Coagulation, angiogenesis and cancer
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Metastasis promoting effects of thrombin inhibitors

Abstract

Both animal and clinical studies show that components of the blood coagulation system contribute to metastasis of cancer. Various anti-thrombotic agents targeting different coagulation factors diminish lung metastasis in pre-clinical models. Interestingly, specific thrombin inhibitors, such as hirudin, inhibit metastasis most efficiently. However, hirudin has a narrow therapeutic window compromising its safety. Consequently, novel direct thrombin inhibitors are being developed. One of these novel thrombin inhibitors is the orally-active reversible inhibitor ximelagatran. We aimed to assess the antimetastatic activity of long term treatment with ximelagatran and hirudin in a mouse pulmonary melanoma metastasis model. Ximelagatran was administered via food pellets and hirudin was administered subcutaneously and the treatment groups were compared to controls. After 7 days of treatment, B16 melanoma cells were injected into the tail vein. Twenty-one days after cancer cell inoculation, mice were sacrificed and lung metastases were counted. Surprisingly, ximelagatran and hirudin treatment both increased the number of lung metastasis compared to the control group significantly. It is concluded that long-term thrombin inhibition is detrimental in a murine model of pulmonary metastasis and increases the number of pulmonary tumor foci. Therefore, antithrombotic therapy may promote tumor metastasis suggesting that anticoagulant treatment of cancer patients should be pursued with great care.
Introduction

Accumulating evidence suggests that coagulation activation is not just an epiphenomenon in cancer but also contributes to cancer progression. Tissue factor (TF), often highly expressed on cancer cells, is the key factor in cancer-induced coagulation resulting in the formation of thrombin and subsequently fibrin deposits. Preclinical studies demonstrated antimetastatic effects of blood coagulation inhibitors such as, low molecular weight heparins (LMWH) and Hirudin\textsuperscript{1,2} and several clinical studies suggested a survival benefit in cancer patients treated with LMWH\textsuperscript{3,9}. The precise mechanisms by which LMWH and hirudin affect cancer progression are still unclear. Various components of the coagulation system, such as TF\textsuperscript{10}, factor Xa\textsuperscript{11}, thrombin\textsuperscript{12}, and fibrinogen\textsuperscript{13} are likely involved in cancer progression. These cancer-promoting activities are induced by blood coagulation-dependent as well as coagulation-independent mechanisms, like the induction and activation of selectins and integrins on platelets and endothelial cells and activation of protease-activated receptors (PARs) on platelets, cancer cells, and endothelial cells\textsuperscript{9,14-18}.

In animal tumor models, thrombin has several functions during the initial phases of cancer cell dissemination. Thrombin stimulates the formation of fibrin-rich platelet tumor emboli that shield cancer cells from cytotoxic elimination by immune cells. In addition, thrombin is involved in the binding of cancer cell complexes to the blood vessel wall\textsuperscript{19,20}, and may contribute to disruption of the endothelial barrier to facilitate cancer cell extravasation\textsuperscript{21}.

Hirudin, a direct thrombin inhibitor, inhibits the metastasis-promoting effects of thrombin in animal models\textsuperscript{12,22}. Hirudin administration just before intravenous cancer cell inoculation resulted in a dramatically reduced number of lung metastases. An important aspect of this model is that hirudin was administered just before cancer cell inoculation, suggesting that thrombin is directly involved in the intravascular phase of metastasis. With indirect thrombin inhibitors, i.e. LMWHs, comparable effects have been observed\textsuperscript{23-25}.

On the other hand, in a secondary metastasis model, where cancer cells were inoculated subcutaneously leading to spontaneous visceral metastasis several days later, hirudin administration for several days during primary tumor growth not only inhibited the formation of secondary lung metastases but also inhibited primary tumor growth\textsuperscript{12}. These data suggest that the tumor-promoting activity of thrombin is not limited to the process of metastasis but also facilitates the growth of primary tumors. The angiogenesis- and tumor growth-stimulating effects of thrombin, as demonstrated in several studies, are in line with these observations.

In the clinical setting, various anticoagulants, including heparin and vitamin K antagonists, have been used for the prevention and treatment of thrombotic disorders. Hirudin is the most potent non-covalent inhibitor of thrombin\textsuperscript{26}. Because
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of its specificity, hirudin does not inhibit other enzymes in the coagulation or fibrinolytic pathways. However, hirudin has a rather narrow therapeutic window. Consequently, novel direct thrombin inhibitors with a broader therapeutic spectrum have been developed. One of these novel direct thrombin inhibitors was the orally-active reversible inhibitor, ximelagatran. Ximelagatran also affects fibrin-bound thrombin.

In the present study, we investigated the effects of the thrombin inhibitor ximelagatran in a murine pulmonary metastasis model and compared the effects with those of hirudin. Ximelagatran was administered orally during 7 days before intravenous cancer cell inoculation. To validate the model and to allow for a comparison with the well known effects on pulmonary metastasis, we treated mice also with various heparin analogues, including the LMWH, nadroparin, a synthetic pentasaccharide (Fondaparinux) and the non-anti-coagulant heparin, N-succ 100 LMWH.

Material and Methods

Tumor model and cultures
Murine B16F10 melanoma cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were grown 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 ºC. Cells were harvested at subconfluence using 0.02% ethylenediaminetetraacetic (EDTA) and washed in phosphate-buffered saline (PBS) prior to counting and inoculation.

Anticoagulants
Different types of anticoagulants were used: LMWH, nadroparin (MW 5000 Da, Fraxiparin®; Sanofi-Synthelabo, Berlin, Germany); 100% N-succinylated LMWH (chemically-modified heparin with reduced anticoagulant activity; MW 4070 Da, Succ 100-LMWH; ITF 1164; kindly supplied by Italfarmaco (Milano, Italy)); PEG-hirudin (MW 7000 Da; Abbott, Ludwigshafen, Germany); pentasaccharide (MW 1728 Da, Arixtra®; Fondaparinux, Sanofi-Synthelabo); ximelagatran (MW 430 Da; AstraZeneca, Mölnadal, Sweden). The various compounds were injected subcutaneously except for ximelagatran which was administered orally via food pellets.

Lung colonization assay and methods of drug administration
Male severe combined immunodeficiency (SCID) mice, 2 months old, were used (average body weight 25 gram, 11-13 mice per group). Single cell suspensions were prepared from 0.02% EDTA-treated monolayers of B16F10 melanoma cells which were washed and diluted in PBS. Cancer cells (3.10^5 cells per animal) in 200 μl PBS were injected into the lateral tail vein of the mice. In the final experiment, 1.5.10^5 cancer
cells were inoculated because metastasis was strongly increased after ximelagatran treatment as was observed in the previous experiment. In all experiments, different forms of anticoagulants (dissolved in 200 μl PBS or in food pellets) were administered before cancer cell inoculation with or without subsequent follow-up doses. After 21 days, mice were euthanized and metastatic foci were counted.

In the first experiment, ximelagatran was applied. One group of mice (n=11) received ximelagatran orally at a dose of 250 μmol/kg during 7 days until 24 hours before cancer cell inoculation. The control group received control food pellets during 7 days, lacking anticoagulants in the food.

In the second experiment, the first group of mice (n=11) was treated subcutaneously with 15 IU nadroparin in 200 μl PBS at 30 min before cancer cell inoculation, followed by repeated subcutaneously administration every 4 hr during 24 hrs. The second group of mice (n=11) received N-Succ 100-LMWH, administered subcutaneously in a single dose of 10 mg/kg at 10 min before cancer cell inoculation. In a third group (n=11), Fondaparinux was administered subcutaneously as a single dose of 10 mg/kg in 200 μl PBS at 10 min before cancer cell inoculation. In the fourth group (n=11),

![Figure 1: Effect of treatment with ximelagatran on the number of B16F10 pulmonary tumors in SCID mice. B16F10 melanoma cells (3.10^5) were injected intravenously into the lateral tail vein of SCID mice. ximelagatran was given orally in food pellets during 7 days (250 μmol/kg) until 24 h before cancer cell inoculation (n=11). Controls received control food pellets before cancer cell inoculation (n=13).](image-url)
hirudin was administered subcutaneously as a single dose of 10 mg/kg in 200 μl PBS at 10 min before cancer cell inoculation. A control group (n=13) received 200 μl PBS subcutaneously injected 10 min before the cancer cell inoculation.

In a third experiment, the first group of mice (n=11) received a single dose subcutaneously of 10 mg/kg hirudin, at 10 min before cancer cell inoculation. The second group (n=11) was treated daily with 1 mg/kg hirudin subcutaneously for 7 days until 24 hours before cancer cell inoculation. The third group (n=11) received ximelagatran orally for 7 days as described in the first experiment. The fourth group (n=13) was not treated and served as controls. Group 2-4 were injected with 200 μl PBS 10 minutes before cancer cell inoculation, whereas group 1, 3 and 4 received 200 μl PBS daily for 7 days.

Statistical analysis
Statistical analysis was conducted using GraphPad Prism version 4.03. Data are expressed as mean +/- SE. Comparisons between two groups were analyzed using the Student t-test. Significance was assumed when p<0.05.

Results
To determine the anti-metastatic properties of ximelagatran, we compared the number of lung metastases of mice fed ad libitum with normal food pellets or pellets containing ximelagatran. As shown in Figure 1, ximelagatran treatment more than doubled the number of tumor foci compared to the control group.

Because of the unexpected increase in the number of lung metastases with ximelagatran, we performed additional experiments. To this end, mice were treated with several anticoagulants that have previously shown to inhibit tumor metastasis in a similar experimental B16F10 lung model25,31. As shown in Figure 2A, a single dose of nadroparin or the non-anticoagulant succ 100-LMWH, at 30 min before cancer cell inoculation, both significantly reduced the number of pulmonary metastases.

Next, mice were injected with a single dose of hirudin, 10 min before cancer cell inoculation. As shown in Figure 2B, hirudin treatment almost eliminated the number of cancer metastases (p=0.01). To further confirm the validity of the model, mice were treated with the pentasaccharide, Fondaparinux, as this FXa inhibitor has previously been shown not to affect metastasis of B16 melanoma cells. Indeed, pentasaccharide treatment did not modify the number of lung metastases (Figure 2B).

The observed adverse effect of ximelagatran may be a specific aspect of this compound. Alternatively, the increased number of metastases may be due to the long-term pre-treatment. To discriminate between these potential explanations, we compared ximelagatran treatment (orally for 7 days) with hirudin either injected for 7 consecutive days or as a single dose just prior to cancer cell inoculation (Figure 3).
As expected, hirudin given in a single dose just before cancer inoculation completely prevented cancer cell metastasis. However, 7 days pre-treatment with hirudin resulted in increased pulmonary metastasis (Figure 3). Seven days pre-treatment with ximelagatran caused again an increased number of metastases (Figure 3). The stimulatory effect of ximelagatran on metastasis was stronger than that of hirudin.

Discussion

We assessed the antimetastatic effects of the direct thrombin inhibitor, ximelagatran, in comparison to Hirudin\textsuperscript{27}. The most important but unexpected finding was that 7 days pre-treatment with ximelagatran resulted in a strong increase in the number of pulmonary metastasis. Increased metastasis does not seem to be an intrinsic effect of ximelagatran but rather an effect of long-term anti-coagulant treatment prior to cancer cell inoculation, since a similar dosing regime with hirudin caused an increase in the number of pulmonary metastases as well.

There are several possible explanations for the metastasis promoting effects of ximelagatran and hirudin. First, technical/experimental problems with our animal model may be the cause. However, hirudin (one dose just before cancer cell inoculation), nadroparin, succ-100-LMWH, and Fondaparinux, all produce similar effects on metastasis as described before\textsuperscript{25,31}. Therefore, we can exclude this possibility.

Second, the metastasis-promoting effects of ximelagatran and hirudin may be schedule-dependent. This seems at least a partial explanation as is shown by the opposite effect on pulmonary metastasis by a single dose of hirudin just before intravenous cancer cell inoculation compared to multiple doses of hirudin during 7 days. These findings suggest that for an antimetastatic effect, thrombin inhibitors must be active during the circulating, intravascular phase, of cancer cell spread. The prometastatic effect of thrombin inhibitors - if administered for several days before cancer cell inoculation - indicate an effect on the integrity of the endothelial barrier and thereby promoting cancer cell extravasation. This hypothesis seems to be in contradiction with studies that demonstrated that thrombin can disrupt the endothelial barrier via protease-activated receptor (PAR)-1 signaling on endothelial cells. However, a recent study\textsuperscript{21} demonstrated that the effect of thrombin on endothelium is thrombin concentration dependent. A high concentration of thrombin disrupts the barrier integrity through PAR-1 activation, whereas a low thrombin concentration can induce barrier-protective signaling through the same receptor. Further proof for the hypothesis that long-term thrombin inhibition compromises endothelial barrier function comes from studies showing that activated protein C (APC) is barrier protective\textsuperscript{43}. As activation of the anticoagulant protein C pathway is triggered by thrombin, inhibition of thrombin also suppresses APC formation and consequently the endothelium is no longer protected by the APC pathway and therefore possible more vulnerable for metastasis.
Figure 2: Effect of treatment with different types of anticoagulants or chemically-modified anticoagulants on the number of B16F10 pulmonary tumors in SCID mice. B16F10 melanoma cells (3.10^5) were injected intravenously into the lateral tail vein of SCID mice. (A) Nadroparin, 15 IU every 4 h during 24 h (first dose 30 min before cancer cell inoculation; n=11) and a single dose of Succ-100-LMWH (10 mg/kg) was administered 10 min before cancer cell inoculation (n=11). Controls received 200 μl PBS before cancer cell inoculation (n=13).
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Irrespective of the actual mechanism by which long-term thrombin inhibition increased the number of lung metastasis, it is intriguing to speculate about the clinical consequences. Overall, our data showed that thrombin inhibitors should be used with restraint in patients with cancer.

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References


