Published in final edited form as:

Cancer Lett. 2011 July 1; 306(1): 106–110. doi:10.1016/j.canlet.2011.02.038.

Endogenous activated protein C is essential for immune-mediated cancer cell elimination from the circulation

GL Van Sluis^{1,2,3,4}, LW Brüggemann⁴, CT. Esmon⁵, PW Kamphuisen¹, DJ Richel⁴, HR Büller¹, CJF van Noorden³, and CA Spek²

¹Department of Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands

²Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam, The Netherlands

³Department of Cell Biology and Histology, Academic Medical Center, Amsterdam, The Netherlands

⁴Department Clinical Oncology, Academic Medical Center, Amsterdam, The Netherlands

⁵Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, and Howard Hughes Medical Institute, Oklahoma City, Oklahoma, USA

Abstract

Fibrinogen and platelets play an important role in cancer cell survival in the circulation by protecting cancer cells from the immune system. Moreover, endogenous activated protein C (APC) limits cancer cell extravasation due to sphingosine-1-phosphate receptor-1 (S₁P₁) and VE-cadherin-dependent vascular barrier enhancement. We aimed to study the relative contribution of these two mechanisms in secondary tumor formation *in vivo*. We show that fibrinogen depletion limits pulmonary tumor foci formation in an experimental metastasis model in C57Bl/6 mice but not in NOD-SCID mice lacking a functional immune system. Moreover, we show that in the absence of endogenous APC, fibrinogen depletion does not prevent cancer cell dissemination and secondary tumor formation in immune competent mice. Overall, we thus show that endogenous APC is essential for immune mediated cancer cell elimination.

Keywords

Activated protein C; coagulation; fibrin; metastasis; thrombin

1. Introduction

Cancer cell dissemination and blood coagulation are related through immune dependent as well as immune-independent mechanisms. Fibrin(ogen) and platelets play a pivotal role in cancer cell survival in the blood stream, providing protection against the host immune system [1-6]. Cancer cell-induced activation of the coagulation cascade by tissue factor (TF) expression leads to fibrin deposition around cancer cells. Subsequently, platelets adhere to

© 2011 Elsevier Ireland Ltd. All rights

Correspondence should be addressed to: C. Arnold Spek, Center for Experimental and Molecular Medicine, H2-257, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands, Phone: +31-20-5667850, Fax: +31-20-6977192, c.a.spek@amc.uva.nl.

Conflicts of Interest Statement None Declared

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

the fibrin-cancer cell complex, thereby inducing thrombin formation which further enhances the formation of the fibrin(ogen) network around cancer cells. The thus-formed complex prevents natural killer (NK) cells, an important component of the innate immune system, from eliminating the cancer cells [4,5].

It is well established that fibrin(ogen) facilitates metastasis, but it is also well known that the more proximal components of the coagulation cascade such as TF and (pro)thrombin are also associated with cancer progression [7,8]. The downstream haemostatic constituents such as fibrin(ogen) and FXIII, have an immune dependent effect on circulating cancer cells, whereas both tumor cell-associated TF and circulating prothrombin are crucial determinants of early cancer cell survival even in the absence of the immune system [5,7,9]. This immune- and thus fibrin(ogen)-independent pro-metastatic effect of thrombin is due to several pro-metastatic and pro-angiogenic effects of thrombin [10-13]. For instance, thrombin induces vascular endothelial leakage through protease activated receptor (PAR) 1 activation on endothelial cells diminishing vascular endothelial (VE)-cadherin expression [14,15]. However, thrombin also induces the activation of the natural anticoagulant protein C. This is particularly relevant, as we recently showed that the cell signaling effects of endogenous APC are essential for the protection against cancer cell-induced vascular leakage and subsequent cancer cell extravasation [2]. Indeed, blocking endogenous APC increased the number of pulmonary tumour foci due to loss of S₁P₁-mediated VE-cadherin-dependent vascular barrier protection.

The blood coagulation cascade in general and thrombin in particular thus plays a dual role in cancer cell extravasation. Thrombin-dependent fibrin formation protects circulating cancer cells from elimination by the immune system, whereas thrombin-dependent APC generation is crucial for barrier protection thereby limiting cancer cell extravasation. The relative importance of these pro- and anti-metastatic effects of thrombin remains to be elucidated *in vivo*. Hence, we aimed to evaluate the effects of fibrin(ogen) on B16F10 cancer cell extravasation and pulmonary tumor formation in the absence or presence of endogenous APC.

2. Materials and Methods

Cells and cell culture

Murine B16F10 melanoma cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in Dulbecco Modified Eagle Medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (Sigma-Aldrich, St Louis, MO), 1% penicillin-streptomycin solution and L-glutamine at 37°C. Single cell suspensions were prepared from 0.02% EDTA-treated monolayers which were washed and diluted in phosphate-buffered saline (PBS) prior to counting and inoculation. Cells were stored on ice until injection.

Animals

Eight to ten week-old C57Bl/6 or severe combined immunodeficient (NOD-SCID) mice (Charles River, Maastricht, The Netherlands) were maintained at the animal care facility of the Academic Medical Centre, Amsterdam, The Netherlands according to institutional guidelines. Animal procedures were carried out in compliance with Institutional Standards for Humane Care and Use of Laboratory Animals. The institutional Animal Care and Use Committee approved all experiments.

Experimental pulmonary metastasis model

Cancer cells (3.5×10^5) suspended in 200 μ l PBS were injected into the lateral tail vein as described before [16-18]. After 14 days, mice were sacrificed and lungs were prepared as before [2]. Tumor foci on the surface of the lungs were counted macroscopically using a binocular in a blinded fashion with respect to the intervention. Experiments were performed with 8 mice per group, however mice that appeared to have had inadequate cancer cell inoculation as documented at the time of injection were excluded from further analysis.

Monoclonal antibodies and fibrinogen depletion

The anticoagulant and signaling properties of endogenous APC were blocked using the MPC1609 monoclonal antibody and the anticoagulant properties of endogenous APC were blocked using the MAPC1591 antibody as described previously [2,19]. A class matched antibody MCO1716 that is targeted against the keyhole limpet hemocyanin (KLH) protein was used as negative control. The various antibodies were injected intraperitoneally (200 μ g in 0.9% NaCl) 30 min before cancer cell inoculation. Antibody administration was repeated at 48 and 96 h after cancer cell inoculation. Depletion of fibrinogen was achieved with an intravenous injection of venom derived ancrod (Sigma-Aldrich, St Louis, MO) which cleaves only the A-chains of fibrinogen, producing soluble degradation products that are removed from the circulation [20,21]. Ancrod was given 1.5 h before cancer cell inoculation in a dose of 0.5 U per mouse as such a dose was previously shown to efficiently deplete fibrinogen levels at [22,23].

Statistical analysis

Statistical analysis was carried out in GraphPad Prism version 4.03 supplies. Data are expressed as mean $\pm$ SEM. For normally distributed data, significance was assessed with the Student t-test. For not normally distributed data, non-parametric testing was performed using the Mann-Whitney test. Multiple comparisons were performed using one-way ANOVA with the Bonferroni test for selected pairs of columns as a post test.

3. Results

Fibrinogen depletion reduces pulmonary melanoma tumor formation only in immune-competent mice

To confirm the effect of fibrinogen on NK cell-mediated cancer cell elimination, intravenous injection of B16F10 melanoma cells into the lateral tail vein of C57Bl/6 mice was combined with fibrinogen depletion by intravenous ancrod administration. The number of pulmonary tumor foci was markedly reduced due to ancrod treatment, as is shown in Figure 1A. To assess the role of the immune system in this process, we repeated the experiment in immune compromised NOD-SCID (mice that lack T cells, B cells, NK cells and circulating complement) and found no significant effect of fibrinogen depletion on pulmonary tumor foci formation after 14 days (Figure 1B).

Fibrinogen depletion reduces pulmonary melanoma tumor formation only in the presence of endogenous APC

We recently reported that blocking endogenous APC enhances pulmonary foci formation in the lung of C57Bl/6 mice through aggravation of cancer cell-induced vascular leakage. Indeed, treatment with anti-PC monoclonal antibody MPC1609 (blocks both anticoagulant and signaling properties) again resulted in a highly significant increased number of pulmonary tumor foci, whereas anti-APC mAb MAPC1591 antibody (blocks anticoagulant properties only) treatment resulted in a similar number of pulmonary tumor foci as in the control group, indicating that the signaling properties of APC rescue cancer cell

extravasation and secondary tumor formation in the lung. In order to assess the relevance of fibrin(ogen) in the absence of endogenous APC, we combined fibrinogen depletion (through anicrod administration) with APC antibody administration before intravenous injection of B16F10 melanoma cells. As shown in Figure 2, fibrinogen depletion no longer reduced the number of pulmonary tumor foci in the presence of either MPC1609 or MAPC1591. In the absence of the anticoagulant properties of endogenous APC, the presence/absence of fibrinogen is irrelevant, emphasizing the importance of APC as an anti-metastatic agent.

4. Discussion

The blood coagulation cascade in general, and thrombin in particular, plays a dual role in cancer progression. Thrombin-dependent fibrin formation protects circulating cancer cells from immune system-dependent clearance, whereas thrombin-dependent APC generation is crucial for barrier protection thereby limiting cancer cell extravasation. We aimed to assess the relative importance of these pro- and anti-metastatic effects of thrombin *in vivo*. Hence, we evaluated the effect of fibrin(ogen) on B16F10 melanoma cancer cell extravasation and secondary tumor formation in the presence or absence of endogenous APC.

Anicrod-dependent fibrinogen depletion dramatically reduced the number of B16F10 pulmonary tumor foci in C57Bl/6 mice. These data are in perfect agreement with previous experiments using fibrinogen null mice thereby confirming the previously shown importance of fibrinogen in experimental metastasis [5,9,24,25] but also showing the efficacy of our anicrod treatment protocol. Moreover, our data showing that anicrod treatment does not limit pulmonary tumor formation of B16F10 cells in NOD-SCID mice confirm and extend previous findings showing that the prometastatic effect of fibrinogen is immune dependent [5,9,24,25]. As previous studies showed that fibrinogen does not affect experimental metastasis of Lewis lung cell carcinoma cells in NK-deficient mice (Ly49A TG mice [26]) [5,9], it is tempting to speculate that experimental metastasis is fibrinogen independent in NOD-SCID due to the absence of functional NK cells in these mice. However, NOD-SCID mice also lack functional T- and B-cells and circulating complement, and a role of these innate immune cells can thus not be excluded. Indeed, the number of B16F10 experimental metastasis is significantly reduced in fibrinogen null mice on a SCID background (i.e. mice that lack T- and B-cells but not NK-cells), although not as efficient as in fibrinogen nulls on a C57B/6 background (4- versus 33-fold reduction in tumor nodules) [24]. The rate limiting role of fibrinogen in immune mediated cancer cell elimination seems thus largely, but not exclusively dependent, on NK-cells.

Importantly, in a model of experimental metastasis, we show that fibrinogen is no longer involved in pulmonary tumor formation in the absence of endogenous APC. The fact that fibrinogen depletion is no longer effective in the absence of APC underlines the crucial role of endogenous APC in preserving the vascular endothelial barrier for the immune system in order to effectively attack cancer cells in the circulation. Remarkably, both the MPC1609 and MAPC1591 antibodies abolish the effect of fibrinogen depletion suggesting that it is not dependent on the signaling properties of APC, but is brought about by its anticoagulant function.

The rather unexpected finding that fibrinogen depletion had no effect in the absence of APCs anticoagulant activity in the MAPC1591 treated animals points at the role of thrombin generation and consequently high thrombin levels in this phenomenon. Indeed, thrombin is known to be barrier disruptive at high levels through PAR-1-dependent sphingosine-1-phosphate receptor-3 (S₁P₃) mediated signaling [8,27]. It is thus tempting to speculate that cancer cell-induced thrombin generation leads to disruption of the endothelial barrier

thereby providing them an alternative mechanism to escape from the host immune system [27,28].

Our results confirm and extend the notion that the endogenous APC pathway plays a potentially crucial clinical and pharmacological role in cancer progression. Next to our previous data on the barrier-protective effects of endogenous APC [2], it was recently shown that repeated administration of exogenous human APC reduced the number of experimental metastasis in mice [29]. Moreover, the same report showed a protective effect of endothelial protein C receptor (EPCR) on experimental metastasis as EPCR overexpressing mice exhibited marked reductions in liver metastases from B16F10 cells compared with wild-type animals. Interestingly, acquired protein C deficiency is observed in cancer patients, especially those using certain types of chemotherapy [30-32]. Importantly, these patients do not only have low APC levels but are also hypercoagulable resulting in enhanced fibrin formation (which is pro-metastatic as it protects cancer cells from the immune system) as well as S₁P₃-mediated vascular barrier disruption. Consequently, preservation and/or restoration of endogenous APC generation may be an interesting target to combat cancer progression as it prevents endothelial barrier disruption via a S₁P₁-dependent mechanism but it also prevents fibrin formation and subsequent protection of cancer cells from the immune system and it prevents thrombin-induced barrier disruption via the S₁P₃ pathway.

Our suggestion that one should preserve or restore endogenous protein C levels in cancer patients may be particularly relevant during anticoagulant treatment. Although anticoagulant treatment has proven to be beneficial in both clinical as well as experimental studies [33,34], it is well-known that anticoagulants also affect endogenous APC generation. At least *in vitro*, it has been suggested that not all anticoagulants affect APC generation to a similar extent [35-37].

Several issues should be kept in mind when interpreting our data. First, the experimental metastasis model used has certain limitations. Although it is routinely used [17,18], it mimics the pathophysiology of the metastatic process only partially since cancer cells are inoculated directly into the bloodstream and are not derived from a primary tumor. This model thus selectively assesses the extravasation step of cancer cell metastasis [38] and allows studies on extravasation without confounding treatment effects on the primary tumor. Importantly, B16F10 cells specifically extravasate in the lung vasculature [38] mimicking the clinical setting in which melanoma preferentially metastasize to the lung. Thus, although our model is appropriate to study cancer cell extravasation, subsequent experiments should validate our findings in spontaneous metastasis models. Second, we used NOD-SCID mice in our experiments focusing on the immune dependence of our results. Although these mice are clearly immunodeficient, a certain level of remnant NK activity has been observed in NOD-SCID mice [39]. However, the observed difference in the number of pulmonary tumors in C57Bl/6 and NOD-SCID mice in the presence or absence of fibrin(ogen) is in agreement with previous experiments showing an important immune-dependent role of fibrin(ogen) [4,5].

In conclusion, our data confirm that fibrinogen depletion prevents pulmonary tumor formation in the B16-melanoma model in an immune-dependent manner. Moreover, fibrinogen depletion prevents pulmonary tumor formation in the B16 melanoma model only in the presence of endogenous APC. The APC pathway seems thus an interesting clinical target to reduce cancer progression and metastasis.

Acknowledgments

C.T.E. is an investigator of the Howard Hughes Medical Institute and received grant support of the Leducq Foundation. We kindly acknowledge the carefully performed animal experiments performed by Joost Daalhuisen

and Marieke ten Brink of the Center for Experimental and Molecular Medicine of the Academic Medical Center in Amsterdam.

References

1. Zheng S, Shen J, Jiao Y, Liu Y, Zhang C, Wei M, Hao S, Zeng X. Platelets and fibrinogen facilitate each other in protecting tumor cells from natural killer cytotoxicity. *Cancer Sci.* 2009; 100:859–865. [PubMed: 19302289]
2. Van Sluis GL, Niers TM, Esmon CT, Tigchelaar W, Richel DJ, Buller HR, van Noorden CJ, Spek CA. Endogenous activated protein C limits cancer cell extravasation through sphingosine-1-phosphate receptor 1 mediated vascular endothelial barrier enhancement. *Blood.* 2009; 114:1968–1973. [PubMed: 19571314]
3. Buller HF, van Doormaal FF, van Sluis GL, Kamphuisen PW. Cancer and thrombosis: from molecular mechanisms to clinical presentations. *J Thromb Haemost.* 2007; 5:246–254. [PubMed: 17635733]
4. Palumbo JS, Barney KA, Blevins EA, Shaw MA, Mishra A, Flick MJ, Kombrinck KW, Talmage KE, Soury M, Ichinose A, Degen JL. Factor XIII transglutaminase supports hematogenous tumor cell metastasis through a mechanism dependent on natural killer cell function. *J Thromb Haemost.* 2008; 6:812–819. [PubMed: 18315549]
5. Palumbo JS, Talmage KE, Massari JV, La Jeunesse CM, Flick MJ, Kombrinck KW, Jirouskova M, Degen JL. Platelets and fibrinogen increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. *Blood.* 2005; 105:178–185. [PubMed: 15367435]
6. Nieswandt B, Hafner M, Echtenacher B, Mannel DN. Lysis of Tumor Cells by Natural Killer Cells in Mice Is Impeded by Platelets. *Cancer Res.* 1999; 59:1295–300. [PubMed: 10096562]
7. Palumbo JS. Mechanisms linking tumor cell-associated procoagulant function to tumor dissemination. *Semin Thromb Hemost.* 2008; 34:154–160. [PubMed: 18645920]
8. Ruf W. Redundant signaling of tissue factor and thrombin in cancer progression? *J Thromb Haemost.* 2007; 5:1584–1587. [PubMed: 17663729]
9. Palumbo JS, Talmage KE, Massari JV, La Jeunesse CM, Flick MJ, Kombrinck KW, Hu Z, Barney KA, Degen JL. Tumor cell-associated tissue factor and circulating hemostatic factors cooperate to increase metastatic potential through natural killer cell-dependent and-independent mechanisms. *Blood.* 2007; 110:133–141. [PubMed: 17371949]
10. Nierodzik ML, Plotkin A, Kajumo F, Karpatkin S. Thrombin stimulates tumor-platelet adhesion in vitro and metastasis in vivo. *J Clin Invest.* 1991; 87:229–236. [PubMed: 1845869]
11. Hu L, Roth JM, Brooks P, Ibrahim S, Karpatkin S. Twist Is Required for Thrombin-Induced Tumor Angiogenesis and Growth. *Cancer Res.* 2008; 68:4296–4302. [PubMed: 18519689]
12. Nierodzik ML, Kajumo F, Karpatkin S. Effect of Thrombin Treatment of Tumor Cells on Adhesion of Tumor Cells to Platelets in Vitro and Tumor Metastasis in Vivo. *Cancer Res.* 1992; 52:3267–3272. [PubMed: 1596884]
13. Hayakawa Y, Kurimoto M, Nagai S, Kurosaki K, Tsuboi Y, Hamada H, Hayashi N, Endo S. Thrombin-induced cell proliferation and platelet-derived growth factor-AB release from A172 human glioblastoma cells. *J Thromb Haemost.* 2007; 5:2219–226. [PubMed: 17958740]
14. Niessen F, Furlan-Freguia C, Fernandez JA, Mosnier LO, Castellino FJ, Weiler H, Rosen H, Griffin JH, Ruf W. Endogenous EPCR/aPC-PAR1 signaling prevents inflammation-induced vascular leakage and lethality. *Blood.* 2009; 113:2859–2866. [PubMed: 19141861]
15. Konstantoulaki M, Kouklis P, Malik AB. Protein kinase C modifications of VE-cadherin, p120, and beta-catenin contribute to endothelial barrier dysregulation induced by thrombin. *Am J Physiol Lung Cell Mol Physiol.* 2003; 285:L434–L442. [PubMed: 12740216]
16. Bruggemann LW, Versteeg HH, Reitsma PH, Spek CA. High factor VIIa levels do not promote tumor metastasis. *Thromb Haemost.* 2008; 99:787–788. [PubMed: 18392341]
17. Bruggemann LW, Versteeg HH, Niers TM, Reitsma PH, Spek CA. Experimental melanoma metastasis in lungs of mice with congenital coagulation disorders. *J Cell Mol Med.* 2008; 12:2622–2627. [PubMed: 18363839]

18. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer*. 2003; 3:453–458. [PubMed: 12778135]
19. Xu J, Ji I, Zhang X, Drake M, Esmon CT. Endogenous activated protein C signaling is critical to protection of mice from lipopolysaccharide induced septic shock. *J Thromb Haemost*. 2009; 7:851–856. [PubMed: 19320827]
20. Ashford A, Ross JW, Southgate P. Pharmacology and toxicology of a defibrinating substance from Malayan pit viper venom. *Lancet*. 1968; 1:486–489. [PubMed: 4170830]
21. Hatton MWC, Ross B, Southward SMR, DeReske M, Richardson M. Pretreatment of rabbits with either hirudin, anicrod, or warfarin significantly reduces the immediate uptake of fibrinogen and platelets by the deendothelialized aorta wall after balloon-catheter injury in vivo. *Arterioscler Thromb Vasc Biol*. 1998; 18:816–824. [PubMed: 9598842]
22. Xu H, Ploplis VA, Castellino FJ. A coagulation factor VII deficiency protects against acute inflammatory responses in mice. *J Pathol*. 2006; 210:488–4896. [PubMed: 17054310]
23. Pawlinski R, Pedersen B, Schabbauer G, Tencati M, Holscher T, Boisvert W, Andrade-Gordon P, Frank RD, Mackman N. Role of tissue factor and protease-activated receptors in a mouse model of endotoxemia. *Blood*. 2004; 103:1342–1347. [PubMed: 14576054]
24. Camerer E, Qazi AA, Duong DN, Cornelissen I, Advincula R, Coughlin SR. Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. *Blood*. 2004; 104:397–401. [PubMed: 15031212]
25. Palumbo JS, Kombrinck KW, Drew AF, Grimes TS, Kiser JH, Degen JL, Bugge TH. Fibrinogen is an important determinant of the metastatic potential of circulating tumor cells. *Blood*. 2000; 96:3302–3309. [PubMed: 11071621]
26. Kim S, Iizuka K, Aguila HL, Weissman IL, Yokoyama WM. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc Natl Acad Sci U S A*. 2000; 97:2731–2736. [PubMed: 10694580]
27. Niessen F, Schaffner F, Furlan-Freguia C, Pawlinski R, Bhattacharjee G, Chun J, Derian CK, Andrade-Gordon P, Rosen H, Ruf W. Dendritic cell PAR1-S1P3 signalling couples coagulation and inflammation. *Nature*. 2008; 452:654–658. [PubMed: 18305483]
28. Riewald M, Petrovan RJ, Donner A, Ruf W. Activated protein C signals through the thrombin receptor PAR1 in endothelial cells. *J Endotoxin Res*. 2003; 9:317–321. [PubMed: 14577849]
29. Bezuhly M, Cullen R, Esmon CT, Morris SF, West KA, Johnston B, Liwski RS. Role of activated protein C and its receptor in inhibition of tumor metastasis. *Blood*. 2009; 113:3371–3374. [PubMed: 19188668]
30. Mewhort-Buist TA, Liaw PC, Patel S, Atkinson HM, Berry LR, Chan AKC. Treatment of endothelium with the chemotherapy agent vincristine affects activated protein C generation to a greater degree in newborn plasma than in adult plasma. *Thromb Res*. 2008; 122:418–426. [PubMed: 18206217]
31. Woodley-Cook J, Shin LYY, Swystun L, Caruso S, Beaudin S, Liaw PC. Effects of the chemotherapeutic agent doxorubicin on the protein C anticoagulant pathway. *Mol Cancer Ther*. 2006; 5:3303–3311. [PubMed: 17172434]
32. Sampson MT, Kakkar AK. Coagulation proteases and human cancer. *Biochem Soc Trans*. 2002; 30:201–207. [PubMed: 12023851]
33. Hu L, Lee M, Campbell W, Perez-Soler R, Karparkin S. Role of endogenous thrombin in tumor implantation, seeding, and spontaneous metastasis. *Blood*. 2004; 104:2746–2751. [PubMed: 15265791]
34. Lazo-Langner A, Goss GD, Spaans JN, Rodger MA. The effect of low-molecular-weight heparin on cancer survival. A systematic review and meta-analysis of randomized trials. *J Thromb Haemost*. 2007; 5:729–737. [PubMed: 17408406]
35. Mattsson C, Menschik-Lundin A, Nylander S, Gyzander E, Deinum J. Effect of Different Types of Thrombin Inhibitors on Thrombin/Thrombomodulin Modulated Activation of Protein C In Vitro. *Thromb Res*. 2001; 104:475–486. [PubMed: 11755958]
36. Linder R, Blombäck M, Egberg N, Grip L L. Thrombin Inhibitors Suppress the Thrombin-Thrombomodulin-Mediated Generation of Activated Protein C. *Thromb Res*. 1999; 95:117–125. [PubMed: 10418800]

37. Linder R, Frebelius S, Jansson K, Swedenborg J. Inhibition of endothelial cell-mediated generation of activated protein C by direct and antithrombin-dependent thrombin inhibitors. *Blood Coagul Fibrinolysis*. 2003; 14:139–146. [PubMed: 12632023]
38. Crissman JD, Hatfield J, Schaldenbrand M, Sloane BF, Honn KV. Arrest and extravasation of B16 amelanotic melanoma in murine lungs. A light and electron microscopic study. *Lab Invest*. 1985; 53:470–478. [PubMed: 4046557]
39. Lin Y, Chen Y, Zeng Y, Wang T, Zeng S. Lymphocyte phenotyping and NK cell activity analysis in pregnant NOD/SCID mice. *J Reprod Immunol*. 2005; 68:39–51. [PubMed: 16309747]

Figure 1. Effect of fibrinogen depletion on the number of B16F10 pulmonary tumor foci in C57Bl/6 and NOD-SCID mice

C57Bl/6 (A) or NOD-SCID (B) mice were treated intravenously with 0.5 U ancrod to eliminate fibrinogen (black bar) or saline as control (white bar) at 90 min prior to the administration of 3.5×10^5 B16F10 melanoma cells into the lateral tail vein. Mice were sacrificed 14 days after cancer cell injection and the number of tumor foci at the surface of the lungs was determined. Error bars represent means $\pm$ SEM (n= 6-8), ** p<0.005.

Figure 2. Fibrinogen depletion prevents pulmonary melanoma tumor formation only in the presence of endogenous APC

C57Bl/6 mice were treated intraperitoneally with 200 μ g of either an antibody that blocks both anticoagulant and signalling properties of APC (MPC1609), an antibody that only blocks the anticoagulant activity of APC (MAPC1591) or an irrelevant antibody (MCO1716) at 30 min prior to the administration of 3.5×10^5 B16F10 melanoma cells into the lateral tail vein. Antibody administration was repeated at 48 and 96 h after cancer cell inoculation. Half of the mice were also intravenously treated with 0.5 U ancrod to eliminate fibrinogen 90 min prior to cancer cell inoculation (black bars). Mice were sacrificed at 14 days after cancer cell inoculation and the number of tumor foci at the surface of the lungs was determined. Error bars represent means $\pm$ SEM (n = 6-8), *, p<0.01.