Mechanisms Linking Hemostatic Factors to Tumor Growth in Mice

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Key Words

Cancer · Coagulation · Fibrinolysis · Thrombosis

Abstract

A persuasive link between hemostatic factors and cancer has been developed through extensive studies of both human and animal malignancies. Coagulation-, platelet- and fibrinolytic-factors all have been tightly associated with tumor growth and/or dissemination. Nevertheless, the mechanisms by which these factors influence tumor growth and progression remain to be fully defined. Recent studies of mice with selected deficits in hemostatic factors have generally supported the hypothesis that hemostatic factors contribute significantly to tumor biology. However, the findings with gene-targeted mice have often been unexpected or tumor model-dependent. Particularly enigmatic was the dual finding that neither fibrin(ogen) nor plasmin(ogen) were significant determinants of Lewis lung carcinoma (LLC) or T241 fibrosarcoma tumor growth following transplantation into the dorsal subcutis of immune-compatible mice. However, follow up studies have revealed that tumor growth is critically dependent on plasminogen when LLC

and T241 tumors cells are transplanted to another anatomical location, the footpad. Furthermore, the negative impact of plasminogen deficiency on footpad tumor growth was effectively relieved by genetically superimposing a deficit in fibrinogen. The likely mechanistic basis for the suppression of tumor growth in plasminogen-deficient mice was revealed through transmission electron microscopy studies of tumor tissues. Widespread microvascular thrombi were found within footpad tumors of plasminogen-deficient mice, whereas no such lesions were observed within tumors from mice that also lacked fibrinogen. These occlusive events were shown to result in a substantial lengthening of tumor cell doubling time in vivo, but increased tumor cell dropout is also likely to contribute to the diminution of footpad tumor growth in plasminogen-deficient mice. Taken together, these studies show that both plasminogen and fibrinogen can serve as critical determinants of tumor growth, but their importance is dependent on anatomical location/microenvironment. Furthermore, one target of plasmin(ogen) relevant to tumor success in vivo is intravascular fibrin.

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Introduction

A critical linkage between hemostatic factors and cancer has been established through detailed studies of both human and animal tumor biology. It is increasingly clear that coagulation system components (e.g., tissue factor, factor X, thrombin, and fibrinogen), fibrinolytic factors (e.g., plasminogen activator, plasminogen activator inhibitor, and plasminogen) and platelets all significantly contribute to tumor success/progression. A particularly compelling link has been made between fibrinogen/fibrin and tumor growth and dissemination. The local deposition of fibrin(ogen) and/or FDPs appears to be a universal and conspicuous characteristic of solid tumors [1]. Tumor cells, endothelial cells, and inflammatory cells are all known to engage fibrin(ogen) through either integrin or non-integrin receptors, and cellular interactions with fibrin(ogen) has been shown to control cell proliferation, migration, apoptosis and the expression of inflammatory mediators [2–5]. Furthermore, both fibrin and FDPs have been shown to support angiogenesis, consistent with the prevailing hypothesis that fibrin and its derivatives may promote tumor stroma formation by mechanisms that are comparable to those employed in normal tissue repair [6]. However, perhaps the most compelling data pointing to a role of fibrin(ogen) in cancer biology has emerged from comparative studies of malignant tumor dissemination in mice with and without fibrinogen [7, 8]. The metastatic potential of tumor cells is greatly diminished, but not eliminated, in fibrinogen-deficient animals. Complementary studies of tumor cell metastasis in mice treated with the thrombin inhibitor, hirudin, have shown that suppression of this key fibrin converting enzyme also results in a major diminution in tumor cell metastatic potential in mice [9]. However, based on the finding that the thrombin inhibitor can further diminish the already low metastatic potential of circulation tumor cells in fibrinogen-deficient mice [7], thrombin probably contributes to tumor cell metastasis through at least one non-fibrinogen target, most likely G-protein coupled protease-activated receptors on platelets and other cell types.

An equally persuasive body of evidence suggests that fibrinolytic factors contributes to tumor growth and progression. The expression of plasminogen activators (PAs), plasminogen activator receptor, and plasminogen activator inhibitor-1 (PAI-1) have been frequently documented within both tumor and stromal cells of malignant cancers [10–13]. Although fibrin might be a significant target related to tumor success, a prevailing hypothesis is that plasmin-mediated proteolysis contributes to the general extracellular matrix degradation required for tumor stroma

formation and tumor cell dissemination. Consistent with this view, detailed studies of mice with deficits in PA or plasminogen have unambiguously shown that there are both physiological and pathological contexts where the plasminogen activation system serves an important biological role *in vivo* independent of fibrinogen/fibrinolysis [14, 15]. Given that plasmin can degrade many common extracellular matrix components, activate protease zymogens (e.g., matrix metalloproteases), and activate latent growth factors, it is possible that plasmin cleavage of multiple non-fibrin targets is biologically important *in vivo*, particularly in the context of complex processes such as tumor development and dissemination.

A causative connection between the plasminogen activation system and tumor growth/dissemination has been suggested from multiple studies using inhibitors and gene-targeted mice [see 12 and 16 for reviews]. However, the findings with genetically modified mice have frequently been unanticipated or tumor-model dependent. Some reports have indicated that either PAI-1 deficiency or PAI-1 overexpression impeded tumor angiogenesis and tumor growth, whereas other equally compelling studies conclude that neither the lack of PAI nor the exuberant expression PAI-1 alters tumor growth [17–21]. The importance of the plasminogen activation system in establishing metastatic potential also appears to vary with experimental setting and route/site (e.g., hematogeneous, lymphatic) of metastatic lesion formation [18, 22–24]. Furthermore, given the substantial body of data available implying that plasmin(ogen) is likely to be important in tumor stroma formation, angiogenesis, and cell invasion, the limited impact of plasminogen deficiency on tumor growth initially observed in the context of both oncogene-driven mammary cancer and Lewis lung carcinoma (LLC) in the dorsal subcutis was an enigma [22, 23]. However, recent studies have revealed that loss of plasminogen dramatically diminished LLC tumor growth in another anatomical location, the footpad. The mechanism underlying this plasminogen- and location-dependent tumor growth remains to be fully explored, but the available data suggest that the restriction of tumor growth in plasminogen-deficient mice results from a failure of vascular patency. Moreover, the available data suggest that at least one target of plasmin relevant to tumor progression *in vivo* is intravascular fibrin.

Results and Discussion

The prevailing view that fibrin deposition and plasmin-mediated proteolysis are important in tumor stroma

formation and growth was significantly challenged by the early findings that robust tumor growth was maintained in mice with genetic deficits in either plasminogen or fibrinogen [7, 8, 22, 23]. However, more recent studies indicate that both plasminogen and fibrinogen are, in fact, important determinants of tumor growth, at least in specific contexts. The growth of both Lewis lung carcinoma (LLC) and T241 fibrosarcoma were shown to be dramatically diminished when transplanted into the footpad, but not the dorsal subcutis, of plasminogen-deficient mice. Furthermore, this impediment in footpad tumor growth in plasminogen-deficient mice was effectively lifted in the absence of fibrin(ogen). The basis for this genotype- and anatomical location-dependent difference in tumor growth was not readily apparent based on either gross or microscopic analysis of tumor tissues. The histological features of tumor tissue collected from both dorsal skin and footpads of plasminogen-deficient mice were similar and unremarkable. Furthermore, the appearance of tumor tissue collected from the footpad of plasminogen-deficient mice was not appreciably different from tumor tissue from control animals when viewed by light microscopy. Regardless of the context, LLC and T241 tumor cells grew as monotonous sheets of cells with relatively modest extracellular matrix and stromal tissue; focal areas of necrosis and small areas of hemorrhage were found, but these features were not obviously different based on genotype. Tumor tissues were well-vascularized with no genotype-related difference in vascular density based on immunohistochemical staining of endothelium. Superficial hemorrhage was more prominent in mice with large tumors in animals of each genotype. However, the restoration of tumor mass in plasminogen-deficient mice lacking fibrinogen was clearly a consequence of increased tumor tissue rather than local bleeding. Immunological analysis of fibrin(ogen) within the LLC footpad tumors of plasminogen-deficient mice indicated that there was relatively sparse fibrin(ogen) deposition, and the limited fibrin(ogen) detected seemed to be primarily peritumoral or associated with small areas of necrosis. This pattern was similar to that observed in tumors grown within the dorsal subcutis, where no major impediment in tumor growth was found in plasminogen-deficient mice. The scant fibrin(ogen) deposition was particularly surprising given the remarkable improvement in footpad tumor growth observed in plasminogen-deficient mice when fibrinogen deficiency was superimposed. Thus, based on histological data, it was not possible to immediately reconcile the spectacular influence on fibrinogen on footpad tumor growth in plasminogen-deficient mice with the level of fibrin deposition.

Fig. 1. Microvascular thrombi are common in LLC footpad tumors derived from plasminogen-deficient mice. Electron microscopic evaluation of footpad tumor vasculature frequently demonstrated occlusive microvascular thrombi (arrowhead in **b**) in tumors derived from plasminogen-deficient mice (**b**), but not tumors collected from control (**a**), $Fib^{-/-}$ (**c**) or $Plg^{-/-}/Fib^{-/-}$ (**d**) mice. The formation of thrombi was not simply the consequence of local necrosis; thrombi in plasminogen-deficient mice were generally found in areas populated with viable tumor cells (*) and where the endothelium (arrows) appeared essentially intact.

Plasminogen is Critical in Maintaining Footpad Tumor Vascular Patency

An important question raised through these initial studies is what feature is distinct about the footpad that makes tumor growth profoundly plasminogen-dependent, whereas tumor growth in the dorsal subcutis using the same tumor line is largely plasminogen-insensitive? One obvious distinction is that footpad tumors are more likely to sustain repeated mechanical trauma secondary to ambulation. Thus, one theory that could explain the observed plasminogen- and location-dependent tumor growth is that, in the context of mechanical trauma, tumor blood supply in plasminogen-deficient mice may be seriously compromised by the formation and persistence of occlusive thrombi. This hypothesis was particularly appealing because relatively modest intravascular fibrin-platelet deposits could significantly limit tumor blood supply and thus dramatically slow tumor growth. Detailed transmission electron microscopy studies revealed that vaso-occlusion is indeed the probable basis for the impediment in tumor growth in the footpad of plasminogen-deficient mice. Occlusive microvascular thrombi were frequently encountered in footpad tumor tissue of plasminogen-deficient mice (fig. 1). These thrombotic lesions were generally in

areas of healthy appearing tumor cells, and were not simply a secondary consequence of tumor necrosis. In contrast, occluded vessels were never encountered within tumor tissue collected from plasminogen-deficient mice if they also lacked fibrinogen, regardless of anatomical location. Thus, the distinct absence of thrombi within the tumor microvasculature of plasminogen-deficient mice lacking fibrinogen directly correlated with the observed restoration of tumor growth.

Tumor Cell Proliferation Rates are Diminished in Plasminogen-Deficient Mice

The presence of microvascular thrombi within footpad tumors of plasminogen-deficient mice, and the secondary restriction in blood supply, is likely to result in a combination of increased tumor cell dropout (necrosis) and slowed tumor cell doubling. Although the precise contribution of tumor cell dropout to overall tumor growth could not be readily established due to the challenge of quantitating 'obliterated cells', genotype-dependent differences in tumor cell cycle times within the footpad appeared to contribute prominently to overall difference in tumor growth; the mitotic index within footpad tumors of plasminogen-deficient mice (~1.1%) was significantly lower than observed within footpad tumors of control and fibrinogen-deficient mice (~1.9%). Assuming that the length of M phase is not sensitive to overall cell cycle time, this would imply that the average tumor cell cycle time in plasminogen-deficient mice is 1.7-fold longer than in control animals. While this difference may seem relatively small, given the exponential expansion of cells, this difference would translate into nearly a 10-fold difference in the total number of progeny cells produced over a period of just 8 doublings for the faster dividing cells. Therefore, much of the 5 to 10-fold difference in footpad tumor growth observed in plasminogen-deficient and control mice over a period of 6 to 13 days could be accounted for by the observed difference in tumor cell proliferation rates. Consistent with the observed restoration of footpad tumor growth in plasminogen-deficient mice following the genetic elimination of fibrinogen, the mitotic index within footpad tumors of plasminogen-deficient mice was restored to that observed in control animals in the absence of fibrinogen.

The available data suggest that tumor growth in the footpad of plasminogen-deficient mice is compromised as a function of mechanical stress (secondary to ambulation) and the formation and persistent of microvascular thrombi that limit tumor blood supply. Of course, the finding that fibrin(ogen) is one important plasmin target related to

tumor growth does not exclude the possibility that plasmin-mediated proteolysis of non-fibrin substrates also contributes to tumor growth. Indeed, biologically significant roles of plasmin outside of fibrinolysis have been firmly established in vivo in both physiological and pathological contexts [14, 15]. However, the available data imply that any benefits (or liabilities) associated with plasmin-mediated proteolysis of non-fibrin targets in the context of footpad tumors are likely to be subtle. The finding that tumor vascular integrity and growth is both plasminogen- and microenvironment-dependent, may provide a partial explanation for the various conflicting reports on tumor growth in mice with deficits in various plasminogen activation system components. Specifically, even subtle differences in the site of tumor formation might result in significant differences in mechanical insults to the tumor tissue, either due to normal activity or the accessibility of the tumor to physical manipulation by the animal. Indeed, any difference in mechanical insult, in combination with a defect in plasmin-mediated proteolysis, could potentially result in substantial differences in vascular patency and tumor growth.

The impediment in footpad tumor growth in plasminogen-deficient mice appears to be the result of the occlusive restriction in nutrient delivery and gas exchange leading to both tumor cell dropout as well as slowed tumor cell proliferation. While this hypothesis may be essentially correct, several additional mechanisms have not been excluded that might also contribute to the diminution in tumor growth, including: (i) diminished in tumor cell retention/seeding, (ii) genotype-dependent differences in the local inflammatory response that control tumor or stromal cell growth and/or survival, and (iii) subtle difference is local vascular architecture. Tumor cell seeding in the footpad remains to be directly compared across genotypes, but it is difficult to imagine how the loss of a key matrix/adhesion protein like fibrin(ogen) would tend to improve tumor cell retention in the footpad of plasminogen-deficient mice and thus restore tumor growth rates. The alternative concept that local differences in inflammatory mediators are coupled to tumor growth in plasminogen-deficient mice is perhaps more attractive in that the generation of these factors may be in part dependent on occlusive events and secondary tissue damage. We found no quantitative genotype-related differences in neutrophil or macrophage infiltrates in our footpad tumor studies, but this does not exclude the possibility of genotype related differences in inflammatory cell *activity*. More detailed studies of leukocyte trafficking and activity, as well as more detailed studies of tumor cell vascular biology, may be revealing.

An important influence of hemostatic factors on tumor growth through a mechanism linked to vascular patency has been established through many previous studies, and even viewed as a potential therapeutic opportunity. A significant number of studies have suggested that targeted thrombosis of tumor vasculature can be used as a means to inhibit the growth of tumors in mice [25–29]. The available data suggest that adjunct therapies focusing on the plasminogen

activation system might be particularly beneficial in suppressing tumor growth under conditions where intravascular thrombosis within tumor tissue can be imposed, pharmacologically or otherwise. Therapeutic strategies designed to restrict tumor vascular patency may limit the need for potentially morbid therapies such as radical surgery, intensive chemotherapy or radiotherapy in controlling and eliminating solid tumors.

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