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Hemostatic factors support metastasis by impeding NK cell-mediated elimination of embolic tumor cells

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A B S T R A C T

Hemostatic factors are critical determinants of tumor cell metastatic potential. Detailed studies of tumor cell metastasis in mice with genetic deficits in key circulating hemostatic factors revealed that deficiencies in prothrombin, fibrinogen, factor XIII and other hemostatic factors greatly reduce the metastatic success of Lewis lung carcinoma (LLC) and other established tumor lines. Metastatic potential was also found to be dramatically reduced in mice with profound deficits in platelet function (e.g., $G\alpha q^{-/-}$ mice), implying that a platelet/fibrin axis supports the metastasis of embolized tumor cells. Recent studies have focused on two fundamental questions: i) what is the interplay between tumor cell-associated tissue factor (TF) and circulating hemostatic factors in establishing metastatic potential; and ii) what are the mechanisms that link the hemostatic system to tumor cell metastatic potential? To develop the means to explore the relationship between tumor cell-associated TF and downstream hemostatic system components, we established hRas-transformed tumor cells from C57Bl/6-derived TF-null embryonic fibroblasts ($TF^{o}/hRas^{+}$ cells). A derivative of these cells was subsequently generated that expresses murine TF (TF+/hRas+ cells) through the stable transfection of a wildtype TF transgene. Both tumor lines supported similar and robust primary tumor growth following subcutaneous transplantation into C57Bl/6 mice. However, $TF^{+}/hRas^{+}$ cells were found to be dramatically more metastatic than $TF^{o}/hRas^{+}$ cells in experimental metastasis assays. Comparative analyses of experimental metastasis following the introduction of $TF^{+}/hRas^{+}$ tumor cells into control and Gαq-deficient mice suggest that tumor cell-associated procoagulant and platelet function are cooperative in establishing metastatic lesions. Recent studies in mice with single and combined deficits in hemostatic factors and NK cells imply that one mechanism whereby hemostatic factors support tumor cell survival/metastasis is by protecting embolized tumor cells from active elimination by natural killer (NK) cells. These studies establish another link between the hemostatic and innate immune systems and suggest that tumor cells exploit hemostatic factors to evade NK cell-mediated immune surveillance mechanisms. These findings also suggest the possibility that therapeutic strategies designed to limit tumor cell association with procoagulants and/or platelets could be effective in restricting tumor cell dissemination.

A substantial body of evidence has accumulated in support of the general hypothesis that both tumor cell-associated and circulating hemostatic factors are important determinants of tumor cell growth and metastasis.[1-6] One early indicator of the linkage between hemostatic factors and malignancy was the finding that the expression of tissue factor by tumor cells dramatically increased the metastatic potential of a variety of tumor cell lines in standard experimental metastasis assays in mice.[7,8] More detailed studies revealed that the extracellular portion of TF that supports fVIIa binding and fX activation is at least one feature of the molecule that is fundamental to supporting tumor cell metastatic success.[8] Inhibitors of TF-mediated thrombin generation (e.g., fVIIai) or direct thrombin inhibitors (e.g., hirudin) were also shown to greatly diminish the metastatic potential of TF-bearing tumor cells, suggesting that thrombin-mediated proteolysis couples TF to tumor cell metastatic potential.[8,9] Recent studies of tumor cell growth and metastasis in gene-targeted mice with deficits in specific hemostatic system components have begun to provide important clues as to which of the many known physiological substrates of thrombin are important mediators of tumor cell metastasis. Comparative studies of C57Bl/6-derived tumor cell lines in immune-competent syngeneic control and fibrinogen-deficient mice revealed that circulating fibrinogen

is one major thrombin substrate that is important in tumor cell metastasis.[2,4] However, other thrombin targets are also likely to contribute to metastasis. This view is supported by multiple observations, including the finding that the pharmacologic inhibition of thrombin further diminishes the already low metastatic potential observed in fibrinogen-deficient mice.[2] It has become increasingly clear that multiple thrombin targets are relevant to tumor cell metastasis *in vivo*, including fibrinogen, factor XIII and protease activated receptors on platelets and other cells.[2, 6-8, 10, 11]

A clear understanding of the role of hemostatic factors in malignancy will require answers to two fundamental questions: i) what is the interplay between tumor cell-associated tissue factor (TF) and circulating hemostatic factors in establishing metastatic potential; and ii) what are the precise mechanisms that link the hemostatic system to tumor cell metastatic potential? Here, we summarize recent work that points to an important link between hemostatic factors and innate anti-tumor immune surveillance mechanisms *in vivo*.[6]

Results and Discussion

Gαq deficiency compromises thrombus formation in vivo and diminishes the metastatic potential of circulating tumor cells

The G protein Gαq is critical to thrombin-mediated platelet activation *in vitro* and platelet thrombus formation *in vivo*. Comparative studies of pulmonary metastasis in control and $G\alpha q^{-/-}$ mice following intravenous injection of either Lewis lung carcinoma or B16 melanoma provided a dramatic demonstration of the importance of platelet activation to malignancy. $G\alpha q^{-/-}$ mice developed two-orders-of-magnitude fewer pulmonary metastases than control animals in experimental metastasis assays. Studies of the more complex context of spontaneous metastasis revealed a similar pattern of reduced metastatic foci within the $G\alpha q^{-/-}$ animals relative to control mice. These studies were recently extended to begin to explore the interplay between tumor cell-associated TF and platelet activation in supporting pulmonary metastasis. Here, hRas-transformed tumor cell lines were generated from tissue factor-null embryonic fibroblasts prepared from C57Bl/6-inbred mice. Derivatives of these TF^{o}/$hRas^{+}$ tumor cells were then generated by stable transfection of murine TF minigenes encoding either wildtype TF (TF^{+}/$hRas^{+}$ cells) or a form of TF lacking the short cytoplasmic tail (TFD^{Δ}tail/$hRas^{+}$cells). Comparative analyses of tumor cell growth following subcutaneous injection indicated that all three tumor cell lines supported robust and comparable primary tumor growth in syngeneic C57Bl/6 mice. However, the capacity to form pulmonary metastases following intravenous injection was found to be far higher with the TF^{+}/$hRas^{+}$ cells relative to the TF^{o}/hRas+ or the $TF^{\Delta-}$tail/$hRas^{+}$ cells. More notably, the metastatic potential of the TF^{+}/$hRas^{+}$ tumor cells was nearly eliminated in mice with a severe defect in platelet activation due to the loss of Gαq. A similar diminution of metastatic potential was observed in comparative studies of TF^{+}/$hRas^{+}$ tumor cells in control and fibrinogen-deficient mice. Thus, tumor cell-associated TF appears to be a determinant of metastasis through a fibrin(ogen)- and platelet-dependent mechanism. A simple hypothesis consistent with these finding is that local, tumor cell-directed thrombus formation increases the chances of metastatic success of circulating tumor cells.

Platelets and fibrinogen support the survival of embolic tumor cells via a mechanism linked to natural killer cells

Given the various challenges confronting tumor cells (e.g., developing a supportive stroma/neovasculature, extravasation, safe transit within the circulation, stabilization within distant vascular beds, transendothelial migration, and secondary tumor growth), fibrin(ogen), platelets, and other hemostatic factors are likely to be beneficial to tumor cells at some steps and a liability at other steps.

Recent studies have shown that one important benefit that the hemostatic system affords to tumor cells is to impede natural killer (NK)-mediated elimination of tumor cells. This was established by short-term tumor cell fate studies and long-term metastasis studies in mice with single and combined deficits in hemostatic factors and NK cells. Specifically, it was shown that the striking diminution in metastatic success observed in fibrinogen- or Gαq-deficient mice relative to control animals was effectively eliminated by either the immunologic or genetic depletion of NK cells. Tumor cell fate studies suggest that the impact of both hemostatic factors and NK cells on tumor cell survival is established within hours of tumor cell entry into the circulation.

The available data provide further evidence for regulatory cross-talk between the hemostatic and innate immune systems and suggest that one mechanism by which tumor cells exploit hemostatic factors is to evade immune surveillance mechanisms.

An important question that remains to be resolved is precisely how hemostatic factors impede NK-mediated clearance of tumor cells.

Several hypotheses are presently under investigation, including: i) tumor cell-associated platelets/fibrin act as a physical barrier between NK and target tumor cells, and/or ii) tumor cell-associated platelets/fibrin pacify NK cells through platelet

immunomodulatory receptors, ligands and/or secreted cytokines. Regardless of the mechanism, one intriguing inference of the available data is that adjunct therapies designed to limit tumor cell-associated procoagulant function could be effective in reducing the risk of metastases in cancer patients.

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