[CANCER RESEARCH 62, 6966–6972, December 1, 2002]

Spontaneous Hematogenous and Lymphatic Metastasis, but not Primary Tumor Growth or Angiogenesis, Is Diminished in Fibrinogen-deficient Mice[1]

Joseph S. Palumbo, Jill M. Potter, Lisa S. Kaplan, Kathryn Talmage, David G. Jackson, and Jay L. Degen[2]

Divisions of Hematology/Oncology [J. S. P.] and Developmental Biology [J. M. P., L. S. K., K. T., J. L. D.], Children's Hospital Research Foundation and University of Cincinnati College of Medicine, Cincinnati, Ohio 45229-3039, and MRC Human Immunology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, United Kingdom [D. G. J.]

ABSTRACT

Previous studies of tumor cell-associated procoagulants and fibrinolytic factors have strongly suggested that local thrombin and plasmin generation may be important in tumor growth and dissemination. Given that one central target of both of these serine proteases is fibrin(ogen), a logical extension of this hypothesis is that local fibrin deposition and dissolution may be key determinants of tumor progression. In this paper, the role of fibrin(ogen) and its degradation products in the growth and spontaneous metastasis of Lewis lung carcinoma was directly examined by comparative studies of control and fibrinogen-deficient mice. Fibrinogen deficiency was found to have no effect on the time required for the formation of palpable tumors, tumor angiogenesis, overall tumor architecture, or primary (s.c.) or secondary (pulmonary) tumor growth. However, fibrinogen deficiency markedly reduced the incidence of spontaneous macroscopic metastases in the lung and regional lymph nodes, a process that occurred relatively late in tumor development. Furthermore, a significant quantitative reduction in pulmonary micrometastases was observed in fibrinogen-deficient mice. Quantitative analyses of pulmonary micrometastases in primary tumor-bearing mice indicated that spontaneous showering of tumor cell emboli into the lung was robust, regardless of animal genotype. Hence, our results suggest fibrin(ogen) plays an important role in spontaneous metastasis, facilitating the stable adhesion and/or survival of metastatic emboli after tumor cell intravasation. These studies suggest that therapeutic strategies focusing on hemostatic factors may be effective in controlling solid tumor metastasis, particularly if used for the treatment of micrometastatic disease.

INTRODUCTION

The potential for an important interplay between hemostatic factors and cancer has been recognized for decades, and the evidence supporting this notion has become increasingly persuasive. Malignant human and experimental animal tumor cells express an impressive collection of procoagulant and fibrinolytic factors that are often either absent or minimally expressed by the normal cells from which the tumor cells are derived. These factors include tissue factor, plasminogen activator, plasminogen activator receptor, plasminogen activator inhibitor, and fibrinogen receptors/binding proteins (*e.g.*, $\alpha v\beta 3$, ICAM-1, thrombospondin, VE-cadherin; Refs. 1–4). In addition, tumor cells often induce the local expression of soluble hemostatic factors or their receptors in adjacent stromal cells (5). On the basis of these findings and the obvious local tissue disruption and necrosis that occurs in association with rapid tumor growth, it is perhaps not surprising that the deposition of fibrin(ogen)-related material in and around solid tumors is commonplace, including cross-linked fibrin and FDPs[3] (6, 7).

A specific role for fibrinogen and/or provisional fibrin matrices in tumor biology is consistent with many previous observations. The local deposition of fibrin(ogen) and FDPs is a universal and conspicuous feature of solid tumors and these provisional matrix components may be biologically significant (6–8). Fibrin(ogen) is specifically recognized by multiple integrin and nonintegrin receptors found on tumor cells, stromal cells, and inflammatory cells, and the cellular engagement of fibrin(ogen) through specific receptors may control cell proliferation, cell migration, apoptosis, and the expression of inflammatory mediators (*e.g.*, cytokines and chemokines; Refs. 9–16). Both fibrin and FDPs have been shown to promote angiogenesis, consistent with the hypothesis that fibrin-rich extracellular matrices may promote tumor stroma formation by mechanisms that are comparable with wound repair (7, 8, 17–19). Substantial experimental evidence is also available pointing to a role for fibrin(ogen) in tumor dissemination. Notably, pharmacological inhibitors of the coagulation system and/or platelet function appear to substantially reduce tumor cell metastatic potential in animal models (20, 21).

Perhaps the most compelling data pointing to an important role for fibrin(ogen) in cancer biology has emerged from studies in gene-targeted mice carrying selected deficits in specific hemostatic factors (22–25). A recent study of tumor dissemination in control and fibrinogen-deficient mice revealed that the formation of pulmonary metastases after i.v. injection of tumor cells (*i.e.*, experimental metastasis assay) was greatly diminished, but not eliminated, in the absence of circulating fibrinogen (26). Although the mechanism(s) linking fibrinogen to metastatic potential remains to be fully defined, the initial studies suggest that fibrinogen supports the metastasis of circulating tumor cells by promoting the sustained adhesion and/or survival of these cells in the pulmonary vasculature. The finding that fibrinogen is a determinant of hematogenous metastasis is consistent with previous reports demonstrating a positive link between prothrombin activation and tumor metastasis. However, based on the finding that the specific thrombin inhibitor, hirudin, further diminishes the already low metastatic potential of circulating tumor cells in fibrinogen-deficient mice, it appears that thrombin contributes to tumor metastasis through at least one fibrinogen-independent mechanism (26).

The studies linking fibrinogen deficiency to diminished metastatic potential have thus far focused solely on experimental metastasis assays (26), whereby the tumor cells are initially introduced into the circulation. Although provocative, this experimental design is arguably artificial and does not provide any information about earlier phases in the metastatic process, including tumor cell migration through tissue barriers, dissociation from the primary tumor mass, and intravasation. In this study, LLC cells were transplanted s.c. into fibrinogen-deficient, gene-targeted mice to directly define the role of fibrin(ogen) and its proteolytic derivatives in tumor growth, angiogenesis and spontaneous metastasis. We report that fibrinogen is an important determinant of spontaneous metastasis to both the lung and regional lymph nodes but that neither fibrin(ogen) nor fibrin(ogen) degradation products are crucial for tumor stroma formation and the growth of established tumors.

Received 4/17/02; accepted 10/3/02.

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[1] This was supported, in part, by NIH Grants F32 CA83299 (to J. S. P.) and HL47826 and HL63194 (to J. L. D.).

[2] To whom requests for reprints should be addressed, at Children's Hospital Research Foundation, Children's Hospital Medical Center, IDR-NRB, Room 2042, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. E-mail: degenjl@chmcc.org.

[3] The abbreviations used are: FDP, fibrin degradation product; LLC, Lewis lung carcinoma; LLC^{GFP}, green fluorescent protein-expressing LLC.

MATERIALS AND METHODS

Transgenic Mice. The generation of fibrinogen (Aα chain)-deficient mice has been described previously (27). All mice enrolled in these studies were inbred into the C57BL/6J background (The Jackson Laboratory, Bar Harbor, ME) for six generations and were previously shown to be histocompatible with transplanted LLC cells (26). Mice were genotyped by multiplex PCR analysis of DNA obtained from ear biopsies as described previously (27). Age- and sex-matched cohorts of fibrinogen-deficient (Fib^-) mice and heterozygous (Fib^+) control mice were enrolled in all experiments. Because the Aα chain of fibrinogen is not the rate-limiting step for fibrinogen biosynthesis in normal mice, heterozygous ($A\alpha^{+/-}$) mice maintain plasma fibrinogen concentrations that are 75% of wild-type mice. The study protocols were approved by the Children's Hospital Research Foundation Institutional Animal Care and Use Committee and were in accordance with the guidelines of the NIH.

Tumor Cell Transplantation. The LLC cells (originally provided by Dr. Michael S. O'Reilly, Boston, MA) were cultured *in vitro* as described previously (26). Tumor cell viability was determined by trypan blue exclusion and was always $\geq$95%. Mice were anesthetized by inhalation of 2% isoflurane (Ohmeda PPD, Liberty Corner, NJ), and 75 μl of cell suspension (2.5×10^5 cells) were injected s.c. into the dorsal skin overlying the lower thoracic spine. In studies where the primary tumors were later eliminated by electron beam irradiation, tumor cells were transplanted in the skin overlying the lumbosacral spine.

Elimination of Primary Tumors by Surgical Resection and Radiotherapy. Twelve to 13 days after transplantation of LLC, the mice were again anesthetized by inhalation of 2% isoflurane. Primary tumors were removed in a sterile fashion using electrocautery to minimize bleeding, and the skin closed using both surgical clips (MikRon, Sparks, MD) and Nexaband (Veterinary Products Laboratories, Phoenix, AZ) surgical adhesive. After surgery, each animal was given a s.c. injection of 1 ml of warm saline and then housed in a 37°C microisolator until recovery from anesthesia. In studies in which the primary tumor was controlled by radiotherapy, anesthetized mice were placed in a lateral decubitus position with the tumor tissue extending into the irradiation field. The head, chest, and upper abdomen were shielded with 6 cm of SuperFlab (Radiation Products Design, Alberville, MN). Irradiation (6500 cGy) was directed at the s.c. tumors using an electron beam irradiator (Siemens Mevatron, Munich, Germany). Primary tumor regrowth was never observed under these conditions.

Generation of LLC^{GFP}. Wild-type LLC cells were transfected with a GFP expression vector, pEGFP-N1, (Clontech Laboratories, Inc., Palo Alto, CA), using LipofectAMINE (Life Technologies, Inc., Rockville, MD) following the manufacture's protocol. Stable transfectants were selected over a 10-day period in culture medium containing 800 μg/ml G418 (Life Technologies, Inc.) and then sorted twice for the most intense green fluorescent population of cells using a FACSVantage SE Sorter (BD Biosciences, San Jose, CA). The fluorescent LLC^{GFP} cells were subsequently maintained in culture medium containing 400 μg/ml G418.

Analysis of Surface Pulmonary Metastatic Foci. Tumor-bearing mice were sacrificed 12–13 days after primary tumor removal/irradiation. The lungs were removed, rinsed in PBS, and placed in Bouin's fixative for at least 24 h to highlight the surface metastases. Surface metastatic foci were counted under a stereomicroscope by an investigator blinded to animal genotype. Metastatic pulmonary foci formed by GFP-labeled LLC cells were counted using fresh lung tissue and a fluorescence-equipped stereomicroscope.

Histological Analysis. Fixed tissue was embedded in paraffin, sectioned, and stained with H&E. Fibrin(ogen) immunostaining was performed using a rabbit antimouse polyclonal antiserum as described previously (26). Anti-LYVE-1 (28) and anti-PECAM (Ref. 22; PharMingen, San Diego, CA) immunostaining were also performed as described previously.

RESULTS

Fibrinogen Is a Determinant of Spontaneous Metastatic Potential. To determine the importance of fibrin(ogen) in the spontaneous metastasis of tumor cells, the formation of pulmonary metastases was explored in immunocompetent fibrinogen-deficient and control mice carrying established s.c. LLC tumors. This specific tumor cell line was chosen because it is both highly metastatic and its metastatic potential has been previously linked to the hemostatic system (22, 26). A single cell suspension of 2.5×10^5 LLC cells was injected s.c. into the mid-dorsal subcutis of mice of each genotype. Twelve days later, the primary tumor was eliminated either by surgical resection (using electrocautery to minimize blood loss) or focal irradiation with an electron beam irradiator (6500 cGy). Consistent with previous findings (26), no genotype-dependent difference was noted in tumor growth. At the time of resection, tumors from Fib^+ mice were a median of 400 mg in size (range, 190–920 mg) compared with a median of 480 mg in Fib^- mice (range, 270-1140 mg; $P > 0.25$, Mann-Whitney *U* test). Similar results were obtained for LLC tumors before irradiation using calipation of length and width to estimate volume (29) (data not shown). However, quantitative evaluation of the number of pulmonary metastases performed 12 days after primary tumor irradiation/resection revealed that fibrinogen-deficient mice developed significantly fewer spontaneous metastases than control mice analyzed in parallel (Fig. 1). Qualitative analyses as well as measurements of the size of individual foci from lungs of both genotypes showed that fibrin(ogen) was neither essential for the

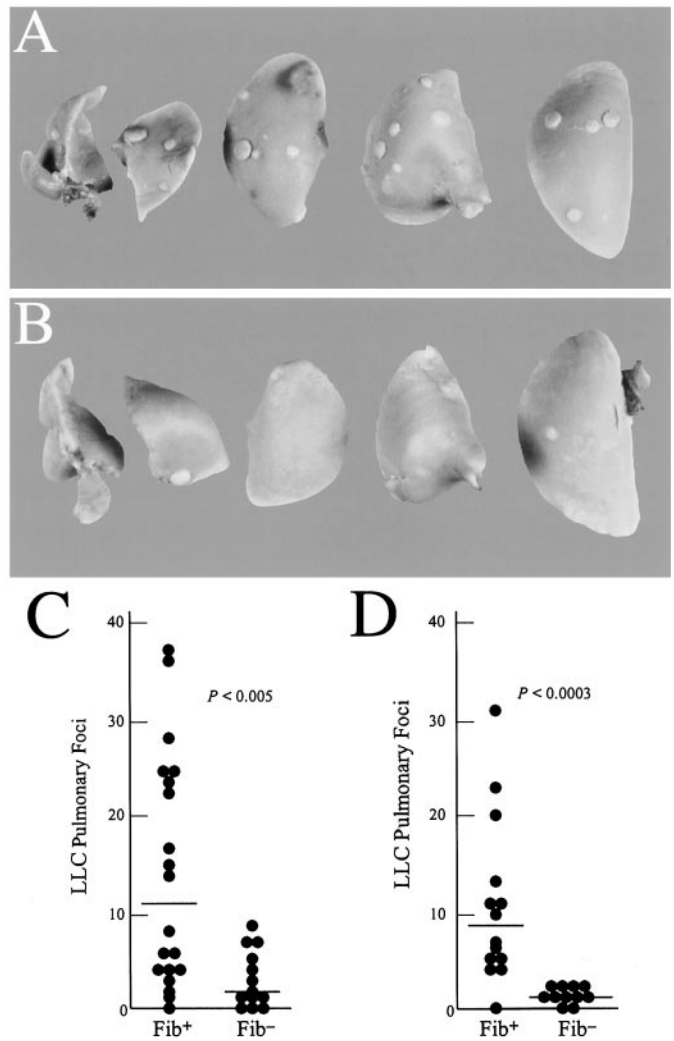

Fig. 1. Analysis of spontaneous pulmonary metastases in fibrinogen-deficient mice. Representative examples of spontaneous pulmonary metastatic foci produced 12 days after surgical resection of ~500 mg s.c. LLC tumors in control (*A*) and fibrinogen-deficient (*B*) mice. Fibrinogen-deficient mice had significantly fewer pulmonary metastases relative to control animals regardless of whether surgical resection (*C*) or irradiation (*D*) was used to control the primary tumor. The *horizontal bars* represent median values. *P*s were generated with the Mann-Whitney *U* test, two-tailed.

formation of metastatic foci nor a significant determinant of the overall appearance or size of established metastatic lesions (Figs. 1 and 2). Furthermore, the distinct quantitative difference in metastatic lesions in control and fibrinogen deficient-mice was not because of an experimental bias based on differences in mouse survival. Only a small fraction of mice of both genotypes died (<20%), and these losses were invariably a result of the surgical/therapeutic challenge rather than metastatic disease. The generally strong survival profile in fibrinogen-deficient mice observed in these studies is consistent with the fact that thrombin generation and platelet activation are not specifically compromised in these animals, and Fib^{-} mice can tolerate some types of surgical challenges, including full skin thickness incisions (30).

Both surgery and radiotherapy were used for local control as complementary methods to address specific experimental issues unique to each approach. For example, the surgical approach raises the potential issue of a genotype-dependent difference in tumor cell shedding during surgical manipulation that might lead to the observed differences in pulmonary metastases. Similarly, the use of primary tumor irradiation has the potential complication that local inflammatory or immunological responses to the necrotic tissue may lead to a genotype-dependent difference in the formation of metastatic lesions. However, these studies show that regardless of the means used to experimentally eliminate the primary tumor, fibrinogen deficiency appears to strongly diminish the formation of spontaneous metastatic foci (Fig. 1). These experiments were performed a total of four separate times (twice using surgical resection and twice using irradiation) all with very similar results.

Fibrinogen Deficiency Diminishes the Establishment of Spontaneous Micrometastases. To explore the impact of fibrinogen on spontaneous tumor metastasis at earlier time points and without the potential complications associated with the elimination of the primary tumor mass, LLC^{GFP} cells were used. Control studies showed that

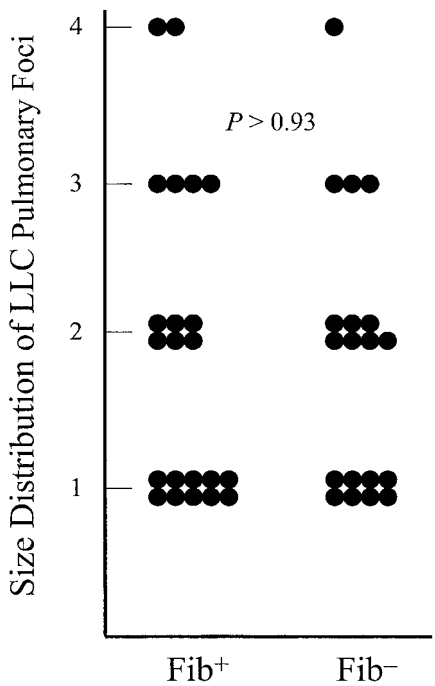

Fig. 2. Fibrinogen does not alter the size distribution of spontaneous pulmonary metastatic foci. Scatter plot of the size distribution of individual metastatic foci produced after surgical resection of s.c. transplanted LLC tumors. The arbitrary units from 1–4 used to score the size of metastatic foci are defined as follows: $1 \leq 0.5$ mm; 2 = 0.6–0.9 mm; 3 = 1–1.4 mm; and $4 \geq 1.5$ mm. *P*s were generated using the Mann-Whitney *U* test, two-tailed.

LLC^{GFP} cells proliferated at the same rate as the parental cell population *in vitro*, and s.c. transplantation into control and fibrinogen-deficient mice resulted in rapidly expanding primary tumors that were similar in growth rate and morphology to those generated by standard LLC cells. Furthermore, these cells displayed the same aggressive metastatic phenotype as the parental cells in control animals when tested in either experimental or spontaneous metastasis assays (data not shown). Most importantly, LLC^{GFP} could be observed at the level of single embolic tumor cells in the lung within 30 min after i.v. injection of a single cell suspension of LLC^{GFP} (data not shown). Following a spontaneous metastasis protocol similar to that described above, a single cell suspension of 2.5×10^5 LLC^{GFP} cells was injected into the dorsal subcutis of control and fibrinogen-deficient mice. Without primary tumor resection or irradiation, lungs were directly evaluated for metastatic lesions 13 days after original tumor cell transplantation. Consistent with our previous results, no genotype-dependent differences were observed in primary tumor size distribution (data not shown). However, the number of micrometastases observed by fluorescence stereomicroscopy was substantially diminished in Fib^{-} mice relative to control animals. Micrometastases were formally quantitated by determining the mean number of fluorescent micrometastatic foci within eight randomly selected high-powered fields (at least one field from each lung lobe). Fibrinogen-deficient mice carried significantly fewer pulmonary micrometastases (Fig. 3) with a median of 22 micrometastases/high-powered field in $Fib^{-/-}$ mice as compared with 75 micrometastases in control mice. A similar pattern was observed in separate experiments using mice harvested 11 or 12 days after tumor cell transplantation, which carried significantly smaller tumors. Mice harvested 12 days after transplantation had tumors ~500 mg in size. In this experiment, fibrinogen-deficient mice had a median of 18 micrometastases/high-powered field compared with 36 in control animals ($P < 0.03$, Mann-Whitney *U* test). When harvested 11 days after transplantation (primary tumors ~300 mg), fibrinogen-deficient mice had a median of 6 micrometastases/high-powered field compared with 25 in control mice ($P < 0.025$, Mann-Whitney *U* test). These findings underscore that fibrin(ogen) is an important determinant of spontaneous metastatic potential and argue that the influence of fibrinogen on metastatic success is exerted at an early stage.

A substantial fraction of the pulmonary micrometastases observed 13 days after s.c. tumor cell transplantation (Fig. 3) are likely to have been established close to the time of lung collection, most within the prior 48 h. This view is consistent with the fact that lungs harvested from similar cohorts of mice analyzed just 2 days earlier (*i.e.*, 11 days after tumor transplantation) had one-third the number of fluorescent micrometastases present within the lungs (see above). Additionally, the spontaneous micrometastases observed were qualitatively identical to the pulmonary foci observed within 30 min after direct i.v. injection (data not shown). The total number of surface pulmonary micrometastases observed in these studies of animals with unresected primary tumors was impressive. However, it is clear that very few of the spontaneous micrometastases detected in animals of both genotypes would have ultimately survived to form macroscopic lesions. The total number of pulmonary micrometastases observed at the day 13 time point (on the order of 5,000–10,000) exceeded by several orders-of-magnitude the number of metastatic outgrowths observed in mice with primary LLC^{GFP} tumors resected at this time point and analyzed 13 days later (on the order of 20–30, see below). Thus, the spontaneous showering of the lung with tumor cell emboli appears to be robust (although not necessarily identical) in both the presence and absence of fibrin(ogen). Notably, studies focusing specifically on circulating tumor cells suggest that fibrin(ogen) may be of primary

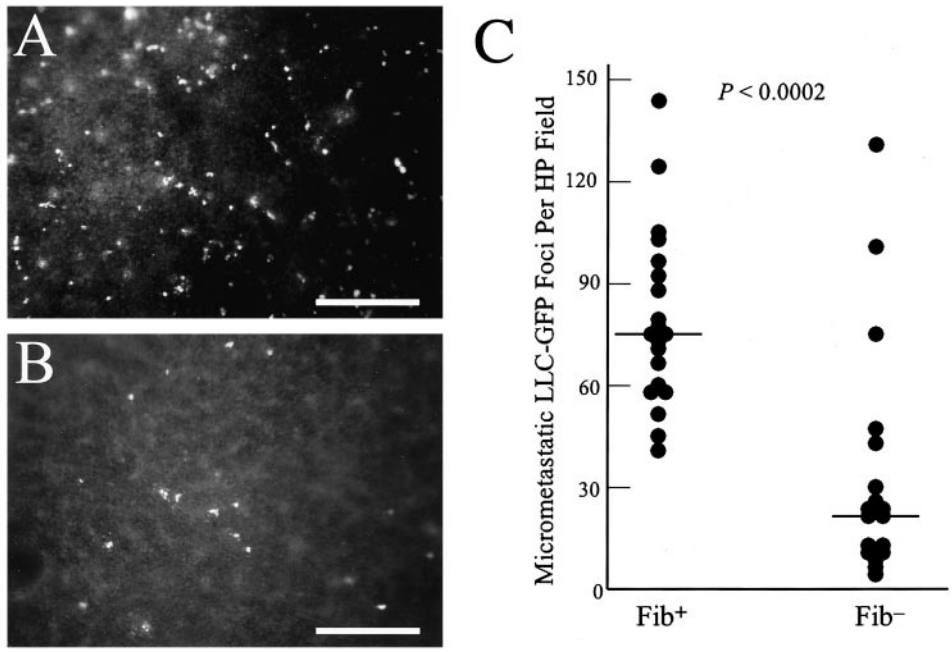

Fig. 3. Fibrinogen deficiency diminishes spontaneous pulmonary micrometastases. LLC^{GFP} cells (2.5×10^5) were transplanted into the dorsal subcutis of Fib^+ (*A*) and Fib^- (*B*) mice. The mice were sacrificed 13 days later (s.c. tumors, ~750 mg), and fluorescent pulmonary micrometastases were visualized using a fluorescence-equipped stereomicroscope. The *white bars* represent 500 μm. Fibrinogen deficiency resulted in a significant diminution in the number of fluorescent micrometastases visualized/high-powered field (*C*). The *horizontal bars* represent median values. *P*s were generated with the Mann Whitney *U* test, two-tailed.

importance in the sustained adhesion and/or survival of tumor cell emboli after arrival and initial adhesion in the lung vasculature (26).

Fibrin(ogen) Contributes to the Spontaneous Lymphatic Metastasis of LLC. LLC is known to spontaneously metastasize to organs other than lung, including regional lymph nodes (22). To determine whether fibrinogen was a determinant of spontaneous metastasis to multiple tissues, s.c. tumors were established in the dorsal subcutis of both fibrinogen-deficient and control mice using easily visualized LLC^{GFP} cells. Twelve days after initial tumor cell transplantation, tumors (median weight of ~500 mg) were surgically excised and after an additional 13 days, the lungs, liver, spleen, and kidneys were evaluated for evidence of metastases. In addition, the axillary and inguinal nodes were dissected and evaluated for the presence of metastases.

Visibly enlarged regional lymph nodes were encountered in tumor-bearing mice of both genotypes, and these were uniformly intensely fluorescent, indicative of LLC^{GFP} cells. Sections from formalin-fixed, paraffin-embedded GFP^+ lymph nodes were stained with H&E. The histological appearance of LLC nodal metastases was identical to that of the primary tumors (see below) with the exception of residual nodal tissue (data not shown). No genotype-dependent differences were noted in the gross or microscopic appearance of lymphatic metastatic foci. However, significantly fewer tumor cell-positive lymph nodes were observed in fibrinogen-deficient mice relative to control animals (see Table 1). Furthermore, control animals were found to frequently carry multiple positive nodes, whereas more than one positive node was rarely observed in Fib^- mice. Although fewer in number, there was no significant genotype-dependent difference in the size distribution of the lymphatic metastatic foci. Cancerous nodes in control mice ranged in weight from 31 to 1190 mg (median, 209 mg) as compared with a range of 24 to 468 mg (median, 125 mg) in fibrinogen-deficient mice ($P > 0.5$; Mann-Whitney *U* test). Closer evaluation of sectioned and H&E-stained lymphatic foci revealed that the tumor tissue was microscopically similar in animals with and without fibrinogen and comparable in appearance to the primary tumor tissue. Occasional fluorescent metastatic foci were observed in the liver, kidney, and spleen of animals with and without fibrinogen, but these were too rare to make any meaningful comparison between genotypes. Consistent with the studies using parental LLC presented above, the number of lung metastases was significantly decreased in fibrinogen-deficient (median of 3) relative to control (median of 25) animals ($P < 0.0001$; Mann-Whitney *U* Test).

Tumor Stroma Formation Is not Dependent on Fibrin(ogen). The deposition and degradation of provisional fibrin matrices in and around malignancies may support tumor stroma formation as well as alter metastatic potential in multiple and even opposing ways. For example, fibrin may promote tumor angiogenesis, thereby providing increased opportunity for tumor cell dissemination. On the other hand, peritumoral fibrin may act as a physical barrier to the migration of tumor cells. To assess the role of fibrin(ogen) in tumor growth, tumor stroma formation, and angiogenesis, the impact of fibrinogen deficiency on primary s.c. tumor architecture was examined in greater detail. No genotype-dependent difference was observed in the growth of LLC tumors in any of the experiments conducted. Furthermore, no significant genotype-dependent differences were noted in the overall microscopic features of tumor tissue (representative data shown in Fig. 4, *A* and *B*). LLC grew in monotonous sheets of highly anaplastic cells. The individual cells had relatively scant cytoplasm and large, angulated nuclei with prominent nucleoli. Numerous mitoses were seen in tumors from mice of both genotypes, some with abnormal configurations. Focal areas of necrosis were seen in tumors harvested from mice of both genotypes. Immunohistochemical analyses for fibrin(ogen) revealed that LLC tumors in control mice contained sparse fibrin(ogen) deposits that were primarily peritumoral or associated with the small areas of focal necrosis (data not shown). Predictably, no fibrin(ogen) could be immunologically detected from tumors collected from Fib^- mice. The tumors harvested from mice of both genotypes were highly vascularized, and no genotype-dependent differences were seen either in vessel density or pattern based on immunohistochemical analyses with an anti-PECAM antibody (Fig. 4,

Table 1 *Effect of fibrinogen deficiency on regional lymphatic metastasis of LLC*[a]

Genotype	Total (*n*)	Presence of lymphatic metastases	One node	Two nodes	Three nodes
Fib^+	41	27 (66%)	12 (29%)	9 (22%)	6 (15%)
Fib^-	26	9 (35%)	7 (26%)	2 (8%)	0

[a] $P < 0.007$; nonparametric analysis (Mann Whitney *U* test) comparing the total number of positive nodes in all mice of each genotype.

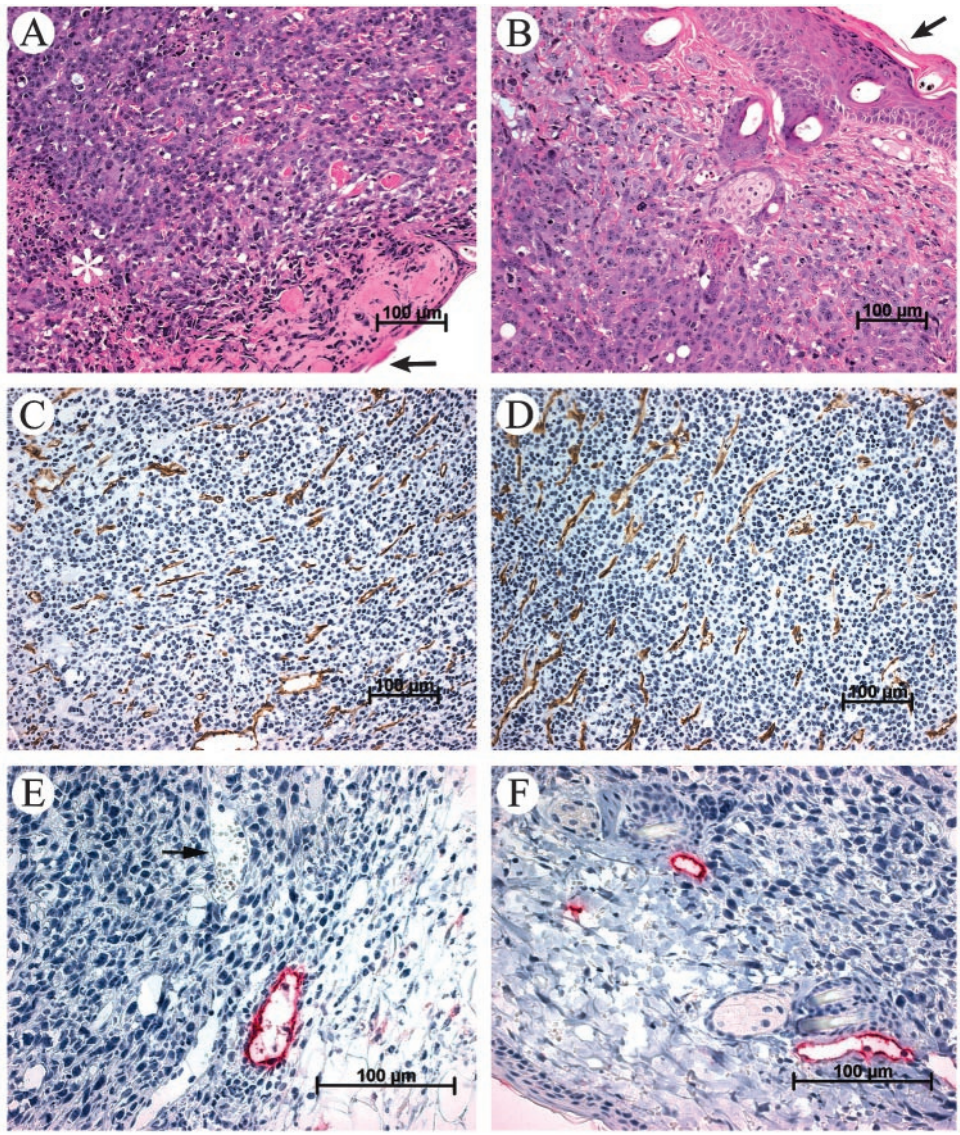

Fig. 4. Histological appearance of primary tumors in fibrinogen-deficient mice. A series of five tumors, 300–400 mg in size, from mice of both genotypes was paraffin embedded and sectioned. Representative images are shown. No genotype-dependent differences in overall tumor architecture were observed based on H&E stains of primary LLC tumors from control (*A*) and fibrinogen-deficient (*B*) mice. The overlying dermis is labeled with *arrows*. Tumors grew as dense cellular masses with small, focal areas of necrosis (*) regardless of genotype. Individual tumor cells demonstrated highly anaplastic features and frequent mitoses in mice of both genotypes. Immunohistological staining for the endothelial cell marker PECAM (*brown staining*) demonstrated robust tumor angiogenesis in both fibrinogen-deficient (*D*) and control (*C*) mice. Furthermore, peritumoral lymphatics, highlighted by immunostaining for LYVE-1 (*red*), were evident and qualitatively similar in density in fibrinogen-deficient (*F*) and control (*E*) mice within the s.c. tissue overlying the tumor mass. The *arrow* in *E* highlights a prominent *red* cell-containing blood vessel that is not stained by the LYVE-1 antibody.

C and *D*). A formal quantitation of the vessel density was done by counting the number of PECAM-staining vessels in several randomly selected ×200 fields in tumors from 6 control mice and 4 Fib^- mice. Tumors harvested from control animals had a mean density of 88 vessels/mm^2 (range, 73–100/mm^2) compared with a mean density of 98 vessels/mm^2 (range, 76–118/mm^2) in tumors from Fib^- mice (not significantly different; $P > 0.28$).

Because fibrinogen deficiency diminished spontaneous regional lymphatic metastasis as well as hematogenous metastasis, a detailed analysis of lymphatic vessels in and around primary tumors was done using mice of both genotypes. One possible explanation for the genotype-dependent difference observed in regional lymphatic metastasis is that fibrin(ogen) may play a role in lymphangiogenesis. Given that the growth of lymphatic vessels and blood vessels can be uniquely influenced by different proangiogenic factors (31, 32), fibrin(ogen) may effect the development of lymphatic vessels in and around tumors while having no significant impact on blood vessel development. Paraffin-embedded sections from a series of four tumors from mice of each genotype were immunohistochemically stained with the lymphatic endothelium-specific antibody anti-LYVE-1 (28). Abundant lymphatic vessels were seen in mice of both genotypes in the s.c. tissue overlying the transplanted tumors. A few lymphatic vessels were also seen in the edges of the tumor tissue near the junction with the overlying skin. No lymphatics were observed deep within tumors harvested from mice of either genotype (Fig. 4, *E* and *F*). Quantitation of the lymphatic vessel density revealed no genotype-dependent differences. Tumors from Fib^- mice had a mean of 4.8 lymphatic vessels/mm^2 (range, 3.7–5.9), compared with 6.9 lymphatic vessels/mm^2 observed in control animals (range, 2.5–7.8; not significantly different, $P > 0.25$).

DISCUSSION

In addition to their critical role in maintaining vascular integrity, hemostatic factors appear to contribute significantly to vascular development, tissue repair, inflammatory processes, cell adhesion, and transendothelial cell migration. This spectrum of biological functions has made hemostatic system components prime candidates as tumor progression factors. Fibrin(ogen), in particular, has been thought to play an important role in tumor stroma formation and angiogenesis. However, contrary to initial expectations, the data presented here demonstrate that neither fibrin(ogen) nor FDPs are essential for primary (s.c.) or secondary (*e.g.*, pulmonary, lymph node) tumor growth. Furthermore, fibrin(ogen) is not strictly required for either tumor

angiogenesis or lymphangiogenesis, and its absence does not substantially alter the overall microscopic features of LLC tumors. However, the data presented here illustrate that the absence of fibrin(ogen) strongly diminishes the spontaneous metastatic potential of an aggressive tumor line, both via hematogenous and lymphatic routes. Furthermore, the data show that fibrin(ogen) is important in malignancy at the level of micrometastatic disease, a crucial phase with regard to cancer pathophysiology and clinical prognosis.

Given the well-established mitogenic, chemotactic, proinflammatory, and angiogenic properties of fibrin and FDPs, a prevailing hypothesis that has emerged is that provisional fibrin matrices may, in part, promote tumor stroma formation and subsequent tumor growth (8). The present studies clearly indicate that fibrin(ogen) deposition is not mandatory for stroma formation, at least in some types of solid tumors. However, a subtle contribution of fibrin matrices to tumor tissue formation (*e.g.,* alterations of tumor and/or stromal cell properties or gene expression profiles, matrix biosynthesis, peritumoral vascular permeability) has not been excluded. In addition, these studies do not rule out the possibility that fibrin(ogen) deposition and dissolution are profound determinants of tumor stroma formation and tumor growth in other forms of cancer, particularly those that either elicit exuberant local fibrin deposition within either the vascular or extravascular compartments or have limited capacity for fibrin clearance. Finally, our findings do not formally challenge the concept that features of tumor stroma/neovasculature formation may be akin to wound repair (7, 8, 33). Indeed, recent studies have shown that fibrin(ogen) is not crucial for the construction of a neovasculature within skin wound fields (30). When taken together with the studies presented here, a new parallel can be drawn between angiogenesis within tumors and wound fields: fibrin(ogen) is not essential for angiogenesis in either context.

These studies show that fibrinogen is a powerful determinant of the spontaneous metastatic potential of LLC tumors, and this finding is reminiscent of those made examining the impact of fibrin(ogen) deficiency on the metastatic potential of tumor cells introduced directly into the circulation (*i.e.,* experimental metastasis; Ref. 26). However, the similarity between experimental and spontaneous metastasis assays could not have been reliably predicted. Conceivably, the absence of fibrin(ogen) might actually increase spontaneous metastatic potential by eliminating one significant physical barrier to cell migration within the primary tumor extracellular matrix. Alternatively, the absence of fibrin in solid tumors might effectively diminish spontaneous metastatic potential by eliminating a provisional matrix component that supports (or drives) tumor cell migration. Taking into account the extremely different challenges confronting tumor cells in processes such as penetration of tissue barriers, transendothelial cell migration, transiting the circulation, stabilization within distant vascular beds and extravasation, fibrin(ogen) might bestow some significant advantages to tumor cells at certain steps of the metastatic process and be a major liability at other steps. In this regard, it is notable that the differences in metastatic potential observed in control and fibrinogen-deficient mice are remarkably similar in magnitude (~10-fold) in both experimental and spontaneous metastasis analyses. If one makes the assumption that the ultimate fate of circulating tumor cells (*i.e.,* success or failure in forming a macrometastatic foci) is not altered by the means by which the tumor cells initially enter the vasculature (*i.e.,* migration/intravasation or direct injection), then the available data suggest that the impact of fibrinogen deficiency on spontaneous metastases to the lung can largely be accounted for by the genotype-dependent differences in tumor cell fate once in the circulation. Taken together with earlier findings (26), it appears that the most important action of fibrin(ogen) in facilitating the formation of spontaneous metastases to the lung occurs at the level of the circulating tumor cells and, specifically, in promoting the sustained adhesion and/or survival within distant vascular beds. However, direct experimental analyses of primary tumor cell shedding into the circulation in control and fibrinogen-deficient mice will be necessary to formally exclude any fibrinogen-dependent difference in this process.

Despite the finding that fibrinogen deficiency strongly diminished spontaneous metastasis via a hematogenous route, the parallel finding that the loss of fibrinogen markedly reduced spontaneous metastases through lymphatics could not have been immediately predicted. No genotype-dependent difference was seen in lymphangiogenesis, suggesting that fibrinogen may influence lymphatic metastasis either by enhancing tumor cell entrance into the lymphatic circulation or by contributing to tumor cell stabilization and survival within the lymph system once the tumor cell has entered the lymphatic circulation. Fibrinogen is known to be present at relatively high concentrations in lymph fluid (~20% of the plasma fibrinogen concentration), as are many other soluble coagulation factors (34). Thus, lymphatic metastasis may be driven by either soluble fibrinogen (*e.g.,* serving as a bridging molecule for cell surface adhesion molecule-mediated engagement of tumor cells and lymphendothelium), local fibrin formation within the lymph vessels, or both. However, unlike hematogenous metastasis, which might be driven, in part, by fibrinogen-mediated interactions between platelets and tumor cells (see below), the lymphatic tumor dissemination data presented here suggest that fibrinogen may contribute significantly to metastasis through platelet-independent mechanisms. Defining the mechanisms by which fibrinogen contributes to tumor cell dissemination through the lymph system will require additional detailed studies, but it is clear that these mechanisms are not necessarily the same as those by which fibrinogen contributes to hematogenous metastasis.

Fibrin(ogen) may promote the metastasis of circulating tumor cells by several possible mechanisms. First, as a dimeric molecule with multiple integrin and nonintegrin binding motifs, fibrinogen might serve as an important molecular bridge between tumor cells, platelets, and endothelial cells, promoting stable adhesion. Second, fibrin-reinforced platelet/tumor microthrombi might help mechanically stabilize tumor cells at distant sites. Third, fibrin might provide a provisional matrix supporting the migration of tumor cells out of the vasculature. Finally, fibrin(ogen)-platelet microthrombi may provide some protection to tumor cells against innate immune surveillance systems (*e.g.,* natural killer cell-mediated tumor cell elimination; Ref. 35). Studies of metastatic disease in mice expressing mutant forms of fibrinogen lacking platelet integrins binding motifs (9), polymerization function, or both, should be informative in additional understanding the relationship of this key hemostatic factor and malignancy. A detailed understanding of the mechanisms by which fibrinogen and other hemostatic factors contribute to tumor dissemination may indicate the most opportune hemostatic target for treating malignant disease without compromising vascular integrity. The availability of viable lines of mice with selected deficits in fibrinogen, platelet signal transduction/adhesion molecules, and other hemostatic factors should be useful in defining the therapeutic potential of this class of pharmacological targets for the treatment of cancer.

ACKNOWLEDGMENTS

We thank Dr. David Witte for his expert assistance in interpreting the histological data. We also thank Alicia Emley for her help with the photographs and Dr. Beth A. Myers for critically reviewing the manuscript.

REFERENCES

1. Gordon, S. G., and Mielicki, W. P. Cancer procoagulant: a factor X activator, tumor marker and growth factor from malignant tissue. Blood Coagul. Fibrinolysis, *8:* 73–86, 1997.
2. Ruf, W., Fischer, E. G., Huang, H. Y., Miyagi, Y., Ott, I., Riewald, M., and Mueller, B. M. Diverse functions of protease receptor tissue factor in inflammation and metastasis. Immunol. Res., *21:* 289–292, 2000.
3. Palumbo, J. S., and Degen, J. L. Hemostatic factors in tumor biology. J. Pediatr. Hematol. Oncol., *22:* 281–287, 2000.
4. Dano, K., Romer, J., Nielsen, B. S., Bjorn, S., Pyke, C., Rygaard, J., and Lund, L. R. Cancer invasion and tissue remodeling: cooperation of protease systems and cell types. APMIS, *107:* 120–127, 1999.
5. Pyke, C., Kristensen, P., Ralfkiaer, E., Grondahl-Hansen, J., Eriksen, J., Blasi, F., and Dano, K. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. Am. J. Pathol., *138:* 1059–1067, 1991.
6. Brown, L. F., Asch, B., Harvey, V. S., Buchinski, B., and Dvorak, H. F. Fibrinogen influx and accumulation of cross-linked fibrin in mouse carcinomas. Cancer Res., *48:* 1920–1925, 1988.
7. Dvorak, H. F., Nagy, J. A., Berse, B., Brown, L. F., Yeo, K. T., Yeo, T. K., Dvorak, A. M., van de Water, L., Sioussat, T. M., and Senger, D. R. Vascular permeability factor, fibrin, and the pathogenesis of tumor stroma formation. Ann. N. Y. Acad. Sci., *667:* 101–111, 1992.
8. Dvorak, H. F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N. Engl. J. Med., *315:* 1650–1659, 1986.
9. Holmback, K., Danton, M. J., Suh, T. T., Daugherty, C. C., and Degen, J. L. Impaired platelet aggregation and sustained bleeding in mice lacking the fibrinogen motif bound by integrin α IIb β3. EMBO J., *15:* 5760–5771, 1996.
10. Katagiri, Y., Hiroyama, T., Akamatsu, N., Suzuki, H., Yamazaki, H., and Tanoue, K. Involvement of α v β 3 integrin in mediating fibrin gel retraction. J. Biol. Chem., *270:* 1785–1790, 1995.
11. Pluskota, E., and D'Souza, S. E. Fibrinogen interactions with ICAM-1 (CD54) regulate endothelial cell survival. Eur. J. Biochem., *267:* 4693–4704, 2000.
12. Suehiro, K., Gailit, J., and Plow, E. F. Fibrinogen is a ligand for integrin α5β1 on endothelial cells. J. Biol. Chem., *272:* 5360–5366, 1997.
13. Ugarova, T. P., Solovjov, D. A., Zhang, L., Loukinov, D. I., Yee, V. C., Medved, L. V., and Plow, E. F. Identification of a novel recognition sequence for integrin αM β2 within the γ chain of fibrinogen. J. Biol. Chem., *273:* 22519–22527, 1998.
14. Smiley, S. T., King, J. A., and Hancock, W. W. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. J. Immunol., *167:* 2887–2894, 2001.
15. Languino, L. R., Duperray, A., Joganic, K. J., Fornaro, M., Thornton, G. B., and Altieri, D. C. Regulation of leukocyte-endothelium interaction and leukocyte transendothelial migration by intercellular adhesion molecule 1-fibrinogen recognition. Proc. Natl. Acad. Sci. USA, *92:* 1505–1509, 1995.
16. Rubel, C., Fernandez, G. C., Rosa, F. A., Gomez, S., Bompadre, M. B., Coso, O. A., Isturiz, M. A., and Palermo, M. S. Soluble fibrinogen modulates neutrophil functionality through the activation of an extracellular signal-regulated kinase-dependent pathway. J. Immunol., *168:* 3527–3535, 2002.
17. Dvorak, H. F., Harvey, V. S., Estrella, P., Brown, L. F., McDonagh, J., and Dvorak, A. M. Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. Lab. Investig., *57:* 673–686, 1987.
18. Thompson, W. D., Smith, E. B., Stirk, C. M., Marshall, F. I., Stout, A. J., and Kocchar, A. Angiogenic activity of fibrin degradation products is located in fibrin fragment Eur. J. Pathol., *168:* 47–53, 1992.
19. Thompson, W. D., Campbell, R., and Evans, T. Fibrin degradation and angiogenesis: quantitative analysis of the angiogenic response in the chick chorioallantoic membrane. J. Pathol., *145:* 27–37, 1985.
20. Esumi, N., Fan, D., and Fidler, I. J. Inhibition of murine melanoma experimental metastasis by recombinant desulfatohirudin, a highly specific thrombin inhibitor. Cancer Res., *51:* 4549–4556, 1991.
21. Gasic, G. J. Role of plasma, platelets, and endothelial cells in tumor metastasis. Cancer Metastasis Rev., *3:* 99–114, 1984.
22. Bugge, T. H., Kombrinck, K. W., Xiao, Q., Holmback, K., Daugherty, C. C., Witte, D. P., and Degen, J. L. Growth and dissemination of Lewis lung carcinoma in plasminogen-deficient mice. Blood., *90:* 4522–4531, 1997.
23. Bugge, T. H., Lund, L. R., Kombrinck, K. K., Nielsen, B. S., Holmback, K., Drew, A. F., Flick, M. J., Witte, D. P., Dano, K., and Degen, J. L. Reduced metastasis of Polyoma virus middle T antigen-induced mammary cancer in plasminogen-deficient mice. Oncogene, *16:* 3097–3104, 1998.
24. Gutierrez, L. S., Schulman, A., Brito-Robinson, T., Noria, F., Ploplis, V. A., and Castellino, F. J. Tumor development is retarded in mice lacking the gene for urokinase-type plasminogen activator or its inhibitor, plasminogen activator inhibitor-1. Cancer Res., *60:* 5839–5847, 2000.
25. Bajou, K., Noel, A., Gerard, R. D., Masson, V., Brunner, N., Holst-Hansen, C., Skobe, M., Fusenig, N. E., Carmeliet, P., Collen, D., and Foidart, J. M. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat. Med., *4:* 923–928, 1998.
26. Palumbo, J. S., Kombrinck, K. W., Drew, A. F., Grimes, T. S., Kiser, J. H., Degen, J. L., and Bugge, T. H. Fibrinogen is an important determinant of the metastatic potential of circulating tumor cells. Blood, *96:* 3302–3309, 2000.
27. Suh, T. T., Holmback, K., Jensen, N. J., Daugherty, C. C., Small, K., Simon, D. I., Potter, S., and Degen, J. L. Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev., *9:* 2020–2033, 1995.
28. Banerji, S., Ni, J., Wang, S. X., Clasper, S., Su, J., Tammi, R., Jones, M., and Jackson, D. G. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J. Cell Biol., *144:* 789–801, 1999.
29. Geran, H., Greenberg, N., MacDonald, M., Schumacher, A., and Abbot, B. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother. Rep., *3:* 1–16, 1972.
30. Drew, A. F., Liu, H., Davidson, J. M., Daugherty, C. C., and Degen, J. L. Wound-healing defects in mice lacking fibrinogen. Blood, *97:* 3691–3698, 2001.
31. Jackson, D. G., Prevo, R., Clasper, S., and Banerji, S. LYVE-1, the lymphatic system and tumor lymphangiogenesis. Trends Immunol., *22:* 317–321, 2001.
32. Stacker, S. A., Caesar, C., Baldwin, M. E., Thornton, G. E., Williams, R. A., Prevo, R., Jackson, D. G., Nishikawa, S., Kubo, H., and Achen, M. G. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat. Med., *7:* 186–191, 2001.
33. Brown, L. F., Lanir, N., McDonagh, J., Tognazzi, K., Dvorak, A. M., and Dvorak, H. F. Fibroblast migration in fibrin gel matrices. Am. J. Pathol., *142:* 273–283, 1993.
34. Miller, G. J., Howarth, D. J., Attfield, J. C., Cooke, C. J., Nanjee, M. N., Olszewski, W. L., Morrissey, J. H., and Miller, N. E. Haemostatic factors in human peripheral afferent lymph. Thromb. Haemostasis, *83:* 427–432, 2000.
35. Nieswandt, B., Hafner, M., Echtenacher, B., and Mannel, D. N. Lysis of tumor cells by natural killer cells in mice is impeded by platelets. Cancer Res., *59:* 1295–1300, 1999.

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Joseph S. Palumbo, Jill M. Potter, Lisa S. Kaplan, et al.

Cancer Res 2002;62:6966-6972.