU5416-treated group was given daily intraperitoneal injections with 25 mg/kg SU5416 for 5 consecutive days, beginning 1 h after the implantation of tumor cells into the window chamber. Control mice were injected with 50 μl of DMSO. Neovascularization was monitored with photographs taken at 24 h, 48 h and 8 days after drug administration. Images from day 8 were quantified using ImagePro software.

Statistical analysis

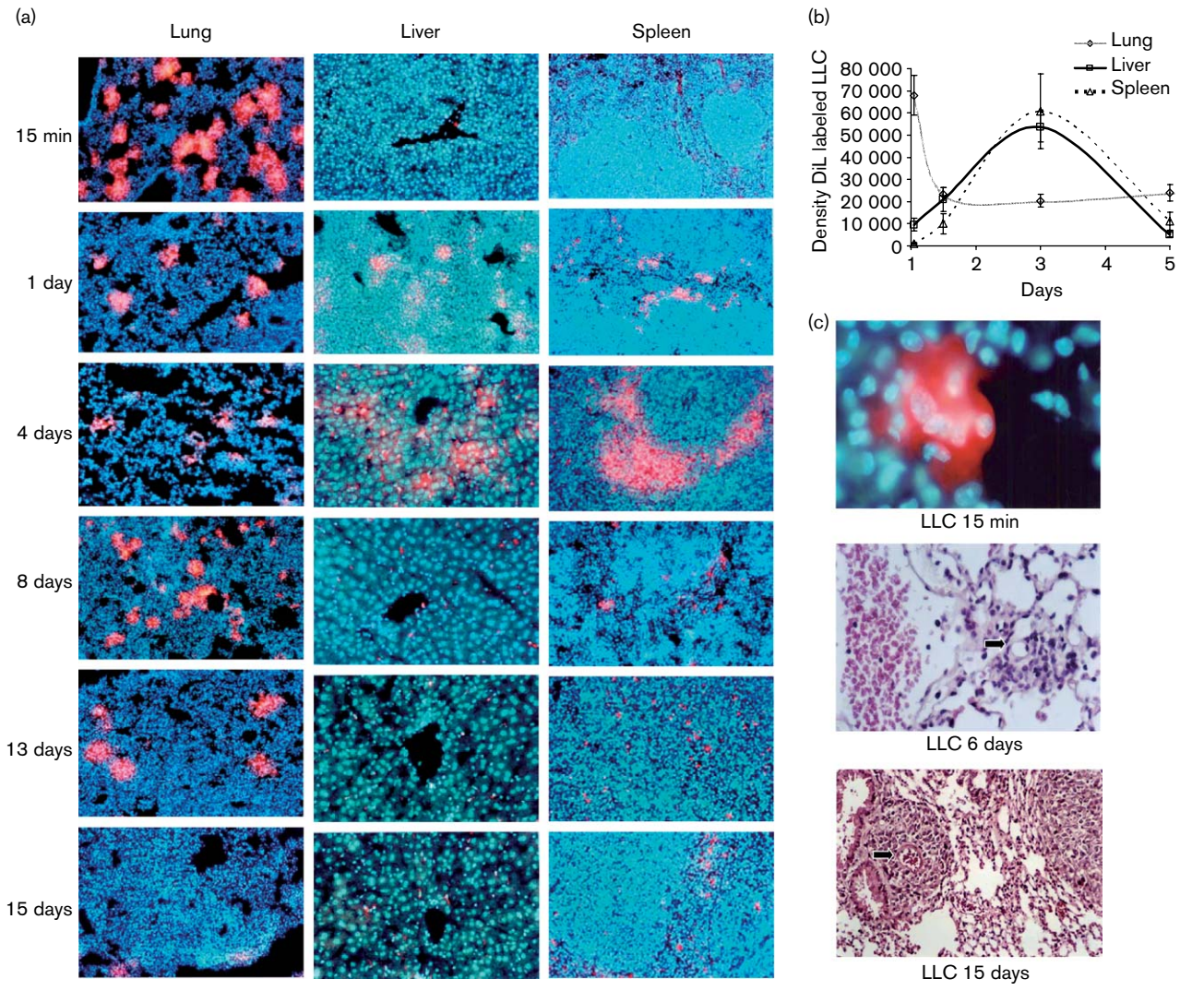
The mean and standard error were calculated for each quantitative experiment using Microsoft Excel software. *P* values were calculated using the Student's *t*-test. *P* values less than or equal to 0.05 were considered statistically significant.

Results

Temporal distribution of injected tumor cells

To determine the temporal distribution of metastatic cells, a lung metastasis model was performed by injecting DiI-labeled LLC cells into the tail veins of mice. Figure 1(a) shows histological sections stained with DAPI (blue) and DiI-labeled LLC cells (red) in the lung, liver and spleen at 15 min, 1 day, 4 days, 8 days, 13 days and 15 days. The number of DiI-labeled LLC cells per unit volume for the first 8 days is shown in Fig. 1(b). Systemically injected tumor cells were abundantly present in the lung at 15 min, declined by a factor of 3.5 over the first 24 h and then remained relatively constant over the following 8 days. Metastatic cells slowly accumulated in the liver and the spleen, reaching a peak density at 4 days and then gradually declined. The peak density reached in the liver and spleen was approximately 80% of the initial density in the lung. Eight days after injection, tumor formation was only evident in the lung.

Fig. 1



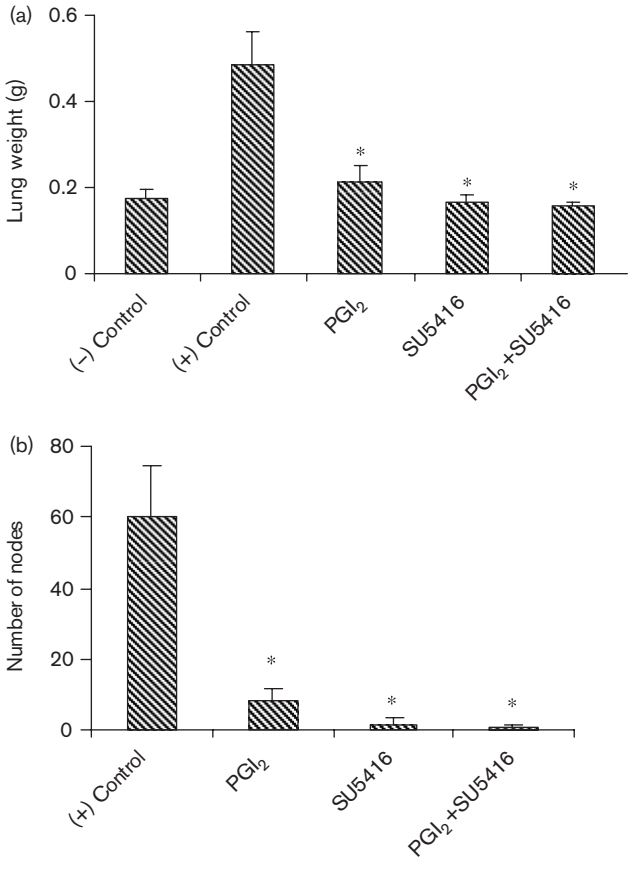
Histology of Lewis lung carcinoma (LLC) metastasis. DiI-labeled LLC cells (red) were injected into the tail veins of mice. At the indicated time points, tissue was harvested, sectioned and stained with 4,6-diamidino-2-phenylindole (blue). (a) Representative sections from the lung, liver and spleen at $\times 10$ or $\times 20$ magnification. (b) Mean density and standard error of DiI-labeled metastatic cells for each time point ($n=5$). (c) Higher magnification images. At 15 min, a cluster of DiI-labeled LLC cells is seen with surrounding lung parenchyma. Hematoxylin & eosin staining at 6 and 15 days demonstrate cell proliferation and early blood vessel formation (arrows).

By 15 days, there was significant tumor growth in the lung whereas the liver and spleen remained histologically normal. Detailed images are shown in Fig. 1(c). At 15 min, a cluster of DiI-labeled LLC cells is seen surrounded by lung parenchyma. Hematoxylin & eosin staining at 6 and 15 days demonstrate tumor proliferation and angiogenesis (arrow) in the lung parenchyma.

Effects of SU5416 and prostacyclin on lung metastasis

The effects of SU5416 and PGI_2 on metastasis were examined using the lung metastasis model described above. Lungs harvested from mice treated with PGI_2 or SU5416 and systemically injected with tumor cells were weighed and examined for gross metastatic node formation (Fig. 2). Lungs from mice injected with metastatic cells and treated with PGI_2 or SU5416 weighed over 50% less than positive controls ($P < 0.001$), indicating an inhibition of metastasis formation (Fig. 2a) with SU5416 being more effective than PGI_2 ($P = 0.020$). Treatment with both compounds was not statistically different from the negative control. Furthermore, lungs treated with PGI_2 had 40–50 times fewer metastatic nodes than the positive control. Use of SU5416 nearly eliminated node formation ($P < 0.001$) and combined treatment resulted in grossly normal lung tissue (Fig. 2b).

Fig. 2



Effect of prostacyclin (PGI_2) and SU5416 on tumor growth and metastatic node formation. Lewis lung carcinoma cells were injected into the tail veins of mice followed by five treatments of 25 mg/kg SU5416 and/or PGI_2 over 5 days. Fifteen days after tumor cell injection, lungs were harvested, weighed and examined under a dissection microscope. Shown are the mean and standard error of lung weight (a) and tumor node count (b). (–) Control indicates normal lung tissue. (+) Control indicates untreated lung with tumor proliferation. *$P < 0.001$ compared with positive control.

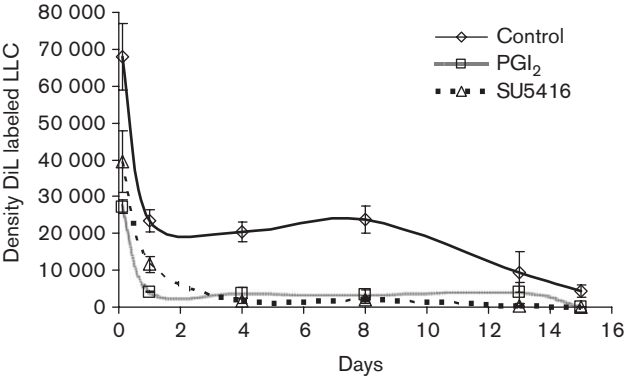
Effect of SU5416 and prostacyclin on the temporal distribution of tumor cells

To further study the effect of these compounds on metastasis development, the temporal distribution of labeled metastatic cells within the mouse was quantified (Fig. 3). Systemically injected DiI-labeled LLC cells accumulated in the lung parenchyma as early as 15 min after injection. Treatment with SU5416 or PGI_2 significantly decreased the amount of tumor cells present at 15 min by factors of 1.8 and 2.3, respectively ($P < 0.05$). Between days 1 and 8 the number of DiI-labeled metastatic cells remained relatively constant in all groups with the PGI_2 and SU5416 treatment groups having 5 times fewer metastatic cells per lung volume during this time. The density of the lung parenchyma was also substantially less at 15 days in the treatment groups with SU5416 being more effective. Lung tissue from mice treated with either PGI_2 or SU5416 appeared histologically similar to normal lung tissue at this time whereas untreated mice showed substantial tumor proliferation.

Effect of prostacyclin on tumor cell adherence to endothelial cells

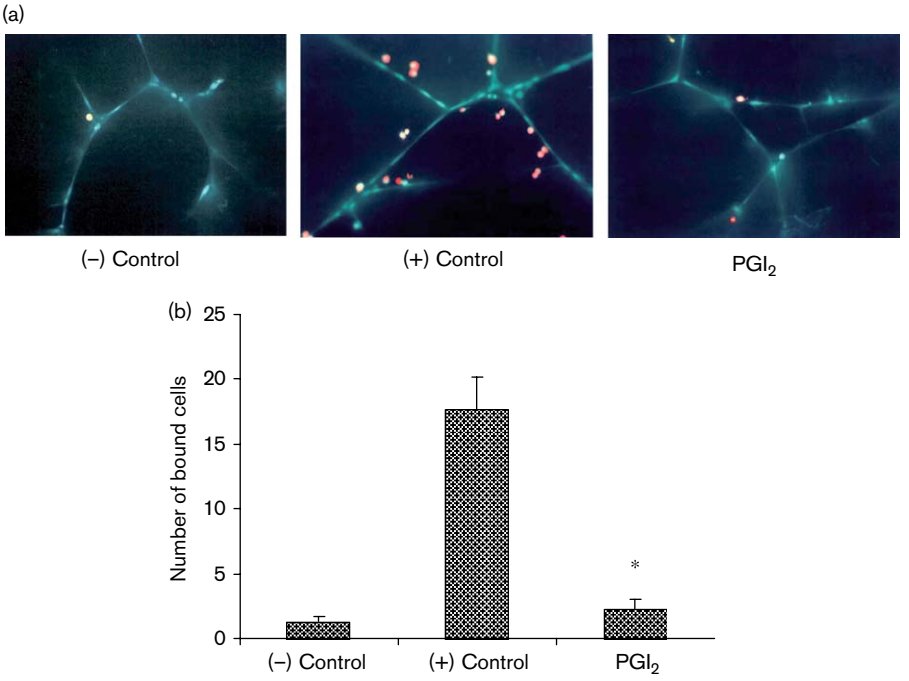
A novel experimental model was developed to study the effect of PGI_2 on LLC adherence to endothelial cells.

Fig. 3



Effect of platelet activation inhibitor prostacyclin (PGI_2) and vascular endothelial growth factor receptor inhibitor SU5416 on lung metastasis formation. Mice were treated with 25 mg/kg SU5416 and/or PGI_2. DiI-labeled Lewis lung carcinoma (LLC) cells were then injected into the tail vein. At the indicated time points, lungs were harvested, sectioned and stained with 4,6-diamidino-2-phenylindole. Images were obtained and quantified. Shown are the mean density and standard error of DiI-labeled metastatic cells for each time point.

Fig. 4



Effect of prostacyclin (PGI_2) on Lewis lung carcinoma (LLC) adherence to the endothelium. DiI-labeled LLC cells were injected into the tail veins of mice treated with PGI_2. After 15 min whole-blood samples were obtained and incubated with preformed endothelial tubules on Matrigel. Shown are photographs of endothelial tubules (blue) with bound LLC cells (red) (a), and the mean and standard error of the number of bound tumor cells (b) for each treatment condition. (–) Control represents labeled LLC cells not injected into the mouse circulation. *$P<0.001$.

DiI-labeled LLC cells were injected into the tail veins of mice with or without PGI_2, harvested minutes later and then incubated with preformed endothelial tubules on Matrigel. In-vivo treatment with PGI_2 resulted in 10 times less metastatic cell adherence to the endothelial tubules compared with the cells harvested from positive control mice ($P < 0.001$) (Fig. 4). Additionally, tumor cells not injected into the mice (negative control) had minimal binding to the tubules indicating that host factors facilitate tumor cell binding and PGI_2 attenuates this process.

SU5416 attenuates angiogenesis in the Lewis lung carcinoma window model

To confirm the effect of SU5416 on angiogenesis, we examined the effect of the VEGFR inhibitor SU5416 on angiogenesis using a vascular window model. In this model, tumor cells were injected into a transparent chamber on the subdermal dorsal skin fold of mice to view vascular formation (Fig. 5a). Intraperitoneal treatment with SU5416 significantly reduced levels of tumor neovascularization by a factor of 10 ($P < 0.001$) confirming that the compound attenuates angiogenesis in this tumor model (Fig. 5b).

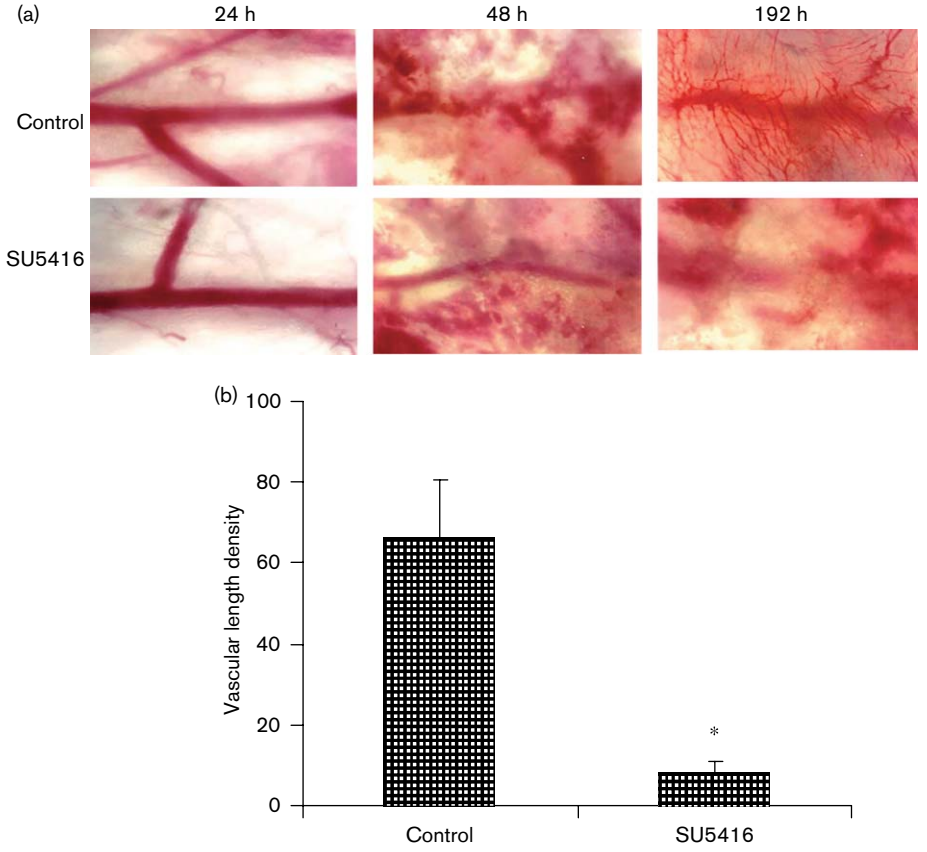
Discussion

In this study, the dynamics of lung metastasis were studied using LLC (murine) cells labeled with the lipophilic marker DiI. DiI is a novel marker of tumor cells with many theoretical advantages over traditionally used methods including GFP labeling. This dye is not metabolized or exchanged between cells *in vitro* or *in vivo* and enables identification of engrafted cells by fluorescence microscopy, flow cytometry or fluorescence-activated cell sorting [32]. DiI labeling does not require transfection and selection, and can be performed on any cell line in approximately 15 min. As this molecule is not a protein, there is also less potential immunogenicity.

It is well known that specific tumors preferentially metastasize to specific organs. Many factors determine the site of metastasis including proximity of the two organs, available transport pathways, host immune system and specific characteristics of the tumor cells. In the model used in this study, systemically injected DiI-labeled metastatic cells initially accumulated in the lung directly downstream from the injection site. Over the next 4 days, the metastatic cells spread and slowly accumulated in the liver and the spleen. Even though a similar density of tumor cells was present in the liver and spleen as in the lung, tumor development only occurred in the lung.

In this study, VEGFR inhibitor SU5416 [11,12] and platelet activation inhibitor PGI_2 [33,34] were used in our model to target specific components of the metastatic

Fig. 5



SU5416 affects neovascularization in the Lewis lung carcinoma (LLC) window model. LLC cells were injected into a subdermal window chamber allowing for visualization of mouse vasculature. Mice were then treated with 25 mg/kg SU5416 for 5 consecutive days. (a) Microscopic images of the window chambers at the indicated time points. (b) Quantification of mean vascular length density and standard error. *$P<0.001$.

process. Both compounds were effective in reducing the number of tumor cells initially deposited in the lung. This finding indicates that these compounds affected the transit and/or extravasation of tumor cells from the systemic circulation to the lung tissue. PGI_2 is known to block the activation of platelets. This process limits adherence to the endothelium and affects immune surveillance [33,34]. Recent reports have further characterized the ability of platelets to enhance MMP-9 secretion promoting invasiveness [35]. SU5416 also affected the number of tumor cells initially present in the lung. Previous studies have characterized the role of VEGF on vascular permeability [36,37] and use of VEGFR inhibitors has been shown to reduce vascular permeability in animal models [38]. Therefore, VEGFR inhibitors have the potential to block metastasis by affecting vasculature permeability in addition to attenuating angiogenesis.

Since Gasic *et al.* reported that metastasis could be reduced by platelet reduction, there have been numerous reports using antiplatelet agents and anticoagulants to inhibit metastasis [14,39]. In this study PGI_2, a known platelet activation inhibitor, was shown to inhibit metastasis formation and decrease tumor cell binding to endothelial cells. Tumor cells were injected into mice, harvested, then incubated *in vitro* with preformed endothelial tubules. Activated platelets facilitated LLC binding to endothelial cells and use of PGI_2 attenuated this process. No binding was seen in negative control tumor cells that were not injected into the mouse circulation. This experiment demonstrates the importance of host factors in tumor cell adherence to endothelial cells.

This study examined two antimetastatic compounds that target host components (vasculature and platelets). Targeting host components versus the tumor cells themselves has many advantages. Host cells are more stable and less prone to mutation when compared with tumor cells. Furthermore, host factors remain relatively constant in different tumor systems allowing for a reliable treatment to be effective in many types of cancer. This fact is especially true in metastasis in which cells have

been selected for and are generally more resistant to treatment. Treating metastasis will require more effective prevention strategies and therapeutics. A better understanding of the transit of metastatic cells and the effect of current therapeutics on this process is needed.

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References

1 Roos E, Dingemans KP. Mechanisms of metastasis. *Biochim Biophys Acta* 1979; **560**:135–166.
2 Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 1994; **79**:185–188.
3 Tang DG, Honn KV. Adhesion molecules and tumor metastasis: an update. *Invasion Metastasis* 1994; **14**:109–122.
4 Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; **285**:1182–1186.
5 Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990; **82**:4–6.
6 Folkman J. How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes Memorial Award lecture. *Cancer Res* 1986; **46**:467–473.
7 Folkman J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 2002; **29**:15–18.
8 Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. *Ann Surg* 1972; **175**:409–416.
9 D'Amore PA. Antiangiogenesis as a strategy for antimetastasis. *Semin Thromb Hemost* 1988; **14**:73–78.
10 Jendraschak E, Sage EH. Regulation of angiogenesis by SPARC and angiostatin: implications for tumor cell biology. *Semin Cancer Biol* 1996; **7**:139–146.
11 Fong TA, Shawver LK, Sun L, Tang C, App H, Powell TJ, *et al.* SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res* 1999; **59**:99–106.
12 Lu H, Lin C, Zheng Z, Li S, Guo S, Zhang X, *et al.* Angiogenesis inhibitor Z24 induces endothelial cell apoptosis and suppresses tumor growth and metastasis. *J Pharmacol Sci* 2005; **97**:533–540.
13 Bergsland EK. Vascular endothelial growth factor as a therapeutic target in cancer. *Am J Health-Syst Pharm* 2004; **61**:S4–S11.
14 Gasic GJ, Gasic TB, Murphy S. Anti-metastatic effect of aspirin. *Lancet* 1972; **2**:932–933.
15 Honn KV, Cicone B, Skoff A. Prostacyclin: a potent antimetastatic agent. *Science* 1981; **212**:1270–1272.
16 Hejna M, Raderer M, Zielinski CC. Inhibition of metastases by anticoagulants. *J Natl Cancer Inst* 1999; **91**:22–36.
17 Honn KV. Inhibition of tumor cell metastasis by modulation of the vascular prostacyclin/thromboxane A2 system. *Clin Exp Metastasis* 1983; **2**:103–114.
18 Crissman JD, Hatfield JS, Menter DG, Sloane B, Honn KV. Morphological study of the interaction of intravascular tumor cells with endothelial cells and subendothelial matrix. *Cancer Res* 1998; **48**:4065–4072.
19 Gasic GJ. Role of plasma, platelets, and endothelial cells in tumor metastasis. *Cancer Metastasis Rev* 1984; **3**:99–114.
20 Honn KV, Tang DG, Crissman JD. Platelets and cancer metastasis: a causal relationship? *Cancer Metastasis Rev* 1992; **11**:325–351.
21 Lin WC, Pretlow TP, Pretlow TG, Culp LA. Bacterial lacZ gene as a highly sensitive marker to detect micrometastasis formation during tumor progression. *Cancer Res* 1990; **50**:2808–2817.
22 Adams JY, Johnson M, Sato M, Berger F, Gambhir SS, Carey M, *et al.* Visualization of advanced human prostate cancer lesions in living mice by a targeted gene transfer vector and optical imaging. *Nat Med* 2002; **8**:891–897.
23 Paris S, Sesboue R. Metastasis models: the green fluorescent revolution? *Carcinogenesis* 2004; **25**:2285–2292.
24 Chishima T, Miyagi Y, Wang X, Yamaoka H, Shimada H, Moossa A, *et al.* Cancer invasion and micrometastasis visualized in live tissue by green fluorescent protein expression. *Cancer Res* 1997; **57**:2042–2047.
25 Torrance CJ, Agrawal V, Vogelstein B, Kinzler KW. Use of isogenic hu7 man cancer cells for high-throughput screening and drug discovery. *Nat Biotechnol* 2001; **19**:940–945.
26 Izycki D, Gryska K, Grabarczyk P, Wysocki PJ, Jarosinska A, Nawrocki S, *et al.* Flow cytometric cytotoxicity assay with GFP gene modified target cells. *Adv Exp Med Biol* 2001; **495**:429–434.
27 Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ. Is green fluorescent protein toxic to living cells? *Biochem Biophys Res Commun* 1999; **260**:712–717.
28 Troyano A, Fernandez C, Sancho P, de Blas E, Aller P. Effect of glutathione depletion on antitumor drug toxicity (apoptosis and necrosis) in U-937 human promonocytic cells. The role of intracellular oxidation. *J Biol Chem* 2001; **276**:47107–47115.
29 Kovacic P, Osuna JA. Mechanisms of anti-cancer agents: emphasis on oxidative stress and electron transfer. *Curr Pharm Des* 2000; **6**:277–309.
30 Steinbauer M, Guba M, Cernaianu G, Kohl G, Getto M, Kunz-Schughart LA, *et al.* GFP-transfected tumor cells are useful in examining early metastasis in vivo, but immune reaction precludes long-term tumor development studies in immunocompetent mice. *Clin Exp Metastasis* 2003; **20**:135–141.
31 Geng L, Donnelly E, McMahon G, Lin PC, Sierra-Rivera E, Oshhinka H, *et al.* Inhibition of vascular endothelial growth factor receptor signaling leads to reversal of tumor resistance to radiotherapy. *Cancer Res* 2001; **61**:241–249.
32 Ledley FD, Soriano HE, O'Malley BW, Lewis D, Darlington GJ, Finegold M. DiI as a marker for cellular transplantation into solid organs. *Bio Techniques* 1992; **13**:581–587.
33 Honn KV, Busse WD, Sloane BF. Prostacyclin and thromboxanes. Implication for their role in tumor cell metastasis. *Biochem Pharmacol* 1983; **32**:1–11.
34 Schneider MR, Tang DG, Schirner M, Honn KV. Prostacyclin and its analogues: antimetastatic effects and mechanism of action. *Cancer Metastasis Rev* 1994; **13**:349–364.
35 Susuki K, Aiura K, Ueda M, Kitajima M. The influence of platelets on the promotion of invasion by tumor cells and inhibition by antiplatelets agents. *Pancreas* 2004; **29**:132–140.
36 Lee YC. The involvement of VEGF in endothelial permeability: a target for anti-inflammatory therapy. *Curr Opin Investig Drugs* 2005; **6**: 1124–1130.
37 Weiss SM, Cheresh DA. Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* 2005; **437**:497–504.
38 Patel N, Sun L, Moshinsky D, Chen H, Leahy KM, Le P, *et al.* A selective and oral small molecule inhibitor of VEGFR-2 and VEGFR-1 inhibits neovascularuzation and vascular permeability. *J Pharmacol Exp Ther* 2003; **306**:838–845.
39 Schirner M, Schneider MR. The prostacyclin analogue cicaprost inhibits metastasis of tumours of R 3327 MAT Lu prostate carcinoma and SMT 2A mammary carcinoma. *J Cancer Res Clin Oncol* 1992; **118**: 497–501.