Microenvironment and Immunology

Plasma Fibronectin Promotes Lung Metastasis by Contributions to Fibrin Clots and Tumor Cell Invasion

Gunjan Malik1, Lynn M. Knowles1, Rajiv Dhir2,3, Shuping Xu1, Shuting Yang1, Erkki Ruoslahti4,5, and Jan Pilch1,3

Abstract

The attachment of circulating tumor cells to the blood vessels of distant organs is an important step in metastasis. We show here that experimental lung metastasis by two cell lines, B16F1 melanoma and 3LL lung carcinoma, is greatly reduced in transgenic mice that lack plasma fibronectin. This multifunctional adhesive glycoprotein becomes cross-linked to fibrin during clotting. Here, we report that eliminating plasma fibronectin from the blood circulation reverses the prometastatic effects of blood clotting and tumor cell integrin αvβ3. In vitro studies showed that fibrin-fibronectin complexes, but not purified fibrin, supported tumor cell attachment and invasion. These functions correlate with the ability of fibrin-fibronectin complexes to induce the activation of integrin αvβ3. Our findings reveal an important contribution of plasma fibronectin in lung metastasis. Furthermore, they suggest that the previously noted effects of blood clotting on lung metastasis might be mediated in part by a fibronectin-αvβ3 integrin axis, in which plasma fibronectin has to be incorporated into the blood clot. Cancer Res; 70(11); 4327–34. ©2010 AACR

Introduction

Metastasis to distant organs is the most critical complication of malignancies, but our understanding of the molecular mechanisms that govern tumor dissemination is still incomplete. After entering the blood circulation at the primary site, metastatic tumor cells attach within the vasculature of the target organ and invade the surrounding tissues (1, 2). Following initial tumor cell arrest in the vasculature of a distant organ, clotted plasma and platelets cooperatively stabilize circulating tumor cells by generating a thrombus that allows tumor cells to attach and spread at the vessel wall (3). Inhibiting blood clotting by blocking platelet activation and fibrin formation reduces metastasis (4). Metastasis is also reduced in knockout mice that lack fibrinogen, as well as in mice that are deficient in coagulation factor XIII, which covalently cross-links fibrin (5, 6).

Plasma fibronectin (pFN) is one of the most abundant adhesion proteins in the blood and a predominant adhesive component of clotted plasma (7, 8). pFN is incorporated into fibrin clots where FXIII covalently links it to fibrin (7). The recruitment of pFN to clotted plasma represents a major functional modification, because pFN is recognized by a large group of cell adhesion receptors, many of which are members of the integrin family (9). Tumor cells are known to express a variety of fibronectin-binding integrins and some of them, such as integrin αvβ3, have been shown to support tumor growth and metastasis (10, 11). Significantly, tumor cells that express activated αvβ3 metastasize aggressively, and inhibiting integrin αvβ3 with antibodies or RGD-containing peptides inhibits metastasis (11–13).

Fibronectin has been found to be upregulated in several types of malignant tumors and its expression positively correlates with an invasive and metastatic phenotype (14–16). Here, we sought to study the role of pFN for tumor metastasis. Although complete loss of fibronectin is embryonal lethal, pFN-deficient mice generated by postnatally deleting the liver fibronectin gene provide an opportunity to do so (17). Using these mice, we show that pFN supports tumor cell retention in the lungs. We further show that pFN is required for prometastatic functions of blood clotting and tumor cell integrin αvβ3. Paralleling the in vivo results, we find that tumor cell adhesion to and invasion in fibrin complexed with fibronectin (FibFN) is predominantly mediated by integrin αvβ3, which in turn, is activated by FibFN. Collectively, our results establish an important role for pFN in lung metastasis.

Materials and Methods

Metastasis model

Transgenic C57BL/6-Fn(+/−) Mx-Cre+ mice become pFN-deficient by postnatally deleting the fibronectin gene in the liver using copolymers of polyinosinic and polycytidylic acids
To induce metastasis, 5 x 10^5 B16F1 melanoma or 3LL Lewis lung carcinoma cells (American Type Culture Collection) were injected into the tail vein (i.v.) of transgenic pFN-deficient Fn(fl/fl) Mx-Cre mice and their wild-type littermates. Mice were euthanized 10 to 14 days after tumor cell injection and tumor nodules were counted on the surface of lungs using a stereomicroscope (Leica M50). To inhibit thrombin function, we injected 500 IU of hirudin (Calbiochem) with the tumor cell suspension. To inhibit PAR1, we added 10 µg/ml of SCH 79797 (Tocris Biosciences) to the tumor cell suspension 30 minutes prior to tail vein injection (18, 19). To inhibit tumor cell integrins, B16F1 cells were incubated with 100 µg/ml of anti-α4 (clones R1-2 and 9C10; BD Biosciences), anti-α5 (clone BMA5; Millipore), or anti-β3 (clone 2C9.G2; BD Biosciences) function-blocking antibodies for 30 minutes at 4°C. Tumor cells were washed with PBS prior to i.v. injection to remove excess antibody. For histologic analysis, tissues were fixed in 4% paraformaldehyde and underwent conventional processing for histology. The blocks were cut at 5 µm and stained with H&E or goat anti-mouse CD31 antibody (Santa Cruz Biotechnology, Inc.).

Tumor cell arrest
To analyze tumor cell arrest, mice were i.v. injected with CytoTracker Green–labeled B16F1 cells (Invitrogen) and perfused through the heart after 1, 4, and 16 hours. Lung tissue was removed, fixed with 4% paraformaldehyde, and analyzed by fluorescence microscopy (Zeiss Axioplan 2). Images were processed with Adobe Photoshop (Adobe Systems Incorporated). Tumor homing was assessed by counting green fluorescent B16F1 cells in random optical fields of lung tissue sections from Fn(fl/fl) Mx-Cre and wild-type mice. To assess colocalization of green fluorescent B16F1 cells with fibrin, paraformaldehyde-fixed lung tissues were stained with biotinylated mouse fibrin(ogen) antiserum (Nordic), followed by streptavidin Alexa Fluor 594 (Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole–containing mounting medium (Vectashield).

Preparation of clot products
Clotted plasma was produced from human citrated plasma (US Biological), pFN-depleted plasma was generated by passing the plasma through Sepharose CL4B connected in tandem with a gelatin-agarose column (20). Thawed plasma containing 0.1 mol/L of ε-aminocaproic acid was added to the column equilibrated with TEC binding buffer (0.05 mol/L Tris-HCl, 0.05 mol/L ε-aminocaproic acid, 0.02 mol/L sodium citrate) and column flow-through was collected as pFN-depleted plasma. Flow-through from the CL4B column was used as a pFN-positive control. Collected plasma was clotted with 20 mol/L of CaCl₂ overnight at 4°C. To produce fibrin, 2 mg/mL of fibrinogen (Enzyme Research Laboratories, Inc.) was mixed with 2.5 mmol/L of CaCl₂, 2.5 units/mL of thrombin, and 25 µg/mL of coagulation factor XIII (Enzyme Research Laboratories). To generate FibFN, 200 µg/mL of pFN (EMD Chemicals, Inc.) was added to the fibrin mixture prior to thrombin. Fibrin and FibFN samples were placed at 4°C and allowed to clot overnight. Then, pFN-deficient plasma, FibFN, and fibrin clots were washed in PBS and solubilized in a buffer containing 8 mol/L of urea, 2% SDS, 2% β-mercaptoethanol, and 0.16 mol/L of Tris (pH 6.8; ref. 21). To eliminate the solubilization buffer, the solubilized clot material was exhaustively dialyzed against PBS.

Cell adhesion assay
Cell adhesion was measured using B16F1, 3LL, or THP-1 cells (American Type Culture Collection). THP-1 cells were treated with 160 mmol/L of phorbol myristate acetate (Sigma-Aldrich) to induce macrophage differentiation (22). Forty-eight–well plates (polystyrene, non-tissue culture–treated; Costar) were coated with 10 µg/mL of fibrinogen or solubilized clot material from plasma (±pFN), FibFN or fibrin, and incubated at 4°C overnight. The plates were then washed with PBS and blocked with 1% bovine serum albumin (1 hour, 37°C). Cells were suspended in HEPES-Tyrode’s buffer containing 0.1% bovine serum albumin and 2 mmol/L of CaCl₂, added to the plate at 2 x 10⁵ in 200 µL/well and allowed to attach for 45 to 60 minutes (37°C; 5% CO₂). Plates were washed to remove floating cells. Attached cells were incubated with para-nitrophenol phosphate (5 mg/mL in 50 mmol/L sodium acetate, 1% Triton X-100; pH 5.2) for 30 minutes and quantified at 405 nm after adding 0.3 mol/L of sodium hydroxide. For integrin inhibition, cells were incubated with 100 µg/mL of function-blocking anti-α4 (clones R1-2 and 9C10), anti-α5 (clone BMA5), anti-β3 (clone 2C9.G2), or isotype controls on ice for 15 minutes prior to plating.

Flow cytometry assay
THP-1 cells were suspended in HEPES-Tyrode’s buffer containing 0.1% bovine serum albumin, 2 mmol/L of CaCl₂, 1 mmol/L of MgCl₂, and incubated with antibody specific for αvβ3 (LM609; Millipore), activated αvβ3 (Wow1; ref. 23), or an isotype control (BD Biosciences) in the presence or absence of FibFN (100 µg/mL) ± 10 mmol/L EDTA for 30 minutes at room temperature. Cells were washed with ice-cold buffer, and incubated for 30 minutes on ice with Alexa Fluor 488 anti-mouse F(ab’)2 (Invitrogen). Cell viability was monitored by staining cells with 5 µg/mL of propidium iodide (Roche Applied Science). Fluorescence was examined on 15,000 viable cells per sample using a tabletop cytometer (Beckman Coulter).

Invasion assay
Cells (5 x 10⁴) were mixed with 2 mg/mL of fibrinogen, 2 mmol/L of CaCl₂, and 25 µg/mL of FXIII with or without 200 µg/mL of pFN. Clotting was induced with 2.5 units/mL of thrombin. Clot-embedded cells were incubated with DMEM supplemented with 10% fetal bovine serum (Mediatech, Inc.) and gentamycin (Invitrogen) at 37°C under a humidified 5% CO₂ atmosphere. At designated time points, clots were analyzed for cell invasion at designated areas by phase contrast microscopy (Nikon Eclipse TS100; ×20 magnification). Invadopodia formation was classified as complete (i.e., elongated or stellate shape) or incomplete (i.e., round shape with or without rudimentary invadopodia) and calculated for completely spread cells as a percentage of the total. Where indicated, cell suspensions were preincubated with 100 µg/mL of anti-β3 or...
anti-hamster isotype antibody for 15 minutes on ice prior to the addition of thrombin and clot formation. Serine protease and matrix metalloproteinase activity was inhibited by adding 1 mg/mL of ε-aminocaproic acid (Sigma-Aldrich), 10 μg/mL of aprotinin, 25 μmol/L of GM6001 (EMD Chemicals), and 1 μg/mL of recombinant TIMP1 or TIMP2 (R&D Systems) to the cell suspension immediately prior to the addition of thrombin, as well as to the culture medium. Cell invasion in the absence of factor XIII was assessed by embedding the cells in clots prepared with FXIII-reduced fibrinogen (“Peak 1” Fibrinogen; Enzyme Research Laboratories).

**Short interfering RNA–mediated gene silencing**

B16F1 cells were plated at $2 \times 10^5$ cells/cm² and grown for 24 hours prior to transfection with 50 nmol/L of integrin β3, MT1-MMP, or nontargeting control short interfering RNA (siRNA; all from Dharmacon On-TARGETplus SMARTpool). Cells were transfected in Opti-MEM medium (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen) according to the instructions of the manufacturer. After 6 hours, cells were placed in normal culture medium and grown for an additional 66 hours. Cells were then harvested using 5 mmol/L of EDTA, washed twice with PBS, and embedded in a clot as described above.

**Statistical analysis**

All values were analyzed using Student’s t test and are expressed as mean ± SE. Treatment differences with a two-sided $P < 0.05$ were considered significantly different.

**Results**

**pFN promotes lung metastasis**

Transgenic C57BL/6-Fn(fl/fl) Mx-Cre mice become pFN-deficient by postnatally deleting the fibronectin gene in the liver (17). We chose B16 melanoma (B16F1) and Lewis lung carcinoma cells (3LL) as metastasis models because blood clotting promotes lung metastasis from these cell lines (24). We found that lung metastasis from B16F1 melanoma or 3LL lung carcinoma cells injected into the tail vein was significantly reduced in pFN-deficient mice (Fig. 1A). pFN had no effect on melanoma metastasis to the liver, adrenal gland, kidney, and ovaries (data not shown). Absence of pFN did not impair initial tumor cell arrest, but 16 hours after the injection, the number of tumor cells was significantly reduced in the lungs of pFN-deficient mice compared with their normal littermates (Fig. 1B). The same pattern of tumor cell retention was observed in fibrinogen-deficient mice compared with wild-type mice (Supplementary Fig. S1). Even though lung metastasis was reduced after 2 weeks in pFN-deficient mice, there was no obvious qualitative difference between lung histologies from wild-type and pFN-deficient mice as lungs in both groups showed a similar pattern of metastatic lesions at typical locations near distal alveoli and bronchioi (Fig. 1C–D). Metastatic lesions were localized outside of blood vessels indicating prior tumor cell extravasation (Fig. 1D). Together, our results indicate that pFN supports metastasis by promoting the retention of tumor cells in the lungs.

![Figure 1](https://cancerres.aacrjournals.org/docinfo/2010/70/11/4329/4329g01a.png)
pFN is required for the prometastatic activity of blood clotting

pFN is an important component of plasma clots, which form a provisional extracellular matrix around tumor cells in the lung vasculature. To determine if pFN facilitates clot formation around tumor cells, tissue sections from lungs of wild-type and pFN-deficient mice were probed for colocalization of tumor cells with fibrin(ogen) in fluorescence microscopy. The results revealed no difference in clot formation around circulating tumor cells in the lung vasculature whether pFN was present or not (Fig. 2A-B). To test if pFN requires clotting activity for its prometastatic function, blood clotting was inhibited with the thrombin antagonist hirudin at the time of tumor cell injection. Hirudin reduced lung metastasis in wild-type mice but did not further reduce the already lower rate of metastasis in pFN-deficient mice (Fig. 2C). This effect of hirudin was the result of inhibiting platelet activation and fibrin formation, because blockade of the thrombin receptor PAR1 on tumor and endothelial cells using SCH 79797 did not diminish lung metastasis (Fig. 2C). Interestingly, thrombin inhibition with hirudin had no effect on liver metastasis (Fig. 2D). Together, these results show that pFN is required for the prometastatic activity of blood clotting in the lung in vivo.

pFN acts through tumor cell integrin αvβ3

To define the role of fibronectin-binding integrins in the formation of lung metastases, we used antibodies to block α4β1, α5β1, and αvβ3 integrins on B16F1 cells and studied the ability of these cells to form metastases. Washing the antibody-treated cells prior to the injection allowed us to block tumor cell integrins without interfering with the adhesive properties of platelets or other blood cells. Only the anti-β3 integrin antibody (αvβ3 blockade) caused significant inhibition of lung metastasis (Fig. 3A-B). Notably, the β3 blockade had no effect on metastasis in pFN-deficient mice, indicating that a pFN-αvβ3 interaction was important to tumor cell retention in the lungs. Anti-β3 treatment had no effect on clot formation around tumor cells (Supplementary Fig. S2). We
next employed *in vitro* assays to analyze the adhesive interactions of tumor cells on surfaces coated with clotted material from complete plasma, plasma depleted of fibronectin, FibFN, or fibrin alone. Paralleling the *in vivo* results, we found that B16F1 cells bound to clotted plasma and fibrin when fibronectin was included, but not in its absence (Fig. 3C). Antibody inhibition showed that B16F1 adhesion to the FibFN complexes was mediated by integrin αvβ3 (Fig. 3D). In contrast, inhibition of integrins α4 and α5 had no effect on B16F1 adhesion to FibFN. Together, our results underscore the important role of integrin αvβ3 in promoting the adhesive interactions of B16F1 cells with FibFN in *vitro* and for pFN-mediated lung metastasis *in vivo*.

**FibFN activates integrin αvβ3**

B16F1 and 3LL cells require integrin activation with manganese to adhere to the αvβ3-specific substrates fibrin or fibrinogen, whereas attachment to FibFN complexes occurred spontaneously (Fig. 4A). Notably, the addition of FibFN complexes to the binding buffer induced the adhesion of B16F1, 3LL, and THP-1 cells to immobilized fibrinogen (Fig. 4B). Soluble pFN or fibrin complexes were not sufficient to induce tumor cell attachment. FibFN-induced cell adhesion was significantly inhibited in the presence of a function-blocking antibody against β3 integrin (Fig. 4C). To further explore the effect of FibFN on αvβ3-mediated cell adhesion, we incubated THP-1 cells with Wow1 antibody, which is specific for activated integrin αvβ3 (23). Analyzing THP-1 cells by flow cytometry, we found that Wow1 binding was significantly increased in the presence of FibFN and was reversed when we added FibFN together with EDTA (Fig. 4D). We did not detect any difference in αvβ3 cell surface expression following FibFN treatment. To identify mediators that resemble the effect of FibFN on αvβ3, we screened a number of established integrin agonists for their ability to induce B16F1 cell adhesion to fibrinogen (Supplementary Fig. S3). In addition to FibFN, B16F1 cell adhesion to fibrinogen was only induced by phorbol myristate acetate and thrombin. The addition of the thrombin inhibitor PPACK reversed thrombin- but not FibFN-induced adhesion. Importantly, out of the group of compounds positive for B16F1 adhesion, only FibFN was sufficient to induce THP-1 cell adhesion as well. Together, our results indicate that FibFN provides a unique stimulus that results in the activation of integrin αvβ3.

**FibFN promotes tumor cell invasion**

To determine the role of FibFN on tumor cell invasion, we embedded B16F1 and 3LL cells in three-dimensional gels of fibrin with or without pFN. After 16 hours, 44% of B16F1 and 28% of 3LL cells formed invadopodia in FibFN, but only 10%...
to 15% in fibrin (Fig. 5A). Invadopodia formation was also significantly reduced when B16F1 cells were embedded in fibronectin-depleted plasma clot (Fig. 5B). FibFN-induced invasion was reduced after β3 integrin inhibition and was abolished in the absence of FXIII, which cross-links fibronectin to fibrin (Fig. 5C-D). FXIII was not necessary for HUVEC and DU145 cells, which do not require the presence of pFN to invade fibrin (Supplementary Fig. S4). We also assessed invadopodia formation in the presence of the matrix metalloproteinase inhibitor GM6001, TIMP2, or MT1-MMP-siRNA, which all caused significant inhibition of FibFN-induced invasion (Fig. 6A-B). TIMP1, as well as the serine protease inhibitors aprotinin and ε-aminocaproic acid, had no such effect. Embedding B16F1 cells in FibFN gels had no effect on cell survival or proliferation (data not shown). Together, our results indicate that FibFN promotes tumor cell invasion into blood clots via integrin αvβ3 and MT1-MMP.

Discussion

We have studied tumor metastasis in pFN-deficient mice and show that pFN supports the retention of tumor cells in the lungs. The prometastatic effect of pFN is linked to clotting activity and mediated by tumor cell integrin αvβ3 in vivo. We attributed this novel function of pFN to the capacity of FibFN to activate integrin αvβ3, and thus, to promote tumor cell adhesion to and invasion into clotted plasma in vitro.

Deposition of fibronectin into the extracellular matrix has been shown to support tumor cell proliferation and angiogenesis, which are crucial steps in the completion of metastasis (25, 26). Our results show that pFN supports lung metastasis at an earlier stage of metastasis when tumor cell fate is determined by the ability of circulating tumor cells to survive in and extravasate from the pulmonary vasculature. Tumor cell survival in the lung vasculature is largely dependent on clot formation, which protects tumor cells from the...
cytotoxic activity of natural killer cells (4, 6). In addition, clotting has been shown to promote tumor cell adhesion and extravasation (3, 27). The pivotal role of clotting has been shown in several models of spontaneous and experimental lung metastasis (6, 28, 29). Blood clots contain large amounts of pFN, which is cross-linked to fibrin (7). Using the thrombin antagonist hirudin to inhibit clot formation, we found that the prometastatic function of clots depends at least in part on the presence of pFN. In agreement with previous studies, this cooperation between pFN and clotting was important for lung metastasis but was not relevant for metastasis to the liver, where circulating tumor cells have direct access to fibronectin and other extracellular matrix components expressed in the endothelial basement membrane of liver sinusoidal blood vessels (30–32). The initial tumor cell arrest in the lung vasculature, in contrast, is mostly initiated by adhesive interactions with the endothelium, suggesting that basement membrane proteins critical for tumor cell survival and extravasation are not immediately accessible (1, 33). In this environment, tumor cells seem to be unable to compensate for the lack of pFN or the inability to form clots.

The association of pFN with fibrin has been shown to promote cell adhesion to clotted plasma (21). Despite this proadhesive function, pFN did not affect the initial tumor cell arrest. This result was not unexpected because soluble pFN is inactive and clots form only after tumor cells adhere to the lung vasculature (3, 34). One of the effects of blood clots is to sustain tumor cell adhesion, which involves integrin activation as tumor cells begin to spread alongside the lung vasculature (3). Our results indicate that adhesive interactions of tumor cells with clotted plasma are uniquely mediated by integrin αvβ3, thus providing a possible explanation for the prometastatic activity of this adhesion receptor. Integrin αvβ3 recognizes a plethora of ligands including fibrin, which is the predominant adhesion protein in clotted plasma (35, 36). Yet, the efficacy of αvβ3 to mediate tumor cell adhesion, invasion, and metastasis decreased significantly when pFN was absent from clotted plasma. Based on this finding, we concluded that incorporation of pFN into blood clots promotes metastasis by enhancing the adhesive function of αvβ3 towards clotted plasma. Moreover, we found that FibFN is important for tumor cells as it leads to the activation of αvβ3. Activated integrins exhibit a conformational state of increased affinity for their ligands (23). The interaction of integrin αvβ3 with its ligands promotes survival, and could also induce the proliferation of metastatic tumor cells (37). However, FibFN had no effect on tumor cell growth, suggesting that the primary function of clotted plasma is to prepare tumor cells for extravasation. Blood clots have been shown to promote endothelial retraction, and thus, to provide access to the pulmonary basement membrane (27). To reach the basement membrane, tumor cells have to invade the surrounding layer of the clot. Our results show that the invasion of blood clots requires FibFN-mediated activation of integrin αvβ3 and the proteolytic activity of MT1-MMP.

In solution, pFN has a compact conformation that limits accessibility to cryptic binding sites for integrins and cell surface proteoglycans buried within the molecule (38). Our results suggest that cross-linking pFN to fibrin via coagulation factor XIIIa gives access to a binding site in pFN that activates integrin αvβ3. It is unlikely that integrin activation is the result of a pFN-binding protein because pFN remained inactive in the absence of FXIIIa, which itself had no effect on αvβ3 (Supplementary Fig. S3). Moreover, we found that FibFN was unique in its ability to activate αvβ3 when compared with a number of known integrin activators or FN-binding proteins (39–44). FibFN-induced cell adhesion and metastasis did not depend on cross-talk with the fibronectin-binding integrins α4β1 and α5β1. However, it is conceivable that FibFN could activate αvβ3 through the ligation of the fibronectin receptors CD26 and CD44, or as a result of cryptic protein disulfide isomerase activity near the COOH terminus of fibronectin (45–50). Identifying the molecular mechanism of FibFN binding to tumor cells will expand our understanding of metastasis and might provide a novel target for the development of antimetastatic treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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