



## Heparin attenuates metastasis mainly due to inhibition of P- and L-selectin, but non-anticoagulant heparins can have additional effects

Jennifer L. Stevenson<sup>a,b</sup>, Ajit Varki<sup>a</sup>, Lubor Borsig<sup>c,\*</sup>

<sup>a</sup>Glycobiology Research and Training Center, Departments of Medicine and Cellular & Molecular Medicine, University of California, San Diego, La Jolla, CA, USA

<sup>b</sup>Currently at Amgen Inc., Thousand Oaks, CA, USA

<sup>c</sup>Zürich Center for Integrative Human Physiology, Institute of Physiology, University of Zürich, Zürich, Switzerland

### Abstract

Heparin and low molecular weight heparin (LMWH) are widely used for treatment of cancer patients with thrombosis, a common complication of malignant disease. Several recent prospective clinical studies indicate that heparin might improve outcomes of human cancer. Meanwhile, experimental evidence from mouse models consistently demonstrates that heparin efficiently inhibits metastasis. We have previously shown that P- and L-selectin play independent roles in supporting the initial stages of hematogenous metastasis. Heparin is a known potent inhibitor of such selectin-mediated interactions. Here we provide evidence that the absence of both P- and L-selectin (PL<sup>-/-</sup> mice) dramatically improved survival in an experimental metastasis model. The use of clinically acceptable amounts of heparin did not further affect metastasis rates in such mice. However, a non-anticoagulant derivative of heparin with P- and L-selectin inhibitory properties reduced metastasis to similar levels as observed in PL<sup>-/-</sup> mice. The virtual elimination of metastasis by a single treatment with a modified heparin without anticoagulant activity strongly suggests that heparin primarily reduces metastatic disease by inhibiting P- and L-selectin interactions. However, such heparins could have further effects at higher doses.

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### 1. Abbreviations

LMWH: low molecular weight heparin;  
NAC-heparin: non-anticoagulant heparin.

### 2. Introduction

During hematogenous metastasis malignant cells enter the bloodstream and initiate a cascade of events resulting in eventual formation of metastatic foci in distant tissues [1]. Tumor cells entering the blood circulation interact with platelets and leukocytes leading to formation of tumor emboli. Although the mechanism of platelet and leukocyte interactions with tumor cells continues to be under investigation, the vascular cell adhesion receptors P- and L-selectin, vascular cell adhesion receptors, are known to be facilitators of these cell contacts. Indeed, the absence

of P-selectin (present on activated platelets and endothelium) lead to decreased platelet-tumor cell interactions resulting in attenuation of metastasis [2]. Mice deficient in L-selectin, exclusively present only on leukocytes, also demonstrated reduced metastasis both in syngeneic and xenogenic models [3]. Mice deficient in both P- and L-selectin (PL<sup>-/-</sup>) have shown further reduction of metastasis, indicating synergistic action in this process [3].

Thromboembolism is a well-recognized complication of malignant disease, where heparins have served as effective anticoagulant treatments for many years [4,5]. Heparin is a natural complex mixture of glycosaminoglycans with variable length, degree of sulfation, only a small fraction of which can mediate anticoagulation by potentiating antithrombin action. While heparins were shown to reduce metastasis in several mouse models, the mechanism of action remains under investigation [6,7]. One of several biological activities of heparin is its ability to block P- and L-selectin binding to natural and

\*Corresponding author. Lubor Borsig, Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. Tel.: +41 44 635 5134; fax: +41 44 635 6814.

E-mail address: lborsig@access.uzh.ch (L. Borsig).

tumor cells ligands [8,9]. A single bolus of 100 U of UFH prior to intravenous injection of colon carcinoma cells attenuated metastasis to a similar extent as the P-selectin deficiency [2,3]. A single injection of UFH was also shown to attenuate metastasis of melanomas, primarily by inhibition of P-selectin interactions [10,11]. Recently was shown that heparins (UFH and LMWH) at clinically relevant doses reduced metastasis by inhibition of P-selectin mediated interactions [12,13]. However, heparin has many other potential anti-metastatic activities, including anticoagulation, heparanase inhibition and binding cytokines and growth factors [14,15]. In the present study heparin the effect of heparin on metastasis was investigated in the absence of both P- and L-selectin, together with evaluation of heparin derivative with no anticoagulant activity.

### 3. Material and Methods

#### 3.1. Cell lines

MC-38GFP, mouse colon carcinoma cell line MC-38 stably expressing GFP was cultured as previously described [3].

#### 3.2. Heparin

Unfractionated heparin sodium – Liquemin (25,000 U/ml, lot number B1041) was obtained from Roche, Reinach Switzerland. Unfractionated heparin sodium from American Pharmaceutical Partners (20,000 U/ml; lot number 333246) was obtained from the University of California, San Diego Medical Center Pharmacy. Heparin derivative with no anticoagulant activity but preserved P- and L-selectin blocking activity was previously characterized [16].

#### 3.3. Mice

C57BL/6J (WT) and P-selectin deficient mice were from The Jackson Laboratories (Bar Harbor, Maine) or from in-house breeding of these mice. Mice deficient in both P- and L-selectin (PL<sup>-/-</sup>) and syngeneic for the C57BL/6J background were previously described [3]. All mice were fed standard chow and water *ad libitum*, and maintained on a 12-hour light/dark cycle.

#### 3.4. Experimental metastasis assay

Experiments were performed in AAALAC-accredited vivariums on a protocol approved by the university's IACUC. In keeping with IACUC recommendations, "survival" studies did not use death as an end point, but instead used euthanasia when the mice reached an obviously moribund state (mostly immobile,

hunched over, breathing rapidly, and not seeking food or water).

Mice were injected subcutaneously with 100  $\mu$ L PBS or heparin (19.68U) in 100  $\mu$ L PBS 30 min prior to intravenous injection of MC-38GFP cells as described [12]. WT mice were euthanized when they appeared moribund. All surviving mice were euthanized 50 or 55 days after injection. For the comparison of heparins with and without anticoagulant activity 300  $\mu$ g were intravenously injected 30 min prior to MC-38GFP cells. Mice were euthanized after 28 days and metastatic foci counted on dissected lungs.

## 4. Results

### 4.1. Combined deficiency of P- and L-selectin markedly extends survival of mice intravenously injected with tumor cells

Decreased formation of metastatic foci in PL<sup>-/-</sup> mice has been demonstrated in experimental metastasis studies [3]. However, these studies were terminated at the time point when the first WT control mouse appeared moribund. Thus, the ability of P- and L-selectin deficiency to improve survival has never been evaluated. We have now intravenously injected WT and PL<sup>-/-</sup> mice with syngeneic mouse colon carcinoma cells and monitored them over a longer period of time, euthanizing individual animals only when they appeared moribund, with the typical necropsy finding being nearly complete displacement of the lung parenchyma by confluent masses of tumor cells. The first WT mice were euthanized at day 33 after tumor cell injection (Figure 1). While the

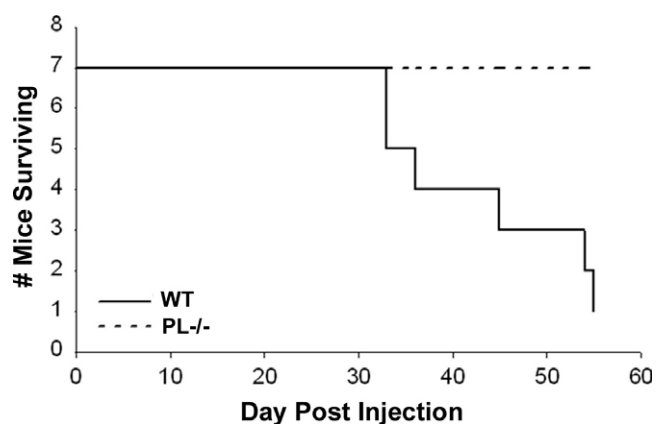


Fig. 1. P- and L-selectin deficiency improves long-term survival in an experimental model of hematogenous metastasis. WT and PL<sup>-/-</sup> mice were injected intravenously with MC-38GFP colon carcinoma cells. Mice were monitored daily for appearance, and were euthanized when moribund to verify the presence of pulmonary metastatic foci. The number of surviving mice is plotted versus time after tumor cell injection. While all PL<sup>-/-</sup> mice appeared normal at the time of termination, 5 of 7 showed visible pulmonary metastatic foci.

number of surviving WT mice continued to decrease over time, no PL<sup>-/-</sup> mice were observed to be moribund at the study's termination on day 55 after tumor cell injection (Figure 1). However we noted that more than half the PL<sup>-/-</sup> mice did have visible lung metastases at day 55. The fact that the long-living PL<sup>-/-</sup> mice still developed some metastatic lesions also allows us to ask whether heparin would have any further effects in these animals [3,17].

**4.2. High dose heparin further reduces metastasis in P- and L-selectin deficient mice**

Previous studies showed that P-selectin is likely playing a role in metastasis at very early time points in the hematogenous metastatic cascade [3]. Based on studies with a function blocking antibody, L-selectin also appears to contribute to metastasis at somewhat later time points, from ~6–18 hours after tumor cell injection [17]. To test whether heparin can attenuate metastasis further beyond blocking P- and L-selectin-mediated interactions, PL<sup>-/-</sup> mice were injected with clinically tolerable levels of heparin 30 min prior to tumor cell injection followed by repeated injection at 6 h and 12 h after tumor cell injection. We previously

optimized the dose of heparin to be 19.68U by studying blood levels in test mice [12]. These mice were kept on test for 50 days, allowing significant metastatic foci to form in at least some animals. As seen by evaluating the number of visible metastatic foci (Figure 2a) there was no significant effect of the clinically relevant heparin injections on metastasis in the setting of P- and L-selectin deficiency. A trend towards a slight improvement with heparin is not statistically significant, either by quantifying the number of visible metastatic foci or by measurement of GFP fluorescence in the lung homogenate (data not shown).

We next asked whether higher doses of heparin have any additive effect in limiting metastasis, beyond inhibition of P- and L-selectin. Therefore, PL<sup>-/-</sup> mice were injected with 100U of heparin at the same three time points, used to inhibit P-selectin and L-selectin (-30 min, +6 h, +12 h). When the visible metastatic foci were counted, a significant reduction was observed in the PL<sup>-/-</sup> mice that received the three heparin injections, as compared to those that received PBS control injections (Figure 2b). Thus, these high dose heparin injections have some additional effects in attenuating metastasis, which are independent of selectin inhibitory activity. However, such effects may not be of practical value in patients.

**4.3. Non-anticoagulant heparin is better inhibitor of metastasis than clinically used unfractionated heparin**

The efficiency of heparin attenuation of metastasis was analyzed either with clinically used heparins or modified heparins with limited characterization of its biological activities [2,11,12,18–21]. To compare the biological activity of unfractionated heparin with a nonanticoagulant derivative – NAC heparin [16], we injected 400µg of the respective heparins (unfractionated heparin – Liquemin and NAC heparin) in WT mice 30 min prior to intravenous injection of colon carcinoma cells MC-38GFP (Figure 3). After evaluation of lungs four weeks later, a clear attenuation of metastasis was detected with both heparins. Interestingly, NAC-heparin was more efficient in attenuating metastasis than UFH (P=0.034), indicating variability in anti-metastatic activity of various preparations of heparins largely dependent on standardization only based on anti-Xa activity. Attenuation of metastasis with both heparin preparations was comparable to the effect of the P-selectin absence. However, unfractionated heparin as well as NAC-heparin were less efficient than the absence of both P- and L-selectins, indicating that single dose of heparin cannot achieve effective

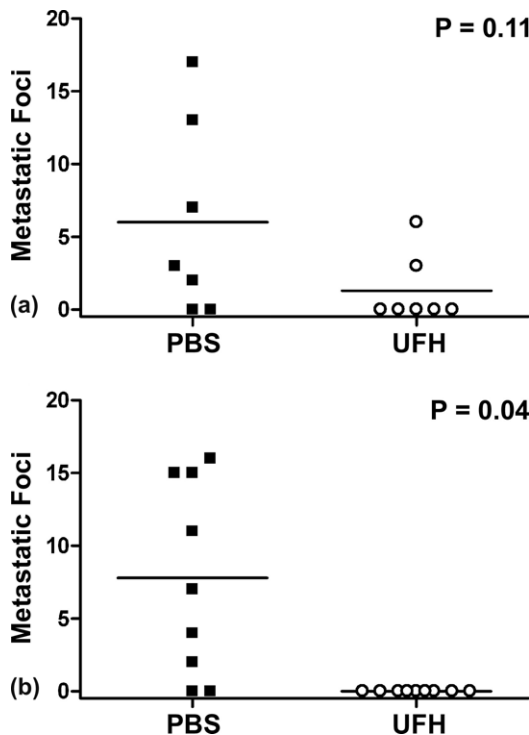


Fig. 2. Only high doses of heparin can further reduce metastasis in mice deficient in P- and L-selectin. PL<sup>-/-</sup> mice were injected intravenously with tumor cells at t=0, and subcutaneously with PBS or unfractionated heparin in PBS at t=-0.5 h, +6 h, and +12 h. (a) 19.68 U of heparin. (b) 100 U of heparin. Mice were euthanized 50 days after injection, and the formation of pulmonary metastases determined by counting the number of visible foci. P-values were determined by performing a Student's T-test.

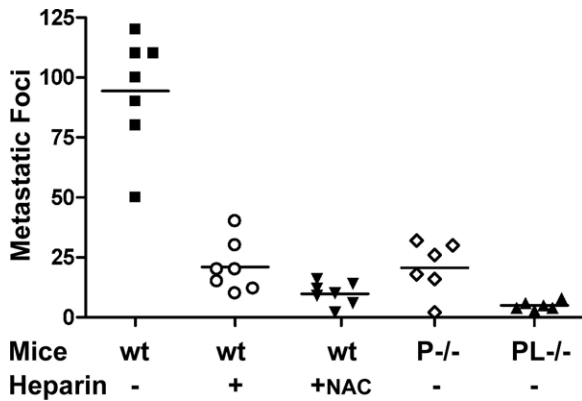


Fig. 3. A single injection of heparin with no anticoagulant activity is equally efficient inhibitor of metastasis as achieved by the absence of P-selectin. Mice were intravenously injected with 400  $\mu$ g of heparin 10 min prior to injection of  $3 \times 10^5$  MC-38GFP cells. Non-anticoagulant heparin (NAC) with selectin inhibitory properties was as previously characterized [16]. For comparison data with P- and P-/L- double selectin deficient mice is shown. The extent of lung colonization was analyzed by counting visible metastatic foci. A 2-way ANOVA multiple compare test was used to determine the statistical significance of NAC heparin versus controls ( $P < 0.01$ ).

inhibition of the apparent synergistic contribution of P- and L-selectin in hematogenous metastasis [3].

## 5. Discussion

Many of heparin's activities, including anticoagulation may reduce metastasis. Other groups have performed studies in which it was demonstrated that administration of hirudin, an antithrombin anticoagulant, can reduce experimental metastasis. One study involved multiple injections of 20 mg/kg of hirudin spanning the first ten days after tumor cell injection [22]. A significant decrease in formation of metastatic foci was observed. Metastatic foci formation was reduced significantly with hirudin administration. A second group observed significant reduction in pulmonary metastases upon administration of 10 mg/kg hirudin prior to injecting tumor cells [23]. However, both of these studies were performed at anticoagulant levels that were likely not clinically relevant, as the activated partial thromboplastin time test results almost all exceeded the assay limit of detection in the second study. Additionally, studies have demonstrated reduction of metastasis with chemically-modified, non-anticoagulant heparins, therefore anticoagulant activity is not required for heparin inhibition of metastasis [16,18–20]. Preparations of heparins with no anticoagulant activity were recently characterized in our laboratory [16]. When directly compared, based on the amount, with the clinically used unfractionated heparin, the NAC heparin seems to be more efficient suggesting that anticoagulant activity of heparin is not contributing to antimetastatic

activity in this model (Figure 3). The synthetic pentasaccharide Fondaparinux, which has no selectin inhibitory activity, was compared to low molecular weight heparins and was found to have no effect on metastasis at clinically acceptable levels giving efficient anticoagulation [12,13]. Thus, while Figure 2 demonstrates some selectin-independent effect on metastasis at this high dose of heparin, no practical conclusion can be drawn about the implications for the use of heparin in the clinical setting. The dose of heparin (19.68 U) has previously been demonstrated to have a dramatic effect on formation of metastatic foci in WT mice [12]. Since no further effect was observed in mice deficient in both P- and L-selectin, we can conclude that clinically relevant doses of heparin attenuate metastasis mainly via inhibition of P- and L-selectin. Of course there is always a possibility that heparin also inhibits one or more additional mechanisms that are within the same linear pathway as the selectin contributions to metastasis. However, in the experimental model of metastasis, in which tumor cells are administered directly into the vasculature and immediately interact with blood cells, the selectins are likely to be involved in some of the earliest steps in the metastatic cascade. Thus, inhibiting these early steps in a cascade would render other downstream effects of heparin to be practically irrelevant. Additionally, as the doses of heparin administered in this experiment are cleared within a few hours, many of the additional effects of heparin (e.g. heparanase and angiogenesis inhibition) are likely not relevant during the time frame studied. It remains possible that heparin binding to chemokines would also be relevant during this time period, and this should be studied further. However, one would not expect this action to necessarily be in the same pathway as the selectins.

Should heparin be administered over a longer period of time during the metastatic process, it is possible that it will also be working by some of its other potential actions. Regardless, all of heparin's other activities should only be beneficial in reducing metastasis and tumor growth. Therefore, the potential for therapeutic use is great. Meanwhile, further studies of non-anticoagulant heparins are warranted, as they could be given at higher doses to patients, providing additional benefits beyond P- and L-selectin inhibition.

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