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Colon Cancer Growth and Dissemination Relies upon Thrombin, Stromal PAR-1, and Fibrinogen

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Abstract

Thrombin-mediated proteolysis is a major determinant of metastasis, but is not universally important for primary tumor growth. Here, we report that colorectal adenocarcinoma represents one important exception whereby thrombin-mediated functions support both primary tumor growth and metastasis. In contrast with studies of multiple nongastrointestinal cancers, we found that the growth of primary tumors formed by murine and human colon cancer cells was reduced in mice by genetic or pharmacologic reduction of circulating prothrombin. Reduced prothrombin expression was associated with lower mitotic indices and invasion of surrounding tissue. Mechanistic investigations revealed that thrombin-driven colonic adenocarcinoma growth relied upon at least two targets of thrombin-mediated proteolysis, protease-activated receptor-1 (PAR-1) expressed by stromal cells and the extracellular matrix protein, fibrinogen. Colonic adenocarcinoma growth was reduced in PAR-1–deficient mice, implicating stromal cell-associated PAR-1 as one thrombin target important for tumor outgrowth. Furthermore, tumor growth was dramatically impeded in fibrinogen-deficient mice, offering the first direct evidence of a critical functional role for fibrinogen in malignant tumor growth. Tumors harvested from fibrinogen-deficient mice displayed a relative reduction in cell proliferative indices, as well as increased

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Disclosure of Potential Conflicts of Interest

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tumor necrosis and decreased tumor vascular density. Collectively, our findings established a functional role for thrombin and its targets PAR-1 and fibrinogen in the pathogenesis of colonic adenocarcinoma, supporting tumor growth as well as local invasion and metastasis.

Introduction

Multiple components of the hemostatic system have been linked to cancer progression, particularly metastasis (1). Previous studies in mice have unequivocally shown that tumor cell–associated tissue factor (TF; refs. 2–4), circulating prothrombin (2, 5, 6), and several downstream thrombin procoagulant targets (i.e., platelets, fibrinogen, factor XIII; refs. 2, 7–10) strongly promote tumor cell metastatic potential. However, at the level of primary tumor growth, the contribution of hemostatic factors has been less clear. Studies showing that inhibitors of thrombin or thrombin generation limit the growth of certain malignancies (e.g., prostate and breast cancer) suggest that at least some cancers depend on thrombin-mediated function for growth (11–15). However, previous analyses in mice using multiple tumor cell lines revealed that genetic alterations at the level of prothrombin expression or thrombin function (2, 5), as well as the genetic elimination of platelet function (9), protease-activated receptor-1 (PAR-1; ref. 16), fibrinogen (8, 9), and factor XIII (FXIII; ref. 7) had no impact on primary tumor growth. These previous findings were surprising, given that each of these thrombin targets has been implicated in the regulation of numerous cellular functions important in tumor growth, including leukocyte functions, endothelial cell migration, and cellular proliferation and survival (17–22). In fact, the potential of hemostatic factors to contribute to the development of a supportive tumor stroma and tumor growth was a fundamental aspect of the hypothesis first put forth by Harold Dvorak almost three decades ago that tumors pathologically replicate the wound-healing process, and are ostensibly “wounds that do not heal” (23).

Colorectal cancer likely represents an important exception where hemostatic system components appear to drive aspects of cancer progression other than the formation of metastases. Homozygous carriers of the prothrombotic factor V Leiden mutation are almost six times more likely to develop colorectal cancer than non-carriers (OR, 5.8), suggesting that thrombin generation is a significant determinant of colorectal tumorigenesis (24). Consistent with this view, studies in mice revealed that a modest 50% decrease in circulating prothrombin significantly impeded the formation of colitis-associated colonic adenomas (25). Furthermore, a significant decrease in inflammation-driven adenoma formation was observed in fibrinogen-deficient mice; an effect coupled to fibrin(ogen)-mediated engagement of the leukocyte integrin receptor $\alpha_M\beta_2$ (26). Although these animal studies reveal an important role for prothrombin and fibrin(ogen) in colonic adenoma formation in a distinctly inflammation-driven setting, they do not speak to the role of hemostatic factors in the growth of fully transformed colorectal adenocarcinoma, or the potential of hemostatic factors to influence colon cancer in settings without an inflammatory colitis component. The latter question is particularly important, as the vast majority of colorectal cancers occur in absence of colitis (27).

Here, we use a combination of genetic and pharmacologic approaches to show that, in contrast with multiple other tumor types, thrombin-mediated proteolysis is a significant determinant of both the metastatic potential and growth of colonic adenocarcinoma. These studies also reveal that thrombin is coupled to colon cancer growth by at least two downstream targets. We show that PAR-1 expressed by tumor stromal cells promotes colon cancer growth *in vivo*. We also show that fibrin(ogen) is a major determinant of colon cancer growth, revealing for the first time an important context where this provisional matrix protein supports the growth of fully transformed cells *in vivo*.

Materials and Methods

Transgenic mice and statistical analyses

$FII^{Lox/-}$ (*F2*$^{Lox/-}$; ref. 28), $Fib^{-/-}$ (*Fga*$^{-/-}$; ref. 29), $FXIII^{-/-}$ (*F13a1*$^{-/-}$; ref. 7), and PAR-$1^{-/-}$(*F2r*$^{-/-}$; ref. 30) mice have been previously described. Sex-matched C57BL/6 mice 8 to 10 weeks of age were used for all studies of MC38 cells. Athymic nude male mice 6 to 8 weeks of age were used for xenograft studies. The Cincinnati Children's Hospital Research Foundation Institutional Animal Care and Use Committee approved all animal studies. Unless otherwise indicated, all data represent the mean and SEM. Statistical comparisons were made using a two-tailed Mann–Whitney *U* test, unless otherwise noted.

Tumor growth and metastasis assays, and ASO-mediated prothrombin depletion

The MC38 (provided by Edith Janssen, University of Cincinnati), HCT116 cells (provided by Janusz Rak, McGill University, Montreal, Canada), and B16-BL6 melanoma cells (originally provided by Isaiah Fidler, University of Texas, Houston, TX) were grown *in vitro* essentially as previously described (31–33). For tumor growth studies, tumor cells were harvested by brief trypsin exposure, suspended in 100 mL cold PBS, and injected into the dorsal subcutis between the scapulae as previously described (MC38 = 2.5×10^5 cells/injection, HCT116 = 8.0×10^5 cells/injection) and tumor growth was tracked by serial caliper measurements. For experimental metastasis assays, MC38 cells suspended in PBS were injected into the mouse tail vein as previously described. Hepatic prothrombin synthesis was suppressed using an established prothrombin-specific ASO "gapmer" as previously described (5). Note that ASO/control oligonucleotide was administered distal to sites of tumor cell inoculation.

Histology

Hematoxylin and eosin (H&E) staining (5-μm sections) was performed on formalin-fixed, paraffin-embedded tissue. Mitotic indices were calculated by blindly counting mitotic figures in telophase, as well as total nuclei, from randomized H&E images of nonnecrotic tumor tissue obtained at ×100 magnification. Quantification of necrotic tumor tissue was obtained by ImageJ analysis (% area = [necrotic area/total area] ×100) by a blinded observer on sequential ×5 performed magnification images of H&E-stained tumor tissue. Fibrin immunostaining was performed as previously described (34). All other histologic analyses were performed on 5-μm frozen tumor tissue sections. Apoptosis measurements ("TUNEL" reaction) were obtained using the Apoptag Plus Fluorescein *In Situ* Apoptosis Detection Kit (Millipore). Immunofluorescence was performed on frozen tumor sections treated with cold

methanol for 10 minutes, washed in PBS, and blocked with 10% donkey serum for 1 hour. Primary (overnight, 4°C) and secondary (1 hour, room temperature) antibodies were diluted in PBS containing 1% donkey serum, and slides were washed 3 × 10 minutes in PBS after each antibody. Antibodies were used at the following dilutions: PECAM/CD31 (Santa Cruz Biotechnology; MEC 13.3) 1:500, secondary (Invitrogen; donkey anti-rat) 1:500; CD68 (Abcam; FA-11), and 1:500, secondary (Invitrogen; donkey anti-rat) 1:500.

Results

Prothrombin promotes the growth and local invasion of colonic adenocarcinoma in mice

To test the hypothesis that prothrombin is a determinant of colon cancer growth, we compared tumor growth following injection of an aggressive C57Bl/6-derived colonic adenocarcinoma cell line (MC38; ref. 35) into the dorsal subcutis of C57Bl/6-derived mice carrying a lifelong diminution in prothrombin levels to approximately 10% of normal ($FII^{Lox/-}$) due to the imposition of a “floxed” prothrombin allele that limits prothrombin expression in the absence of any other genetic modification (i.e., Cre-mediated recombination; ref. 28) and wild-type controls (WT). Colon cancer growth was significantly impeded in female $FII^{Lox/-}$ mice relative to WT control animals based on estimates of tumor volume as determined by serial caliper measurements (Fig. 1A, left). Tumor mass determined at sacrifice 16 days after inoculation was decreased almost 3-fold in $FII^{Lox/-}$ mice relative to WT animals (Fig. 1A, right). Colonic adenocarcinoma growth was also significantly diminished in male $FII^{Lox/-}$ mice, but the genotype-dependent difference was not as profound (Supplementary Fig. S1A). All data presented hereafter use female mice unless otherwise stated. These experiments were completed three times with similar results.

Consistent with previous reports showing that $FII^{Lox/-}$ mice never suffer spontaneous bleeding events and can survive significant hemostatic challenges (28), tumor tissue from $FII^{Lox/-}$ mice showed no obvious signs of hemorrhage. Detailed histologic analyses of H&E-stained tumor sections revealed sporadic, small areas of intratumoral hemorrhage that were not genotype dependent. Focal areas of necrosis were also observed in tumors harvested from mice of both genotypes, and no genotype-dependent difference in the degree of necrosis was observed (Fig. 1B and Supplementary Fig. S1B, respectively). There were also no obvious genotype-dependent differences in overall tumor architecture (Fig. 1B). The tumors consisted of sheets of highly anaplastic appearing cells with vesicular nuclei with nucleoli, regardless of genotype. The presence of apoptotic cells, identified using the TUNEL assay, was not different between genotypes (Supplementary Fig. S1C). However, detailed analyses at high-power magnification revealed that mitotic figures were less common in tumors harvested from $FII^{Lox/-}$ mice (Fig. 1C). Quantitation of mitotic indices confirmed that tumors harvested from $FII^{Lox/-}$ mice were less proliferative than those harvested from WT mice (Fig. 1C). Here, approximately 4,000 total tumor cells were evaluated within each genotype by a blinded observer (10 individual tumors per genotype from two experiments with equivalent outcomes). These results suggest that thrombin promotes events in the colonic adenocarcinoma microenvironment that drive tumor cell proliferation.

To gain a better understanding of the processes underlying the prothrombin-dependent growth of colonic adenocarcinoma, a series of detailed histologic analyses were completed on tumors harvested from WT and $FII^{Lox/-}$ mice. Immunostaining revealed fibrin(ogen) deposits that were primarily peritumoral underlying the adjacent dermal tissue, as well as sparse fibrin(ogen) deposits associated with areas of focal necrosis, both of which were similar between genotypes (Fig. 1D and data not shown). Analyses of tumor-associated macrophages (TAM) in MC38 tumor tissue were carried out by immunofluorescence staining for CD68. A large proportion of cells within the tumor microenvironment were CD68-positive, regardless of genotype (Supplementary Fig. S1D). Quantitative analyses of vascular density in MC38 tumors based on immunofluorescent staining for PECAM (CD31) were also similar between genotypes (Fig. 1E).

Histologic analyses further revealed that prothrombin supports tumor cell invasion into surrounding tissue. Growth of MC38 tumors into the adjacent panniculus muscle was much more common in tumors harvested from WT mice relative to those harvested from $FII^{Lox/-}$ mice (Fig. 1F). Eighteen of the 19 tumors analyzed from WT mice showed signs of panniculus invasion, whereas only 8 of 17 tumors from $FII^{Lox/-}$ mice had any noticeable signs of panniculus invasion ($P < 0.005$, Fisher exact test). To rule out the possibility that the observed difference in tumor invasion was simply secondary to genotype-dependent differences in tumor growth rather than invasion, data were further stratified into groups of tumors with similar volumes (mean tumor volumes: WT = 459 ± 72 mm^3, $n = 12$; $FII^{Lox/-}$ = 488 ± 85 mm^3, $n = 6$; $P = 0.81$). For this subgroup, all 12 tumors harvested from WT mice showed signs of panniculus invasion, whereas only 2 of 6 tumors harvested from $FII^{Lox/-}$ mice had any evidence of invasion ($P < 0.005$, Fisher exact test).

To address whether diminished prothrombin expression conferred by the $FII^{Lox/-}$ genotype would broadly affect subcutaneous tumor growth, we injected B16 melanoma cells into the dorsal subcutis of WT and $FII^{Lox/-}$ mice in parallel. In contrast with what was observed following injection of colonic adenocarcinoma cells, tumor growth following melanoma cell injection was indistinguishable between genotypes. These results further highlight the malignancy-specific nature of the role of thrombin in driving primary tumor growth.

Prothrombin is a major determinant of the metastatic potential of colon cancer cells

Previous studies on the contribution of coagulation system components to tumor biology unequivocally demonstrated that prothrombin and multiple procoagulant thrombin substrates promote tumor cell metastasis across multiple types of cancers (1, 2, 5, 7–10). To determine whether the metastatic potential of colorectal cancer is prothrombin dependent, we i.v. injected cohorts of WT and $FII^{Lox/-}$ mice with GFP-expressing MC38 cells in parallel. Thirty minutes after injection, GFP-expressing micrometastatic pulmonary foci were similarly abundant in lungs harvested from WT and $FII^{Lox/-}$ mice (Fig. 2A). However, by 20 hours post-injection lungs harvested from $FII^{Lox/-}$ mice had approximately 3-fold fewer pulmonary foci than those harvested from WT mice (Fig. 2B). Analyses 2 weeks after tumor cell injection demonstrated that the majority of the lungs harvested from $FII^{Lox/-}$ had no discernable pulmonary metastases, whereas penetrance of colon cancer metastases was nearly 100% in control mice (Fig. 2C). Together, these results reveal that prothrombin plays

a broad role in the progression of colonic adenocarcinoma that involves promoting both the growth of established tumors, and the potential of circulating tumor cells to form distant metastases.

Pharmacologic inhibition of hepatic prothrombin production impedes murine and human colon cancer growth

As a complementary approach to explore the role of prothrombin in colon cancer growth, hepatic prothrombin expression was pharmacologically reduced using a highly selective murine pro-thrombin anti-sense oligonucleotide (ASO) "gapmer" (5, 25). Beginning 2 weeks before inoculation, cohorts of WT immunocompetent C57BL/6 mice were given weekly s.c. injections of a prothrombin-specific ASO (or a sequence-irrelevant control oligonucleotide) at a dose documented to lower circulating pro-thrombin levels to approximately 5% of normal (5). Paralleling results in $FII^{Lox/-}$ mice, pharmacologically lowering prothrombin significantly impeded MC38 tumor growth (Fig. 3A). An identical approach was used to compare the growth of a human-derived colonic adenocarcinoma cell line (HCT116) in male athymic nude mice treated with prothrombin-specific ASO. Similar to results with murine-derived colonic adenocarcinoma cells, pharmacologically decreasing prothrombin expression significantly impeded the growth of HCT116 human colonic adenocarcinoma (Fig. 3B).

Stromal cell–associated PAR-1 promotes colon cancer growth

To determine whether expression of the thrombin receptor PAR-1 by tumor stromal cells plays a role in colon cancer growth, we inoculated MC38 cells into the dorsal subcutis of $PAR\text{-}1^{-/-}$ and WT mice in parallel. Palpable tumors developed in the same time frame in both genotypes, but tumors growing in $PAR\text{-}1^{-/-}$ mice grew significantly slower than those in WT mice, resulting in a significant genotype-dependent difference in tumor volume and tumor mass 3 weeks after injection (Fig. 4A). These experiments were completed twice with similar significant outcomes. Overall tumor architecture was similar between genotypes based on analyses of H&E-stained tumor sections (data not shown). No significant differences in tumor vessel density (Fig. 4B) or the number of TAMs (Fig. 4C) were seen based on immunofluorescence analyses, suggesting that PAR-1 expression in the stromal compartment is not critical for tumor angiogenesis or recruitment of TAMs to the tumor microenvironment.

Fibrin(ogen) is a major determinant of colon cancer growth

To determine whether prothrombin-dependent colonic tumor growth was linked to clotting function, we enrolled mice with genetic deletions of FXIII or fibrinogen in analyses of colon cancer growth. MC38 tumors grew similarly in $FXIII^{-/-}$ and control mice over the course of a 3-week evaluation period (Supplementary Fig. S2A), indicating that FXIII-transglutaminase activity is not crucial for colon cancer growth. In contrast, studies where MC38 cells were inoculated into $Fib^{-/-}$ (Fib^{-}) and $Fib^{+/-}$ (Fib^{+}) mice revealed that deletion of fibrinogen dramatically limited colon cancer growth, resulting in an approximately 3-fold difference in tumor mass by the end of the 20 day experiment (Fig. 5A). Three independent experiments were completed with similar results. Intriguingly, the modest gender-dependent effect of MC38 tumor growth observed in mice with low prothrombin (Fig. 1A and

Supplementary Fig. S1A) was not observed in Fib^{-} mice. Fibrinogen deficiency resulted in a drastic reduction in tumor progression in both female (Fig. 5A) and male mice (Supplementary Fig. S2B). Detailed microscopic analyses revealed that the total percentage of necrotic tumor tissue relative to healthy appearing tissue was higher in tumors harvested from Fib^{-} mice relative to control animals (Fig. 5B). Despite the lack of clotting function, areas of hemorrhage were generally small and uncommon in tumors from both Fib^{+} and Fib^{-} mice, even in areas of necrosis. High-power analyses of H&E-stained sections also revealed that mitotic figures in areas of healthy tumor tissue were more common in tumors harvested from control mice relative to those harvested from Fib^{-} mice (Fig. 5C). Apoptotic cells were relatively uncommon and similar between genotypes based on TUNEL stains (Supplementary Fig. S2E). The presence of TAMs within colonic adenocarcinoma tumors was also similar in tumors harvested from Fib^{-} and control mice based on immunofluorescence staining for CD68 (Supplementary Fig. S2C and S2D).

Overall, the findings of diminished tumor growth in Fib^{-} mice were similar to those observed in mice with reduced prothrombin expression with two notable exceptions. First, tumors from Fib^{-} mice displayed a relatively higher degree of necrotic tumor tissue than tumors grown in $FII^{Lox/-}$ mice as noted above. Second, in contrast with the results observed in $FII^{Lox/-}$ mice, vascular density was decreased in tumors harvested from Fib^{-} mice relative to those from Fib^{+} mice based on immunofluorescence staining for PECAM (Fig. 5D and E). Note that H&E-stained parallel frozen sections were evaluated to ensure that tumor vasculature analyses were limited to areas of healthy appearing tumor tissue (Fig. 5D). To determine whether these fibrinogen-dependent differences in tumor vascular density were present throughout the process of tumor growth, we compared vascular density in MC38 tumors harvested from Fib^{-} and Fib^{+} mice at an earlier stage of tumor development (14 days after inoculation) in separate experiments. As expected at this early time point, tumors were relatively small in both genotypes and had just begun to show a genotype-dependent difference in growth (Fig. 5F). There was also no significant necrosis in tumors from either genotype (data not shown). At this early time point, vascular density was similar between genotypes (Fig. 5F and G). These data indicate that the differences in tumor vasculature between Fib^{+} and Fib^{-} mice observed at the 3-week time point were acquired over time.

Discussion

The capacity of hemostatic system components to regulate multiple cellular functions important in tumor pathobiology contributed to the longstanding hypothesis that hemostatic factors are determinants of tumor growth and stroma formation (1, 23). However, this hypothesis was significantly challenged by studies showing that thrombin and multiple thrombin targets were crucial for metastasis formation, but were dispensable for primary tumor growth across multiple cancers (1, 5, 7–9). The findings presented here reveal that colonic adenocarcinoma represents one important malignancy wherein thrombin drives not only experimental metastasis, but also tumor growth and local invasion. These studies further reveal that colon cancer represents an important context where thrombin supports tumor growth through multiple mechanisms involving distinct downstream targets, including fibrinogen and PAR-1.

Previous studies have shown that inhibitors of thrombin or thrombin generation are capable of slowing the growth of certain malignancies (e.g., prostate and breast cancer; refs. 11–15), suggesting that a supportive role for thrombin in tumor growth is not limited to colonic adenocarcinoma. However, thrombin and multiple thrombin targets, including fibrinogen, are clearly not universally important for primary tumor growth as evinced by previous studies of lung cancer (7–9), fibrosarcoma (2), and the studies with melanoma presented here. It is also worth noting that, with one exception (15), these studies demonstrating thrombin-dependent or -independent growth used experimental settings where tumor cells were injected s.c. into mice (2, 7–9, 11–14), suggesting that the role of thrombin in tumor growth is dependent more on the tumor tissue of origin than tumor location. It is also conceivable that certain cancers "evolve" the capacity for thrombin-independent growth over time as malignant cells collect additional transforming mutations. Defining the molecular differences between cancers exhibiting thrombin-dependent and -independent growth could significantly add to our understanding of the role of hemostasis in cancer progression. The view that colon cancer represents one important malignancy that is strongly thrombin dependent for multiple aspects of progression is compatible with several observations. Patients homozygous for the prothrombotic factor V variant, fV^{Leiden}, carry an almost 6-fold increased risk of developing colon cancer (24), suggesting that thrombin drives colonic tumorigenesis. Consistent with this view, previous studies showed that thrombin and fibrin(ogen) are important determinants of adenoma formation in the context of inflammatory colitis (25). The data presented here reveal that thrombin also supports the growth and dissemination of fully transformed colonic adenocarcinoma in the absence of investigator-imposed inflammation. In sum, these studies suggest that thrombin plays a very broad role in the pathogenesis of colonic adenocarcinoma, influencing events important in tumorigenesis, tumor growth, local invasion, and the ability of circulating tumor cells to form metastases.

The thrombin growth dependence of a given cancer does not appear to be simply due to the presence or absence of tumor cell–associated TF expression, as TF expression is common among aggressive malignancies, including colon cancer (1, 3, 36). Although TF expression by tumor cells is dispensable for tumor growth in some contexts (1, 2), tumor cell–associated TF clearly plays a major role in tumorigenesis and tumor growth in multiple contexts, including colon cancer (37, 38). Tumor cell–associated TF has been proposed to promote tumor growth through thrombin-independent mechanisms involving regulation of cell signaling events coupled to PAR-2 (39), as well as secretion of an alternatively spliced variant of TF (asTF), known to induce integrin signaling (40). Although the source of TF driving thrombin generation in these studies remains to be definitively determined, we favor the view that one mechanism coupling TF expressed by colon cancer cells to tumor growth involves local thrombin generation, raising the distinct possibility that colon cancer represents an important context where tumor cell-associated TF supports tumor growth through thrombin-dependent and -independent mechanisms.

Thrombin has over a dozen recognized substrates (41), making it capable of influencing colon cancer pathogenesis through multiple distinct mechanisms. Previous *in vitro* studies suggested that activation of tumor cell–associated PAR-1 promotes colon cancer

proliferation and invasion (42, 43). Although this view is consistent with the data presented here, these studies also reveal that stromal cell–associated PAR-1 promotes colon cancer growth. Multiple tumor stromal cells important in colon cancer progression express PAR-1 (e.g., endothelial cells, fibroblasts, multiple leukocyte subsets; refs. 44, 45). Although the findings here show that stromal cell–associated PAR-1 is not critically required for colon cancer angiogenesis or TAM recruitment, further study will be needed to determine whether PAR-1 signaling alters endothelial and/or macrophage functions in a manner that promotes colon cancer growth. It also remains to be determined whether PAR-1 expressed by other cell types known to promote colon cancer progression (e.g., fibroblasts, other leukocyte subsets) influence colon cancer progression. The ability to eliminate PAR-1 in a cell-type–specific manner would provide the means to answer this question as well as dissect the potential mechanisms coupling this thrombin receptor to other disease pathologies.

The findings presented here represent the first unambiguous example of a context where fibrin(ogen) drives the growth of fully transformed malignant cells *in vivo*. The relative reduction in tumor vascular density observed in Fib^{-} mice at a later stage in tumor progression (day 20) suggests a role for fibrin(ogen) in tumor angiogenesis in colonic adenocarcinoma. This concept is consistent with studies showing that fibrin(ogen) binds VEGF (46), as well as studies showing that fibrin and fibrin degradation products support endothelial migration in some contexts (47). However, fibrinogen-deficient mice manifest no abnormalities in vascular development (29), and fibrinogen is dispensable for angiogenesis in the growth of other tumor types and in incisional wound healing (8, 48). Although colon cancer may represent an important context where fibrin(ogen) is important for angiogenesis, it is also possible that the fibrinogen-dependent difference in tumor vascular density observed here late in the experimental timeframe represents a by-product of the significant impedance in tumor growth resulting from fibrinogen deficiency. This conclusion is consistent with the finding that tumor vascular density was indistinguishable between fibrinogen genotypes at an earlier time point (day 14), when tumors were much smaller overall. Furthermore, the data presented here do not address whether fibrin(ogen) supports vascular functions in the setting of colon cancer. Indeed, any loss of vascular integrity that may result from fibrin(ogen) deficiency would be expected to restrict nutrient delivery and/or gas exchange, and could explain the fibrin (ogen)-dependent differences in tumor necrosis and tumor cell proliferative rates observed in these studies.

Fibrin(ogen) could also support colon cancer growth through numerous mechanisms independent of angiogenesis. Fibrin(ogen) can serve as a scaffold for the delivery of numerous accessory proteins capable of shaping the tumor microenvironment in addition to VEGF (e.g., FGF-2, tPA, plasminogen, PAI-2, TGF-b1; refs. 49, 50). Fibrin(ogen) also possesses binding motifs for numerous integrin and non-integrin receptors (e.g., cadherins, α_{IIb}/β_3, $\alpha_V\beta_3$, $\alpha_X\beta_2$, $\alpha_M\beta_2$, $\alpha_5\beta_1$, $\alpha_V\beta_1$, Toll-like receptors; refs. 51–53), making it capable of influencing numerous cell types in the colon cancer microenvironment, including endothelial cells, platelets, leukocytes, fibroblasts, and tumor cells themselves. Notably, previous studies showed that fibrin(ogen)-mediated engagement of the leukocyte integrin $\alpha_M\beta_2$ supports the development of adenomas in the specific context of inflammatory colitis (26). Determining whether fibrin(ogen)-mediated integrin engagement modulates the

function of leukocytes within the tumor microenvironment in a manner that supports colonic adenocarcinoma growth will be a significant focus of future studies. Given the recognized importance of colonic microflora in colon cancer pathogenesis (54), as well as the fact that the vast majority of human colon cancers arise in the absence of colitis (27), pursuing such studies in an experimental setting where fully malignant colonic adenocarcinoma develops *in situ* in the absence of investigator-imposed inflammation would be ideal.

In contrast with fibrinogen, elimination of FXIII had no effect on colon cancer growth. One explanation for this finding is that soluble fibrinogen supports colon cancer growth. Soluble fibrinogen retains numerous biologic functions, including the ability to engage the platelet integrin $\alpha_{IIb}\beta_3$, enzymes, and other matrix proteins (e.g., fibronectin), making it conceivable that soluble fibrinogen in the colon cancer microenvironment could support tumor growth. Alternatively, fibrin monomer within the colon cancer microenvironment may be sufficient to support colon cancer growth in the absence of FXIII-mediated stabilization. Given the importance of prothrombin in colon cancer growth, we favor the view that one mechanism coupling thrombin to colon cancer growth involves fibrin polymer formation. Ultimately, the ability to genetically alter native fibrinogen in a manner that prevents thrombin-mediated polymer formation would yield the ideal tool for defining the potential of fibrinogen to support colon cancer pathogenesis and other disease processes.

The present findings illuminate one important context where hemostatic factors are important determinants of tumor growth as well as metastasis formation, but multiple additional contexts may ultimately be identified. Given that thrombin functions appear to play a broadly important role in colon cancer pathogenesis, supporting events important in tumorigenesis (25, 26), as well as tumor cell proliferation, local invasion, and the metastatic potential of circulating tumor cells, therapies targeting thrombin function/generation, or downstream thrombin targets, may have a role as adjuvants in the prevention and/or treatment of colonic adenocarcinoma. Indeed, the recent development of approved and emerging anticoagulants with multiple advantages over vitamin K antagonists and heparins, including less bleeding risk (55), makes this possibility particularly exciting. Of course, additional preclinical and clinical studies will be needed to determine whether the potential benefits of this approach outweigh the bleeding risks associated with anticoagulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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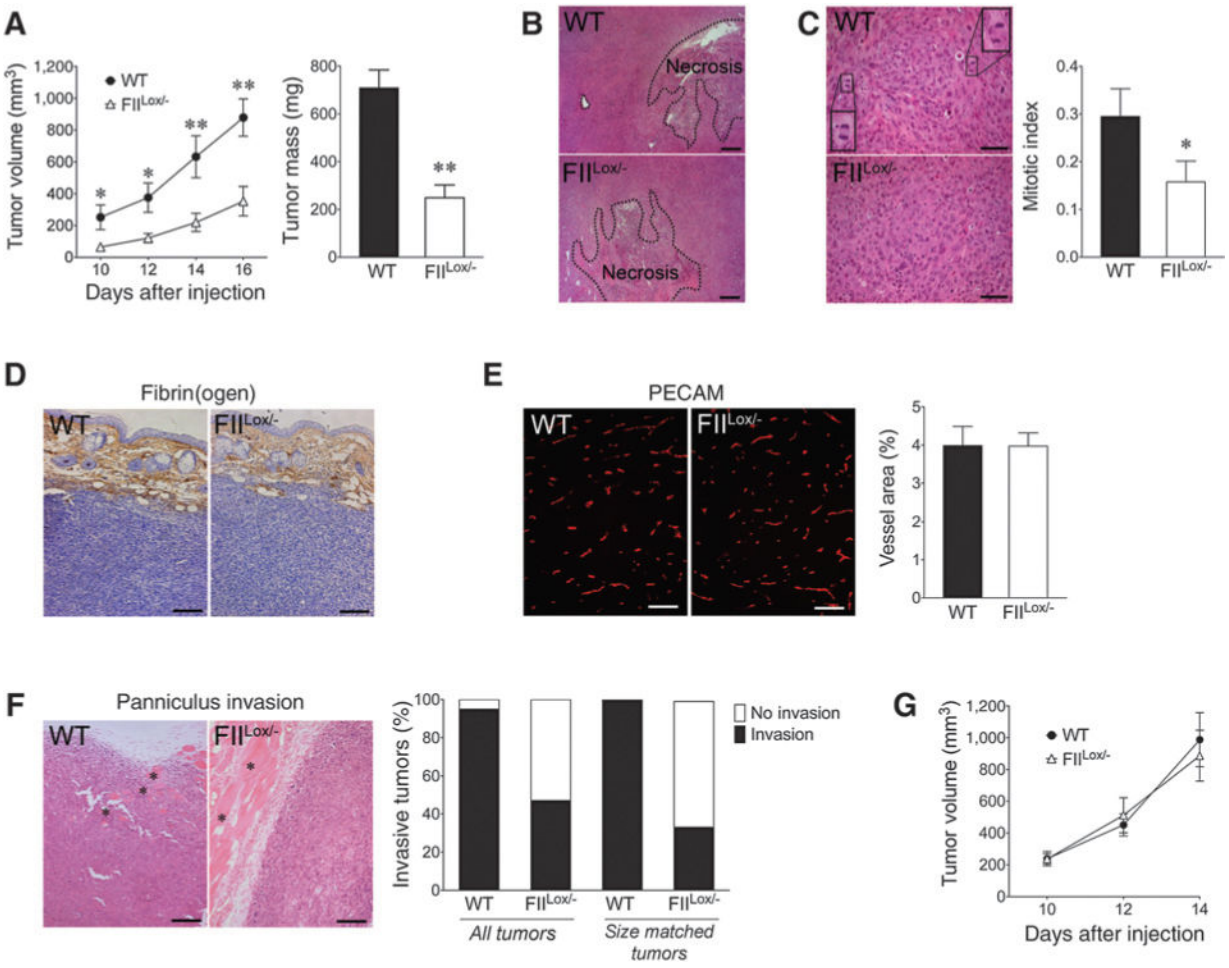

Figure 1.

Prothrombin promotes the growth and local invasion of colonic adenocarcinoma in mice. A, tumor growth in WT and $FII^{Lox/-}$ mice (n = 5/genotype) following injection of MC38 cells into the dorsal subcutis based on serial caliper measurements and tumor mass at the time of sacrifice (*, $P < 0.05$; **, $P < 0.01$). B, shown are representative H&E-stained sections of MC38 tumors harvested from WT and $FII^{Lox/-}$ revealing sheets of tumor cells and focal areas of necrosis (dotted lines) in both genotypes. C, analyses at higher magnification revealed that mitotic figures (insets) were more common in tumors harvested from WT mice. This was confirmed by quantitation of mitotic indices (see text for details). D, fibrin(ogen) deposits in tumor tissue (brown immunostaining) were indistinguishable between genotypes. Fibrin(ogen) deposits were generally peritumoral adjacent to overlying dermal tissue, with occasional sparse deposits associated with small areas of necrosis (not shown). E, tumor vascular density was similar between genotypes based on ImageJ analyses of frozen tissue sections immunofluorescently stained for PECAM/CD31. The data shown represents the results of analyses of 6 individual images per tumor from 10 tumors harvested from WT mice and 8 tumors harvested from $FII^{Lox/-}$ mice from two experiments with similar outcomes. F, detailed analyses of the tumor/skin junction revealed that local invasion of panniculus muscle fibers (*) was more common in tumors harvested from WT mice relative to those from $FII^{Lox/-}$ mice. Also shown are quantitative analyses revealing that

local invasion of the panniculus muscle was more common in tumors harvested from WT mice relative to those from $FII^{Lox/-}$ mice. This was true even when the analyses were limited to tumors from each genotype of comparable size (~450 mm^3). ($P < 0.005$ for each comparison, Fisher exact test. See text for details.) G, in contrast with observations with colon cancer cells, tumor growth in WT and $FII^{Lox/-}$ mice ($n = 7$/genotype) was indistinguishable following injection of B16 melanoma cells into the dorsal subcutis. Size bars, 100 μm (B, D–F) or 50 μm (C).

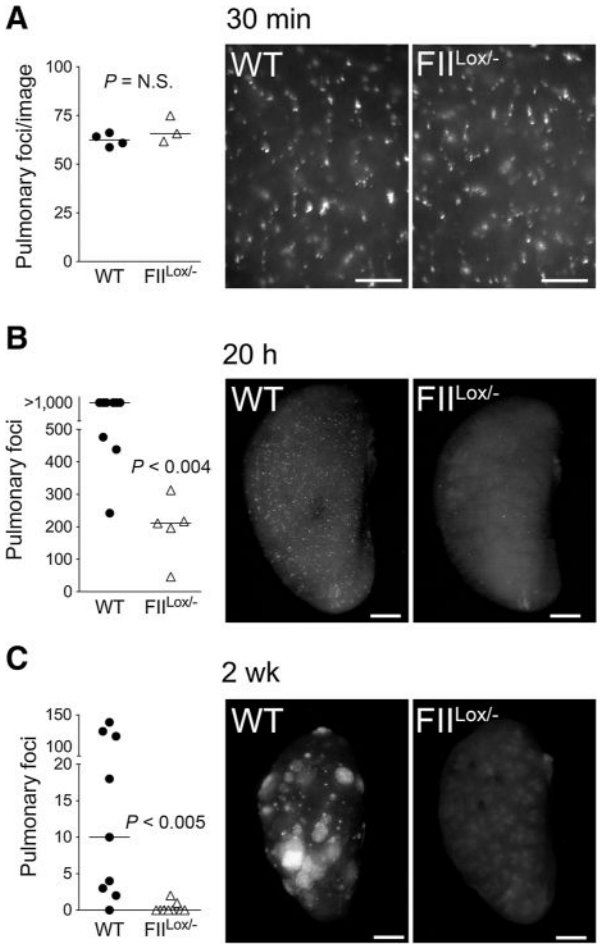

Figure 2.

Prothrombin is a major determinant of the metastatic potential of colon cancer cells. Cohorts of WT and $FII^{Lox/-}$ mice were i.v. injected in parallel via the tail vein with 5×10^5 GFP-expressing MC38 cells. A, shown are representative high-power views of micrometastatic pulmonary foci visualized using a fluorescence-equipped stereomicroscope from WT and $FII^{Lox/-}$ mice 30 minutes after injection. Quantitative analyses of micrometastases from 10 high-powered fields per lung demonstrated similarly abundant foci in both genotypes at this early time point. B, gross appearance of representative lung lobes harvested 20 hours after tumor cell injection. Here, quantitation of total surface pulmonary micrometastases revealed significantly fewer foci in lungs harvested from mice with low prothrombin expression relative to control animals. C, analyses 2 weeks after injection demonstrated multiple macroscopic pulmonary foci in the majority of lungs harvested from WT mice, whereas 8 of 10 lungs harvested from $FII^{Lox/-}$ mice had no discernable metastatic foci whatsoever. Horizontal bars represent medians; size bars, 250 μm (A) or 2 mm (B and C).

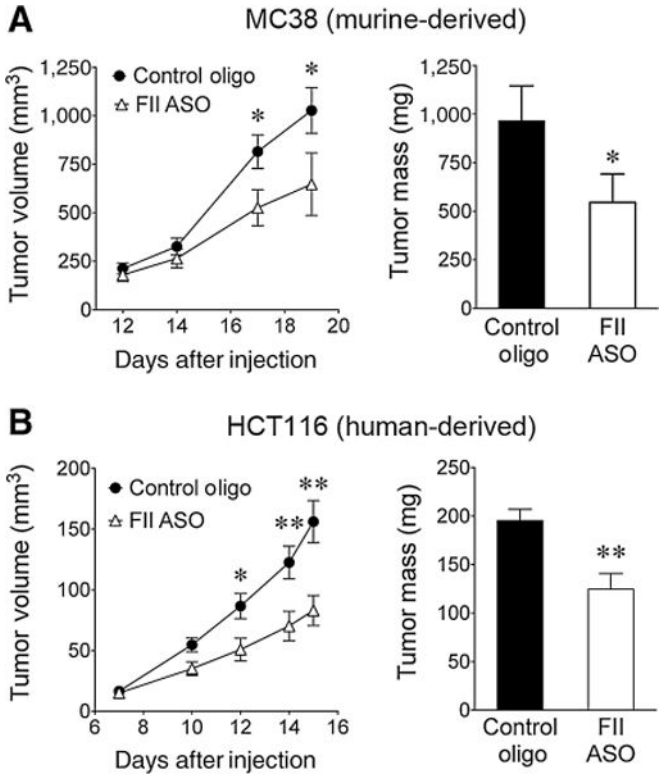

Figure 3.

Pharmacologic inhibition of hepatic prothrombin production impedes murine and human colon cancer growth. A, diminution of prothrombin levels to approximately 5% of normal with a prothrombin-specific ASO (n = 10) significantly impeded the growth of MC38 cells in C57Bl/6 mice relative to administration of a control oligonucleotide with no homology in the murine genome (n = 13) as determined by serial caliper measurements estimating tumor volume and tumor mass determined at sacrifice. B, diminishing prothrombin expression in male athymic nude mice (n = 13) also significantly impeded the growth of the human colon cancer cell line HCT116 relative to administration of a control oligonucleotide (n = 9; *, P < 0.05; **, P < 0.01).

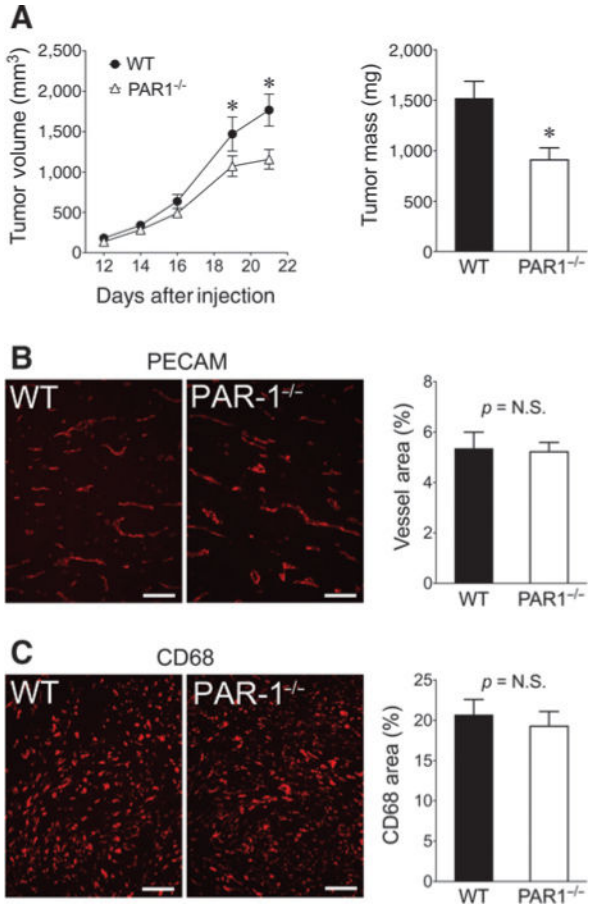

Figure 4.

Stromal cell–associated PAR-1 promotes colon cancer growth. A, comparison of tumor growth following injection of MC38 cells into the dorsal subcutis of WT and PAR1$^{-/-}$ mice; n = 8/cohort; *, $P < 0.05$. B, qualitative tumor vasculature as visualized by immunofluorescence staining using an anti-PECAM antibody as well as quantitative measurements of vascular density based on ImageJ analyses of 10 images per tumor were similar between genotypes. C, qualitative and quantitative analyses of TAMs based on immunofluorescent staining of CD68 were also similar in tumors harvested from WT and PAR1$^{-/-}$ mice. Note that TAMs were quantitated as a function of the total tumor tissue area from 8 images taken from each tumor using ImageJ software; size bars, 100 μm.

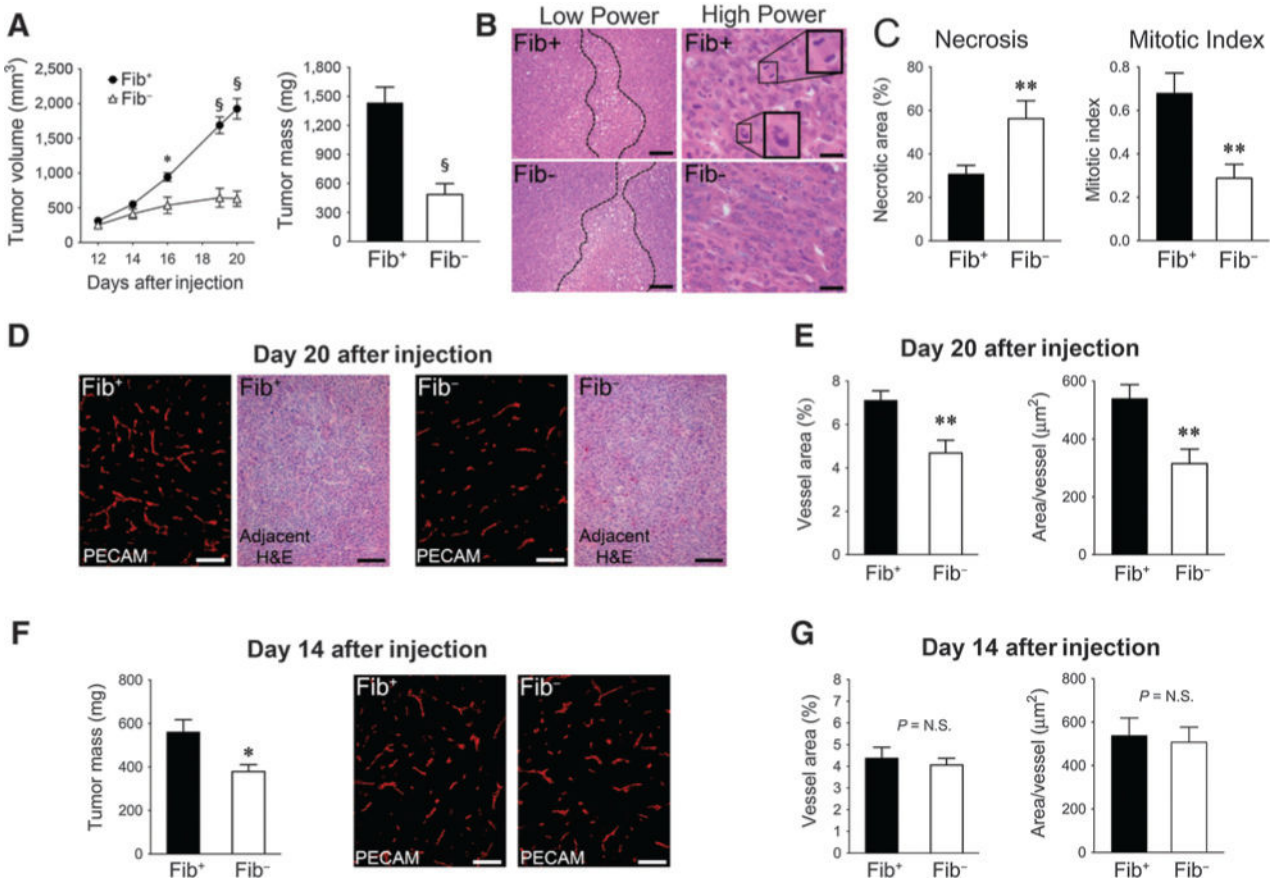

Figure 5.

Fibrinogen promotes tumor progression. A, tumor growth was significantly impeded following injection of MC38 cells into the dorsal subcutis of female Fib^{-} mice ($n = 6$) relative to Fib^{+} mice ($n = 10$). B, shown are representative low- and high-power images of H&E-stained tumor sections harvested from Fib^{+} and Fib^{-} mice 20 days after inoculation. Note that areas of necrosis (dotted line in low power images) were more common in tumors harvested from Fib^{-} mice and that mitotic figures (insets in high power images) were more prevalent in tumors harvested from Fib^{+} mice. C, quantitation of necrotic tumor tissue as a function of tumor tissue surface area analyzed (see Materials and Methods) and mitotic indices confirmed that colonic adenocarcinomas harvested from Fib^{-} mice were more necrotic and less proliferative than tumors from control animals. D, shown are representative sections of tumor tissue harvested 20 days after tumor cell injection immunofluorescently stained for PECAM as well as the adjacent H&E-stained section revealing healthy appearing tumor tissue. E, tumor vascular density and mean vessel size assessed by quantitation of PECAM-staining area from 7 images per tumor using ImageJ software were significantly diminished in tumors harvested from Fib^{-} relative to those harvested from Fib^{+} mice. F, results of a separate experiment showing the mass of MC38 tumors harvested from female Fib^{+} ($n = 8$) and Fib^{-} mice ($n = 7$) 14 days after inoculation. Shown are representative sections of tumor tissue harvested at this earlier time point immunofluorescently stained for PECAM. G, in contrast with analyses performed after a longer period of tumor growth, tumor vasculature density and vessel size were similar in tumors harvested from Fib^{+} and

Fib^{-} mice at this early time point; *, $P < 0.05$; **, $P < 0.01$; §, $P < 0.001$; size bars, 200 μm (B low power, D and F) or 20 μm (B high power).