Colitis-Associated Cancer Is Dependent on the Interplay between the Hemostatic and Inflammatory Systems and Supported by Integrin αMβ2 Engagement of Fibrinogen

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Abstract

A link between colitis and colon cancer is well established, but the mechanisms regulating inflammation in this context are not fully defined. Given substantial evidence that hemostatic system components are powerful modulators of both inflammation and tumor progression, we used gene-targeted mice to directly test the hypothesis that the coagulation factor fibrinogen contributes to colitis-associated colon cancer in mice. This fundamental provisional matrix protein was found to be an important determinant of colon cancer. Fibrinogen deficiency resulted in a dramatic diminution in the number of colonic adenomas formed following azoxymethane/dextran sodium sulfate challenge. More detailed analyses in mice expressing a mutant form of fibrinogen that retains clotting function, but lacks the leukocyte integrin receptor αMβ2 binding motif (Fibγ390-396A), revealed that αMβ2-mediated engagement of fibrin(ogen) is mechanistically coupled to local inflammatory processes (e.g., interleukin-6 elaboration) and epithelial alterations that contribute to adenoma formation. Consistent with these findings, the majority of Fibγ390-396A mice developed no discernable adenomas, whereas penetrance was 100% in controls. Furthermore, the adenomas harvested from Fibγ390-396A mice were significantly smaller than those from control mice and less proliferative based on quantitative analyses of mitotic indices, suggesting an additional role for fibrin(ogen) in the growth of established adenomas. These studies show, for the first time, a unique link between fibrin(ogen) and the development of inflammation-driven malignancy. Given the importance of antecedent inflammation in the progression of numerous cancers, these studies suggest that therapies targeting fibrin(ogen)-αMβ2 interactions may be useful in preventing and/or treating this important subset of malignancies.

Introduction

Chronic inflammation is an established major risk factor in the development and progression of cancer (1). One of the strongest associations between inflammation and cancer is seen in the link between chronic colitis and colon cancer (2). Although a mechanistic link between colitis and colon cancer is generally accepted, the factors that support this process are not fully defined. Several lines of evidence suggest that hemostatic factors, traditionally associated with the maintenance of vascular integrity and control of blood loss, also play a central role in the regulation of inflammatory processes (e.g., bacterial infection, arthritis, neuroinflammatory disease, and wound healing; refs. 3–6). A logical extension of the findings underscoring the importance of hemostatic system components in reparative and inflammatory processes is the hypothesis that hemostatic factors are likely to be significant determinants of the progression of colitis and colon cancer. In support of this notion, colitis and colon cancer in humans are associated with activation of coagulation and an increased risk of thromboembolism (7). Increased plasma fibrinogen and D-dimer levels have been associated with a poor prognosis in patients with colon cancer (8). Furthermore, the immunologic blockade of tissue factor was shown to limit the severity of colitis in mice, implicating thrombin generation in the pathogenesis of colitis (9). However, direct and definitive studies establishing a role of fibrin(ogen) and other targets of thrombin-mediated proteolysis in colitis, colitis-associated colon cancer (CAC), and other inflammation-driven malignancies remain necessary.

The concept that fibrin(ogen) may influence the progression of CAC is compatible with multiple recent studies pointing to a role for hemostatic factors in tumor proliferation, tumor stroma formation, and metastasis in mice (10).
Fibrinogen is particularly intriguing in the context of CAC because of the potential of this key hemostatic protein to influence colitis and CAC through several independent mechanisms. In addition to supporting the local maintenance of vascular integrity, provisional fibrin matrices are likely to support local cell proliferation and tissue reorganization as well as control inflammatory events in the setting of inflammation-induced colonic injury (11). Here, fibrinogen could influence the progression of colitis or CAC through interactions with multiple integrin and nonintegrin receptors that mediate innate immune cell function, cellular proliferation, migration, and apoptosis (e.g., cadherins, αβ3, α3β2, αMβ2, α2β1, α4β1, Toll-like receptors; refs. 4, 12–17). Fibrin matrices are also a prominent feature of many human and murine tumors and have been proposed to promote tumor growth by supporting tumor proliferation, angiogenesis, and the expression of key inflammatory mediators (10, 12, 13).

Here, we directly test the hypothesis that fibrinogen is a determinant of CAC progression in mice challenged with azoxymethane (AOM)/dextran sodium sulfate (DSS). We report that the genetic elimination of fibrinogen significantly diminished colonic adenoma formation. However, fibrin deposition per se was not sufficient to drive CAC. Mice expressing a mutant form of fibrinogen retaining full clotting function but lacking the fibrinogen leukocyte integrin receptor αMβ2 binding motif (Fib390–396Δ) revealed that the mere loss of fibrinogen-αMβ2 interactions was sufficient to dramatically diminish both adenoma formation and growth.

**Materials and Methods**

**Transgenic mice and statistics.** Fibrinogen-deficient and Fib390–396Δ mice were previously described (5, 14). C57Bl/6-derived male mice ages 8 to 12 wk were used in all experiments. Fibrinogen−/− mice were paired with Fibrinogen−/− siblings (referred to as Fib− and Fib−, respectively). Homozygous Fibrinogenγ390–396Δ mice were similarly paired with wild-type mice generated within the same breeding colony (referred to as Fibγ390–396Δ and FibWT, respectively, denoting the protein expressed). Study protocols were approved by the Cincinnati Children’s Hospital Institutional Animal Care and Use Committee in accordance with NIH guidelines. Unless otherwise indicated, data are presented as mean SEM and P values were calculated using the Mann-Whitney U test.

**Induction of colitis and CAC.** Acute colitis was induced using 1.5% DSS (MP Biomedicals) in drinking water for 5 d (15). Chronic inflammation was induced using two cycles of 1.5% DSS (days 1–7 and 21–28). Colitis-associated adenomas were induced by i.p. injection of 10 μg/kg AOM (Midwest Research Institute) on day 0 followed by 1.5% DSS on days 7 to 14 and days 28 to 35 (15). Colon segments were snap frozen in liquid nitrogen homogenized and analyzed using the NIH ImageJ software.

**Measurement of cytokines and MPO activity.** Frozen distal colonos were homogenized in Trizol (Invitrogen) and RNA was extracted. Further purification of RNAs, cDNA synthesis, and real-time PCR were carried out as previously described (6). The data obtained were analyzed using the ΔCT threshold cycle method in which GAPDH was used as the normalizing gene. Cytokine protein levels from cultured colonic explants were measured as previously described (16) using ELISA per the manufacturer’s recommendations (eBioscience). Plasma cytokines were measured using an in vivo cytokine capture assay as described in ref. (19). For the measurement of colonic MPO activity, colon segments were snap frozen in liquid nitrogen, homogenized, and analyzed using an established protocol (6). MPO activity was expressed in units based on dilutions of a known standard MPO preparation from human leukocytes (Sigma-Aldrich).

**Results**

**Fibrinogen supports CAC progression through interaction with the leukocyte integrin αMβ2.** To test the hypothesis that fibrinogen is a determinant of CAC, we challenged cohorts of Fib− and Fib− mice in parallel using a well-established protocol consisting of one i.p. injection of AOM followed by two week-long courses of DSS (15). Despite the obvious bleeding risk associated with colitis and the loss of clotting function (10 of 21 Fib− mice and 16 of 20 Fib− mice exhibited gastrointestinal bleeding), no control animals and just three Fib− mice died, providing an opportunity to formally compare adenoma formation. Analyses of colonic tissue 12 weeks after AOM injection revealed that fibrinogen deficiency significantly diminished adenoma formation. Indeed, a substantial fraction of Fib− mice (35%) exhibited no overt or microscopic evidence of adenomas, whereas control mice uniformly developed adenomas (Fig. 1). The data presented are the combined results of two independent experiments yielding similar outcomes. Adenomas harvested from Fib− mice also tended to be smaller than those from control mice; however, these results did not achieve statistical significance (data not shown). Thus, in addition to an expected role of fibrinogen in the maintenance of vascular integrity following an inflammatory insult, these results suggest that this key hemostatic factor drives colitis-associated adenoma formation/outgrowth.

A detailed microscopic evaluation of tissue sections of each lesion confirmed the classification as adenoma. Adenomas harvested from Fib− and Fib− mice displayed a variable histologic appearance, including low- and high-grade lesions (Fig. 1). Low-grade lesions consisted of a generally well-defined nodular...
thickening of the mucosa characterized by elongated colonic glands with only slight nuclear pleomorphism and no loss of polarity in the mucosal epithelium. High-grade lesions frequently exhibited a loss of cellular orientation with some glands characterized by epithelial cell piling. Colonocytes showed moderate to occasionally severe pleomorphism, including large vesicular nuclei with nucleoli. Adenomas also showed a mixed inflammatory cell infiltrate consisting primarily of neutrophils, macrophages, and lymphocytes in both genotypes (data not shown). Fibrin(ogen) deposition was readily detected in immunohistochemical analyses of adenomas harvested from Fib* mice but was predictably absent in colonic tissue harvested from unchallenged mice or adenomas harvested from Fib* mice (data not shown).

Fibrin(ogen) could promote CAC through several nonmutually exclusive mechanisms, including providing a provisional matrix that supports epithelial proliferation, tumor stroma formation, or local inflammatory processes. Given that fibrin(ogen) interactions with the leukocyte integrin αMβ2 are an important determinant of inflammation in other contexts, we hypothesized that fibrin(ogen) supports adenoma formation/outgrowth in CAC by binding to αMβ2. To test this hypothesis, we initiated comparative studies of CAC in control mice and “knock-in” mice expressing a mutant form of fibrinogen lacking the αMβ2 binding motif on the fibrinogen γ chain (Fibγ390-396A; ref. 5). Fibγ390-396A mice express normal fibrinogen levels and retain normal clotting function and thrombus formation in vivo (5). Therefore, experimental interpretation of CAC findings in Fibγ390-396A mice is simplified over fibrinogen-null mice in that the Fibγ390-396A mutation imposes no inherent bleeding risk (5). Indeed, the loss of the proinflammatory property might be expected to reduce bleeding risk. Consistent with this, parallel analyses of AOM/DSS–challenged cohorts of control and Fibγ390-396A mice revealed that 58% of wild-type mice (11 of 19) developed overt gastrointestinal hemorrhage, whereas bloody diarrhea was observed in appreciably fewer Fibγ390-396A mice (26%, 5 of 19; P < 0.05, Fisher’s exact test). These data represent combined results from two experiments yielding similar outcomes. The comparative findings were even more dramatic when adenoma development was evaluated. Although the phenotypic penetrance of adenoma formation in control mice was 100%, the majority of Fibγ390-396A mice had no discernable adenomas and those that did had few lesions (Fig. 2A). No evidence of microadenomas was observed in either genotype based on histologic analyses of serial colonic sections. Paralleling observations in Fib* mice, a detailed microscopic analysis of each adenoma harvested from FibWT and Fibγ390-396A mice revealed a spectrum of low- and high-grade lesions (Fig. 2B). Immunohistochemical staining of representative sections from each genotype for neutrophils (MPO) and macrophages (F4/80) showed a mixed inflammatory cell infiltrate that was similar in both genotypes (data not shown). Predictably, fibrin(ogen) deposition was readily seen by immunohistochemistry in adenomas harvested from both FibWT and Fibγ390-396A mice (Fig. 2B).

Fibrin(ogen)-mediated αMβ2 engagement supports the proliferation of established adenomas. In addition to a significant diminution in adenoma number, the genetic imposition of Fibγ390-396A resulted in a significant decrease in average adenoma size, resulting in an even more dramatic diminution in the total tumor burden (Fig. 3A and B). The basis for the difference in adenoma size was not readily apparent based on microscopic analyses of adenoma architecture (see above). Furthermore, quantitative comparisons of tumor cell apoptosis using an anti–cleaved caspase-3

**Figure 1.** Fibrin(ogen) supports colitis-associated adenoma formation. A, quantitation of the total number of adenomas observed per animal following AOM/DSS challenge. Note that fibrinogen-deficient mice developed significantly fewer adenomas than control mice challenged in parallel. Shown are representative H&E-stained sections of adenomas harvested from Fib* (B) and Fib* mice (C). High-power views (inset) show the loss of epithelial cell polarity, cell piling, and nuclear pleomorphism typical of adenomas. D, an unchallenged colon polarity in the same plane is shown for comparison. Bars, 100 or 25 μm (insets).
antibody revealed relatively comparable and few positively staining cells in adenomas harvested from both genotypes (data not shown), suggesting that genotype-dependent differences in apoptosis were not responsible for the differences observed in adenoma size. To determine whether inflammatory processes supported by the fibrinogen αMβ2 binding motif resulted in increased adenoma cell proliferation, mitotic indices were quantitated in tissue sections from 16 randomly chosen adenomas harvested from 10 wild-type mice and 14 adenomas harvested from 8 Fibγ390-396A mice. Approximately 1,200 tumor cells from each adenoma were evaluated. The Fibγ390-396A mutation resulted in a significantly lower (~1.8-fold) tumor cell mitotic index relative to control mice (Fig. 3D). Assuming that the length of M phase in tumor cells is a consistent fraction of the cell cycle regardless of fibrinogen status, this would indicate an ~2-fold longer tumor cell doubling time in Fibγ390-396A mice relative to wild-type animals, a difference that would be expected to result in about a 4-fold difference in total progeny tumor cells (tumor mass) generated over a period of just eight doublings. Collectively, these data suggest that alterations in inflammatory cell function within tumor tissues resulting from fibrinogen(ogen)-mediated αMβ2 engagement supports the growth of colonic adenomas. Furthermore, the crucial importance of inflammation in the development of adenomas in this context lends support to the hypothesis that local fibrin-leukocyte interactions through αMβ2 mediate earlier inflammatory processes that support the establishment of adenomas.

The fibrinogen αMβ2 binding motif drives experimental colitis. To define the importance of fibrinogen as a ligand for αMβ2 in the context of the antecedent colitis, we challenged cohorts of Fibγ390-396A and FibWT mice with chronic DSS using a multicourse treatment regimen analogous to that used to induce CAC (see Materials and Methods; ref. 15). Mice were sacrificed on day 28, a time point in which the colitis would be expected to be advanced. The degree of colonic pathology in Fibγ390-396A mice was dramatically diminished relative to control animals based on multiple parameters. Consistent with more severe colitis in DSS-challenged wild-type mice, the majority of Fibγ390-396A mice had no discernable adenomas. This difference was due, at least in part, to differences in individual adenoma size (B). C, mitotic figures (arrowheads) seemed qualitatively more commonplace in adenomas harvested from FibWT mice relative to the Fibγ390-396A cohort. D, quantitative analyses revealed a significantly higher tumor cell mitotic index in adenomas harvested from control mice compared with Fibγ390-396A animals. Bars, 25 μm.

**Figure 2.** Fibrinogen is mechanistically coupled to colitis-associated adenoma formation through the leukocyte integrin αMβ2 binding motif. A, quantitative analysis of the number of adenomas per animal. Note that the majority of Fibγ390-396A mice had no discernable adenomas. B, representative H&E-stained sections of adenomas with typical features associated with cellular transformation shown in the high-power insets. Adenomas harvested from both genotypes exhibited significant fibrinogen deposits (brown) based on immunostaining. Bars, 100 μm (H&E), 50 μm (α-Fib), or 25 μm (insets).

**Figure 3.** Fibrinogen-adenoma formation through the leukocyte integrin αMβ2 binding motif. A, quantitative analysis of the number of adenomas per animal. Note that the majority of Fibγ390-396A mice had no discernable adenomas. B, representative H&E-stained sections of adenomas with typical features associated with cellular transformation shown in the high-power insets. Adenomas harvested from both genotypes exhibited significant fibrinogen deposits (brown) based on immunostaining. Bars, 100 μm (H&E), 50 μm (α-Fib), or 25 μm (insets).
mice, colons harvested from these animals were significantly shorter than those harvested from Fibγ390-396A animals. Colons harvested from control animals had a mean length of 50.6 mm (47–63 mm) compared with colons from Fibγ390-396A mice, which averaged 61.3 mm in length (49–78 mm; P < 0.03). Because colon length was shown to inversely correlate with the severity of inflammation-induced damage (20), this gross parameter inferred an appreciable attenuation of disease in Fibγ390-396A mice. Diminished inflammatory disease was affirmed in Fibγ390-396A animals through microscopic analyses. Colons harvested from FibWT mice exhibited significant inflammatory cell infiltration, large areas of crypt loss and severe ulceration, and substantial inflammatory edema between the mucosal and muscularis layers. In contrast, ulceration and crypt loss were much less severe in Fibγ390-396A mice and overall disease involvement was blunted (Fig. 4A).

Fibrinogen immunostaining of DSS-challenged colons revealed that the extent of fibrinogen deposition correlated with the severity of tissue damage within each genotype. Significant fibrinogen deposition was generally seen in underlying areas of severe ulceration and located primarily between the mucosal and muscularis layers, corresponding to areas of inflammatory edema. Consistent with the overall diminution in tissue damage observed in Fibγ390-396A mice relative to controls, sections harvested from Fibγ390-396A animals had qualitatively less fibrinogen staining (Fig. 4A, insets). As expected, based on the intact hemostatic function of Fibγ390-396A mice, the occasional areas of severe tissue damage/ulceration seen in Fibγ390-396A animals exhibited fibrinogen deposition that was generally comparable with lesions in control animals (data not shown). No appreciable fibrin deposition was observed in unchallenged colons (Fig. 4A). The significant attenuation in disease severity in Fibγ390-396A mice relative to FibWT mice was confirmed using an established, semiquantitative histopathologic scoring system (see Materials and Methods; ref. 16). Fibγ390-396A mice showed significantly diminished disease scores for each individual parameter assessed relative to wild-types, resulting in a dramatic diminution in the overall pathology score (data not shown; Fig. 4B). These experiments were completed twice with similar results.

Consistent with the genotype-dependent differences in inflammatory cell infiltration and epithelial damage, the relative mRNA levels of interleukin-6 (IL-6) and IL-1β, two cytokines known to contribute to colitis severity (21, 22), were significantly diminished in chronically DSS-challenged colons harvested from Fibγ390-396A mice relative to those from control animals based on reverse transcription-PCR (RT-PCR; Fig. 4C). No significant differences in the relative expression of IFN-γ or tumor necrosis factor-α (TNF-α) were observed between genotypes (data not shown). As expected, the mRNA levels for each cytokine were similar in colons harvested from unchallenged FibWT and Fibγ390-396A mice and significantly lower than those in colons harvested from animals after DSS challenge (data not shown; Fig. 4C). Complementary analyses of cytokine expression at the protein level using an established ex vivo colonic explant culture technique (16) essentially mirrored these results (data not shown). Similarly, plasma IL-6 levels measured using an in vivo cytokine capture ELISA assay (19) were significantly elevated in samples harvested from DSS-challenged control mice relative to Fibγ390-396A mice (data not shown). Plasma IFN-γ and TNF-α levels in chronically DSS-challenged mice were not greater than those observed in unchallenged mice (data not shown).

To more fully define the effect of loss of the fibrinogen-α5β1 interaction on the nature of the inflammatory cell infiltrate in chronically DSS-challenged colons, sections of colonic tissue were immunostained for monocytes/macrophages using an F4/80 antibody. Wild-type colons revealed qualitatively more F4/80+ cells than those prepared from Fibγ390-396A animals analyzed in parallel (data not shown). Complementary measurements of neutrophil accumulation into chronically inflamed colonic tissue were made using MPO as a biomarker. MPO activity in colons from DSS-challenged mice of both genotypes was predictably elevated relative to unchallenged animals. More importantly, the MPO activity of colonic tissue harvested from DSS-challenged wild-type mice was ∼5-fold greater than that detected in DSS-challenged colons from Fibγ390-396A animals (Fig. 4D), suggesting substantially genotype-dependent differences in neutrophil accumulation. These experiments were completed twice with similar results.

Fibrinogen (fibrinogen)-α5β1 interactions promote proinflammatory cytokine elaboration and epithelial stress in the early phases of colitis. To examine the role of fibrinogen-α5β1 interactions in the early events in colitis development, we challenged cohorts of Fibγ390-396A and FibWT mice with 1.5% DSS for just 5 days. Analyses of H&E-stained sections of the distal colon revealed no evidence of ulceration or significant crypt loss in either genotype at this early time point (Fig. 5A). When colon sections were evaluated microscopically using a more comprehensive semiquantitative histopathologic scoring system, the total disease scores were predictably low, regardless of genotype. However, the total disease scores were statistically greater in the wild-type cohort relative to Fibγ390-396A mice (Fig. 5B) and driven primarily by genotype-dependent differences in immune cell infiltration. To better determine which inflammatory cells accounted for these differences, macrophage infiltration was quantitated by ImageJ analyses of tissue sections immunofluorescently stained for F4/80 (Fig. 5A). Colons harvested from control mice at this early time point exhibited greater numbers of macrophages relative to colons harvested from Fibγ390-396A mice (Fig. 5C). In contrast, neutrophil infiltration evaluated by the quantitation of MPO-staining cells in colons harvested after 5 days of DSS exposure was indistinguishable between genotypes (Fig. 5A), and was confirmed by the quantitation of MPO activity in colonic extracts (Fig. 5D).

The relatively modest genotype-dependent differences in inflammatory cell infiltration noted early in the process of colitis seemed to belie the much more profound differences noted later in the disease course (see above). An attractive hypothesis is that fibrinogen-mediated differences in inflammatory cell function early in the process of colitis underlie, at least in part, the much more profound genotype-dependent
differences noted later in the disease and ultimately CAC. Notably, an important mechanism of mucosal changes in experimental colitis is cytokine-driven epithelial cell activation and death (16, 18, 23). Consistent with this hypothesis, protein levels of IL-6, IL-1β, IFN-γ, and TNF-α from cultured colonic explants were all 10-fold greater in colons harvested from wild-type mice relative to Fibγγ390-396A mice after only 5 days of DSS exposure (Fig. 6A). The diminished early cytokine production observed in Fibγγ390-396A mice suggests genotype-dependent differences in cytokine stress placed upon epithelial cells early in the disease process.

To investigate this possibility, colon sections from mutant and control animals challenged with 5 days of DSS were immunostained with antibodies to phosphorylated cJun and RelA/p65, which served here as markers of cytokine-induced epithelial cell activation. Epithelial cells staining positive for either marker (Fig. 6B) were significantly elevated in colons harvested from wild-type mice relative to Fibγγ390-396A animals (Fig. 6C).

**Discussion**

These studies establish for the first time that the key hemostatic factor fibrin(ogen) is a determinant of CAC. This provisional matrix protein contributes to adenoma formation through a mechanism that is specifically dependent on the leukocyte integrin αMβ2 binding motif known to control both leukocyte activation events and apoptosis in vivo (24, 25). Fibrin(ogen) supports early inflammatory events (e.g., local elaboration of cytokines and epithelial damage) in AOM/DSS–challenged mice that are coupled to epithelial hyperplasia and secondary transformation events leading to CAC. The presence of fibrin matrices is not sufficient to advance the progression of CAC. Rather, fibrin(ogen)-αMβ2 interactions are critical to promote colitis and CAC, and this seems to be achieved through mechanisms that control both the incidence and ultimate growth of adenomas.

Fibrin matrices are a common feature of many tumors and have been proposed to support tumor stroma formation and

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**Figure 4.** Fibrin(ogen)-mediated engagement of αMβ2 promotes experimental colitis. A, H&E-stained sections of colons harvested from FibWT mice after two courses of DSS revealed large areas of ulceration (arrowhead) and edema (*) as well as significant fibrin(ogen) deposition (brown immunostaining in inset) generally associated with areas of severe epithelial damage. In contrast, colons harvested from Fibγγ390-396A mice generally had intact mucosa and little fibrin(ogen) deposition (inset). An unchallenged colon cut in the same plane is shown for comparison. Bars, 100 μm. B, genotype-dependent differences in colitis histology were confirmed by a multiparameter histopathologic scoring system. The imposition of Fibγγ390-396A resulted in a significant diminution in each disease parameter analyzed. Shown are results for crypt loss and ulceration, the most severe manifestations of colitis. Consequently, Fibγγ390-396A mice showed a dramatically diminished total disease score (bars, median). C, Fibγγ390-396A mice exhibited diminished relative levels of colonic IL-6 and IL-1β mRNA after two courses of DSS based on quantitative RT-PCR (n = 8 per group). D, quantitative analysis of MPO activity in DSS-challenged colons (n = 8 per group).
tumor growth (26). This notion is consistent with studies showing that fibrin(ogen) can control numerous cellular processes important for tumor growth, including cell proliferation, apoptosis, growth factor and cytokine elaboration, and cell migration (26, 27). However, prior studies in fibrinogen-deficient mice established the somewhat enigmatic finding that fibrin(ogen) was entirely dispensable for the robust growth of transplantable tumor cell models (e.g., Lewis lung carcinoma, B16 melanoma; refs. 17, 28). Here, we directly show that fibrin(ogen) is a crucial determinant of tumor development/outgrowth in the context of a distinctly inflammation-driven tumor model developing in situ. Therefore, a primary distinguishing feature determining the fibrin(ogen) dependence of tumor formation/growth in some contexts (e.g., CAC) but not others (e.g., transplantable tumor models) may be the fundamental requirement for local, fibrin-supported inflammatory events. Although inflammatory cells may contribute to the growth of transplantable tumors, this may be driven by the direct production of inflammatory mediators by fully transformed tumor cells independent of peritumoral fibrin (29). An intriguing concept that remains to be fully explored is that fibrin indirectly supports the accumulation of tumor cell transformation events and growth through the control of local inflammation-associated proliferative signals.

The data presented here show that the fibrin(ogen) αMβ2 binding motif promotes early inflammatory events in colitis coupled to the later development/outgrowth of adenomas. Fibrin(ogen)-αMβ2 interactions could support the early inflammatory response in colitis through several mechanisms. Neither fibrin(ogen) nor αMβ2 are strictly required for leukocyte trafficking (5, 6, 30), but multiple studies have shown that fibrin(ogen) and αMβ2 can control key leukocyte activation events, including calcium mobilization, cytokine elaboration, degranulation, and NF-κB–dependent transcription (25, 31, 32). The data presented here are compatible with the concept that genotype-dependent differences in leukocyte activation are primarily responsible for the dramatic differences in cytokine elaboration observed following short-term DSS exposure. Indeed, proinflammatory cytokine elaboration

Figure 5. Early inflammatory cell trafficking in DSS-challenged colons. A, representative H&E and immunofluorescently stained tissue sections of colons harvested from FibWT and Fibγ390–396A mice after 5 d of DSS. Leukocyte infiltrates (arrowhead) were more readily apparent in colons harvested from control mice. The difference in leukocyte infiltration seemed to be primarily due to differences in macrophages (F4/80-staining red cells) rather than neutrophils (MPO-staining green cells). B, the differences observed in H&E-stained sections resulted in a genotype-dependent difference in histopathologic disease scores (n = 8 per group; bars, median). C, qualitative observations regarding macrophage infiltration were confirmed by ImageJ analyses of the percentage of colonic tissue staining for F4/80 (n = 6 per genotype). In contrast, quantitation of MPO activity in the distal colon (D) revealed no significant differences between genotypes (n = 8 per group). As expected, MPO activity in colons from unchallenged mice was similar between genotypes (n = 3 per group) and significantly less than that observed after DSS challenge (P < 0.03). Bars, 100 μm (H&E and α-F4/80) or 50 μm (α-MPO).
mediated by fibrin(ogen)-αMβ2 engagement may be a major factor driving the genotype-dependent differences in colitis severity and adenoma outgrowth observed later in the disease process. Proinflammatory cytokines are known to drive epithelial cell turnover, consistent with the genotype-dependent differences in markers of epithelial cell stress (i.e., phosphorylated cJun and RelA/p65) observed here after short-term DSS exposure. Furthermore, fibrinogen(ogen)-αMβ2-dependent cytokine elaboration would be expected to support secondary leukocyte recruitment, ultimately resulting in the pronounced genotype-dependent differences in inflammatory cell infiltration and epithelial damage observed following long-term DSS exposure.

Although a mechanistic role for fibrinogen-mediated αMβ2 engagement in colitis and CAC coupled to cytokine elaboration seems likely, the relative importance of fibrinogen-supported elaboration of any specific cytokine remains to be determined. The fact TNF-α and IFN-γ were shown to be fibrinogen dependent in the acute but not the chronic phase of colitis, whereas IL-6 and IL-1β were fibrinogen dependent in both phases, does not necessarily represent their relative importance in disease progression. Fluctuations in cytokine levels during the transition from acute to chronic colitis are well described and, on an individual basis, may not always reflect the degree of local histopathology (33, 34). Regardless, the working hypothesis that fibrinogen-mediated proinflammatory cytokine elaboration through αMβ2 engagement is generally crucial in the pathogenesis of CAC is consistent with studies showing that the elimination or inhibition of key inflammatory cytokines significantly diminishes the number and size of adenomas formed after AOM/DSS challenge (35, 36).

The data presented here directly establish that one mechanism coupling fibrinogen to colitis and colon cancer is limited to this single integrin. Fibrinogen(ogen) is a ligand for several other receptors found on a diverse array of cell types (5, 37–41). Many of these interactions are likely to support reparative and inflammatory processes and, thus, could also

![Figure 6](image-url)
contribute to the progression of colitis and CAC. Even traditional fibrinogen-mediated stabilization of occlusive platelet thrombi within highly vascularized inflamed colonic tissues (a common pathologic finding in humans with colitis; ref. 42) could promote the progression of colitis by increasing ischemic tissue necrosis and secondary inflammation. Here, it is notable that mice immunologically depleted of platelets exhibited diminished early leukocyte recruitment in DSS colitis (43). Fibrinogen could also play an important role in colitis by supporting hemostasis and wound healing (11). In this regard, provisional fibrin matrices may have opposing effects on the progression of colitis. Fibrin may simultaneously tend to limit disease by limiting blood loss and supporting tissue repair, and tend to aggravate disease by promoting inflammatory processes and supporting counterproductive vaso-occlusive events. This notion is consistent with the observation presented here that complete fibrinogen deficiency, while limiting adenoma formation, seemed to worsen the bleeding events associated with colitis. Of course, the appreciably more selective elimination of only the fibrin (ogen) αMβ2 binding motif provides both potent anti-inflammatory benefits and a reduction in adenoma formation without the liability of any loss of hemostatic function.

Given that pathologic inflammation has been strongly implicated in the development and progression of multiple malignancies (e.g., cervical, lung, gastric, ovarian, and prostate; ref. 44), it would seem likely that the importance of fibrinogen in tumor development/outgrowth is not limited to colon cancer. Therefore, fibrinogen-αMβ2 interactions represent an attractive therapeutic target for treating and/or preventing a significant number of cancers without incurring the potential bleeding risks associated with anticoagulants or other modalities targeting fibrin deposition.

Disclosure of Potential Conflicts of Interest

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References

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