Differential Metastasis Inhibition by Clinically Relevant Levels of Heparins—Correlation with Selectin Inhibition, Not Antithrombotic Activity

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Abstract

Purpose: Unfractionated heparin reduces metastasis in many murine models. Multiple mechanisms are proposed, particularly anticoagulation and/or inhibition of P-selectin and L-selectin. However, the doses used are not clinically tolerable and other heparins are now commonly used. We studied metastasis inhibition by clinically relevant levels of various heparins and investigated the structural basis for selectin inhibition differences.

Experimental Design: Five clinically approved heparins were evaluated for inhibition of P-selectin and L-selectin binding to carcinoma cells. Pharmacokinetic studies determined optimal dosing for clinically relevant anticoagulant levels in mice. Experimental metastasis assays using carcinoma and melanoma cells investigated effects of a single injection of various heparins. Heparins were compared for structural relationships to selectin inhibition.

Results: One (Tinzaparin) of three low molecular weight heparins showed increased selectin inhibitory activity, and the synthetic pentasaccharide, Fondaparinux, showed none when normalized to anticoagulant activity. Experimental metastasis models showed attenuation with unfractionated heparin and Tinzaparin, but not Fondaparinux, at clinically relevant anticoagulation levels. Tinzaparin has a small population of high molecular weight fragments not present in other low molecular weight heparins, enriched for selectin inhibitory activity.

Conclusions: Heparin can attenuate metastasis at clinically relevant doses, likely by inhibiting selectins. Equivalent anticoagulation alone with Fondaparinux is ineffective. Clinically approved heparins have differing abilities to inhibit selectins, likely explained by size distribution. It should be possible to size fractionate heparins and inhibit selectins at concentrations that do not have a large effect on coagulation. Caution is also raised about the current preference for smaller heparins. Despite equivalent anticoagulation, hitherto unsuspected benefits of selectin inhibition in various clinical circumstances may be unwittingly discarded.

P-selectin and L-selectin are C-type lectins that recognize sialylated, fucosylated, sulfated ligands. P-selectin is stored within resting platelets and endothelial cells and translocates to the cell surface upon activation. L-selectin is constitutively expressed on most leukocyte types and mediates their interactions with endothelial ligands. Both selectins promote the initial tethering of leukocytes during extravasation at sites of inflammation. P-selectin also plays a role in hemostasis. Endogenous ligands for P-selectin and L-selectin (such as PSGL-1) are expressed on leukocytes and endothelial cells (for general reviews on selectins and their ligands, see refs. 1–6).

P-selectin and L-selectin also have pathologic roles in many diseases involving inflammation and reperfusion (7–9), as well as in carcinoma metastasis. Many tumor cells express selectin ligands and an inverse relationship between tumor selectin ligand expression and survival has been reported (reviewed in ref. 10). Syngenic and allogenic mouse models have shown that metastasis of selectin ligand-positive adenocarcinomas to the lungs is P-selectin and L-selectin dependent (11–15).

Many classic studies documented an inhibitory effect of unfractionated heparin in animal models of cancer metastasis (16–19), and retrospective analyses indicated that heparin may have similar effects in human cancer (20–24). A large body of literature also discusses the well-documented relationships of cancer and venous thrombosis (reviewed in ref. 25) and the inhibition of metastasis via blocking fluid-phase coagulation, either with heparin or hirudin (16–18, 21, 22, 26–30). However, human trials using vitamin K antagonists as an alternate mode of anticoagulation showed no effect on survival in most carcinomas (18, 30–32). Thus, it should not be assumed that heparin efficacy in metastasis is primarily based on its anticoagulant activity.

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Unfractionated heparin has been in clinical use for decades based on its ability to inhibit fluid phase coagulation by enhancing antithrombin inactivation of factors IIa and Xa (33–35). However, unfractionated heparin is a natural product containing a complex polydisperse mixture of highly sulfated glycosaminoglycan chains ranging from 5,000 to 30,000 Da, only some of which actually bind antithrombin (34, 36). Early studies showed that P-selectin could bind to immobilized heparin (37). We and others subsequently showed that various heparins and heparinoids could inhibit binding of both P-selectin and L-selectin to their natural ligands (38–42). We have, therefore, hypothesized that the heparin effect on metastasis is not primarily due to anticoagulation, but rather to inhibition of P-selectin and L-selectin binding to tumor cell ligands (10).

Of course, heparins have many other biological effects potentially relevant to solid tumor spread, including inhibition of heparinases involved in degrading basement membranes, modulatory effects on various heparin-binding growth factors or extracellular proteases, alteration of integrin functions in cell adhesion, inhibition of angiogenesis, etc. (for general reviews, see refs. 17, 19, 43). Of all these potential nonanticoagulant mechanisms, P-selectin and L-selectin inhibition is the first one likely to be relevant when tumor cells initially enter the bloodstream. This effect also stands at the beginning of a cascade of events involved in survival of tumor cells, before their eventual extravasation and establishment as metastatic foci. As with any cascade, blocking the first step can make all subsequent mechanisms practically irrelevant. Indeed, we have shown previously that effects of single-dose unfractionated heparin given before i.v. tumor cell introduction can be explained by inhibition of P-selectin and L-selectin because heparin had no further effects on metastasis in mice with a combined deficiency of both selectins (14). A similar result was seen regarding heparin effects in attenuating inflammation, with the relevant activity again limited to P-selectin and L-selectin inhibition (44).

Overall, our working model to explain heparin action in solid tumor metastasis has been inhibition of P-selectin and L-selectin, combined with an unknown degree of blockade of intravascular fibrin formation by the fluid-phase coagulation pathway. However, the relatively high doses administered in most previous studies would be impractical to use clinically because of excessive anticoagulation. Unfractionated heparin also generally has poor bioavailability, requires multiple daily dosing, and has side effects, such as heparin-induced thrombocytopenia (34, 35). To circumvent this, many low molecular weight heparins have been created by degrading unfractionated heparin using a variety of methods, including chemical depolymerization and enzymatic digestion (34, 45). Whereas low molecular weight heparins are also a mixture of fragment sizes, with molecular weight profiles ranging from 3,000 to 9,000 Da, they have better kinetics and bioavailability, typically requiring only single daily doses. Taken together with a similar efficacy in clinical anticoagulation (via anti-Xa activity) and a lower incidence of side effects, such as heparin-induced thrombocytopenia, they have become favored in clinical practice (35, 46). Further benefits are claimed for Fondaparinux, a synthetic heparinoid pentasaccharide of defined structure (33) that specifically binds to antithrombin (47, 48).

Recently, one low molecular weight heparin was reported to give improved survival in a subset of patients with advanced cancer (49). Meanwhile, we have suggested the use of heparin to interdict metastasis during the period between initial diagnosis of early-stage carcinomas and soon after their surgical removal (10), an idea supported by the recent finding that patients with primary tumors (but no metastases) who were treated with a low molecular weight heparin had increased survival (30).

Translating all these promising ideas into clinical practice now requires experimental evaluation of the potential for clinically acceptable levels of the various kinds of heparins to block P-selectin and L-selectin and attenuate metastasis. Here, we compare five heparins/heparinoids (hereafter collectively called heparins) for their ability to inhibit P-selectin and L-selectin. After defining pharmacokinetics in mice, we then compare a subset of these heparins for their ability to inhibit metastasis in two different tumor models at clinically relevant doses. Finally, we investigate the structural differences between the low molecular weight heparins in light of their differential selectin inhibition activity and address the relative roles of anticoagulation and selectin inhibition in attenuating metastasis.

Materials and Methods

Materials. The following materials were from the University of California, San Diego, Medical Center Pharmacy: unfractionated heparin sodium from American Pharmaceutical Partners (Schaumburg, IL; 20,000 U/mL; lot numbers 302523, 333246); Innohep (Tinzaparin) from Pharmion (licensed from LEO Pharmaceutical Products, Ballerup, Denmark, 20,000 IU/mL; lot numbers E9867A and G3371A); Fragmin (Dalteparin) from Pharmacia (Kalamazoo, MI; 5,000 IU/0.2 mL; lot numbers 94250A51, 94683A02, and 94802A01); Lovenox (Enoxaparin) from Aventis (Bridgewater, NJ; 30 mg/0.3 mL; lot numbers 30324, 9367, and 9369); and Arixtra (Fondaparinux) from Sanofi-Synthelabo (West Orange, NJ; 2.5 mg/0.5 mL; lot numbers 0010000003 and 0170000010). Unless otherwise noted, all remaining chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Cell lines. LS180 human colonic adenocarcinoma cells and MC38GFP cells (a mouse colon carcinoma cell line stably transfected with enhanced green fluorescent protein) were cultured as described (13, 14). Mouse melanoma B16F1 cells were cultured in DMEM with 10% FCS. All media and additives were from Life Technologies (Invitrogen, Carlsbad, CA) except for FCS, which is from HyClone (Logan, UT). All cells were incubated at 37°C with 5% CO_2. Before use, cells were released by incubation in PBS with 2 mmol/L EDTA at 37°C for 5 to 10 minutes, and washed in PBS with Ca^{2+}, Mg^{2+}, and glucose before suspending in the same buffer for i.v. injection.

Mice. C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME) were fed standard chow and water *ad libitum* and maintained on a 12-hour light/dark cycle. Some mice were obtained from in-house breeding of these C57BL/6J mice. All purchased mice were allowed to acclimate in the vivarium for a minimum of 1 week following arrival before beginning experiments. All experiments were done in Association for Assessment and Accreditation of Laboratory Animal Care–accredited vivariums on a protocol approved by the Institutional Animal Care and Use Committee of the university.

Heparin inhibition of LS180 binding to selectins. Levels of heparin were normalized based on their anti-Xa activity. Binding of cells to immobilized human selectin-Fc chimeras was studied as previously described (44), except that Calcein AM–loaded LS180 cells were used. Results are expressed as percent of control binding, calculated using the following formula: 100 (heparin value − EDTA value) / (buffer alone value − EDTA value). Each anticoagulant was tested in triplicate wells at each relative concentration.

Titrating heparin dosage via plasma anti-Xa levels. Mice were injected subcutaneously with 100 μL unfractionated heparin, Tinzaparin, or Fondaparinux diluted in PBS at various final concentrations. Thirty minutes later, blood was collected by cardiac puncture into 1 mL syringe containing 30 μL of 10 mmol/L EDTA. Samples were centrifuged twice at 2,000 relative centrifugal force at 24°C to collect the plasma, which was stored at −80°C until analysis for anti-Xa activity. Human Antithrombin III (3.3 μg/well; Enzyme Research Laboratories, South Bend, IN) and human factor Xa (0.02 μg/well; Enzyme Research Laboratories), in 155 μL of 25 mmol/L HEPES/150 mmol/L NaCl (pH 7.5), were added to 1.25 μL plasma samples, which were then incubated with 25 μg/well of a synthetic factor Xa chromogenic substrate (Diapharma, Columbus, OH). The reaction was stopped after 15 minutes by adding 50 μL/well 20% acetic acid. The resulting chromophore was measured at 405 nm. Plasma heparin levels were calculated in anti-Xa units/mL by comparing against a standard curve of heparin-spiked mouse plasma samples. Standards and samples were analyzed in triplicate. Final amounts used for 1× dosing were 6.56 units unfractionated heparin, 7.32 IU Tinzaparin, and 0.0033 mg Fondaparinux. The 3× dosing used thrice the amount.

Carcinoma experimental metastasis assay. Mice were injected subcutaneously with 100 μL PBS or heparin in 100 μL PBS. Thirty minutes afterwards, 500,000 MC38GFP cells were injected i.v. into the lateral tail vein. Mice from each studied group were injected in alternating order and cells were resuspended by gently flicking the tube before aspirating the sample for each injection. Twenty-seven days after injection, the mice were euthanized, the lungs were removed, and enhanced green fluorescent protein fluorescence in lysates was quantified as described (14).

Melanoma experimental metastasis assay. Mice were injected with heparin and 500,000 B16F1 cells using the protocol described for the carcinoma metastasis assay. Seventeen days after injection, mice were euthanized, tracheal perfusion with 10% buffered formalin was done, and the lungs were removed and placed into 10% buffered formalin. Lungs were allowed to fix for a minimum of 24 hours, removed from formalin individually, and photographed using a digital camera. Lung weights were determined by removing the lungs from formalin, briefly setting them on filter paper to remove excess liquid, and then weighing them on a Sartorius analytic scale.

Heparin disaccharide analysis. Disaccharide analysis was done by the University of California, San Diego, Glycotechnology Core Facility. Briefly, 5 μg of each heparin were dried down, resuspended in 100 mmol/L sodium acetate, 0.1 mmol/L calcium acetate (pH 7.0), and incubated with 5 milliunits each of heparin lyases I, II, and III for 18 hours at 37°C. Samples were boiled for 2 minutes and run through a prewashed Microcon 10 filter. Samples were then dried down, resuspended in MilliQ water, and separated by high-performance liquid chromatography (HPLC) on a Dionex ProPac PA1 anion exchange column using MilliQ water (pH 3.5) with a sodium chloride gradient of 50 to 1,000 mmol/L over 60 minutes. Postcolumn derivatization with fluorescence detection was achieved by mixing 2-cyanoacetamide (1%) with 250 mmol/L NaOH in the eluent stream using an Eldex dual-channel pump. The eluent was then passed through an Eppendorf TC-40 reaction coil heated to 130°C, followed by a cooling bath, and then to a Jasco fluorescence detector set at an excitation of 346 nm and an emission of 410 nm. The sensitivity of this method is ~5 pmol (50).

Heparin sizing. A TosoHaas TSKG2000SW HPLC column was run at 0.4 mL/min in 10 mmol/L KH_2PO_4, 0.5 mol/L NaCl, and 0.2% Zwittergent (Calbiochem, San Diego, CA). The void volume was determined using blue dextran. Cytidine monophosphate (0.5 μg) was spiked into all samples for use as an internal control to mark the included volume. Two different lots of each heparin were evaluated. Each sample was brought up to 10 μL total volume with MilliQ water. UV absorbance was monitored throughout the 45-minute runs at a wavelength of 206 nm. In an additional run, a larger aliquot of Tinzaparin (19.5 μL Tinzaparin and 2.5 μg cytidine monophosphate marker) was run on the same column, and 200 μL fractions were collected and evaluated for their inhibitory activity against P-selectin binding to Sialyl Lewis X (sLe^x; see assay details below). The amount of uronic acid in each fraction was quantified using a standard carbazole assay (51).

Assay for inhibition of P-selectin binding to sialyl Lewis X. High-binding 96-well ELISA plates were coated overnight with 2 μg/mL sLe^x-PAA (Glycotech, Gaithersburg, MD) in 50 mmol/L carbonate buffer (pH 9.5). Plates were rinsed twice with a 1:5 dilution of HPLC running buffer (final concentration of 2 mmol/L KH_2PO_4, 0.1 mol/L NaCl, and 0.04% Zwittergent), and then blocked for 1 hour in a 1:5 dilution of HPLC running buffer + 0.5% bovine serum albumin. Human P-selectin chimera was precomplexed with goat anti-human IgG-AP (0.25 μg:0.25 μL; Bio-Rad, Hercules, CA) in the presence of 1:5 dilutions of collected HPLC fractions (or dilutions of those fractions in column buffer) or heparin standards for 1 hour at room temperature with mixing. Samples were added to the blocked plate and incubated at room temperature for 2 to 3 hours. The plate was rinsed twice with 1:5 dilution of HPLC buffer + 0.5% bovine serum albumin, and then twice with a 1:5 dilution of HLPC buffer. AP substrate solution [150 μL; 10 mmol/L *p*-nitrophenyl phosphate, 100 mmol/L Na_2CO_3, 1 mmol/L $MgCl_2$ (pH 9.5)] was added to the plate and allowed to develop at room temperature. The absorbance at 405 nm was read on a SpectraMax 250 plate reader. One unit of inhibitory activity was arbitrarily defined as 1% inhibition of selectin binding within the linear range of the assay. Results are expressed as total inhibitory units, which is calculated using the following formula: 100 [(max binding − unknown binding) / (max binding − min binding)] (200/μL fraction tested in inhibition assay), where "max binding" is the amount of binding in the presence of a fraction that eluted before heparin elution and "min binding" is the amount of binding in the presence of 0.5 IU/mL Tinzaparin.

Results

***Clinically approved heparin formulations have widely varying abilities to inhibit P-selectin and L-selectin* in vitro.** Clinical-grade preparations of unfractionated heparin, three types of low molecular weight heparin (Tinzaparin, Dalteparin, and Enoxaparin), and the synthetic pentasaccharide Fondaparinux were obtained from the University of California, San Diego, Medical Center Pharmacy, representing all heparins currently marketed for clinical use in the United States (source: Physician's Desk Reference). Notably, the low molecular weight heparins are prepared by different methods of unfractionated heparin degradation: Tinzaparin, by β-eliminative cleavage with heparinase; Dalteparin, by deaminative cleavage with nitrous acid; and Enoxaparin, by β-eliminative cleavage with alkali (45). P-selectin or L-selectin chimeras were immobilized on Protein A–coated plates and fluorescently labeled tumor cells allowed to bind in the presence of varying amounts of heparins. When normalized to anti–factor Xa activity (anti-Xa is a good predictor of *in vivo* anticoagulant activity), unfractionated heparin was the best inhibitor of both selectins (Fig. 1). Much variation was observed among the three low molecular weight heparins, with Tinzaparin having higher inhibitory activity than Dalteparin and Enoxaparin. Interestingly, Fondaparinux, while synthetically designed specifically for its potent anticoagulant activity, had no ability to inhibit either P-selectin or L-selectin. Dalteparin and Enoxaparin were capable of inhibiting P-selectin binding at higher anti-Xa concentrations (Fig. 1, *top*), but had only minimal ability to inhibit L-selectin binding (Fig. 1, *bottom*). Whereas inhibition of P-selectin was obtained at lower relative doses than L-selectin, the overall rank order of inhibition

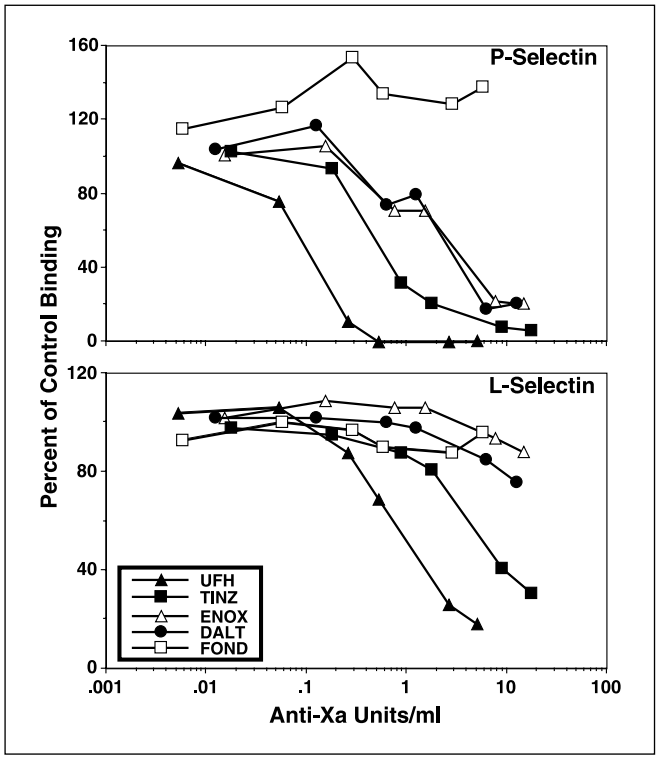

Fig. 1. Clinically utilized heparin preparations show marked differences in their ability to inhibit P-selectin (*top*) and L-selectin (*bottom*) binding to carcinoma ligands. Binding of human colon carcinoma cells to immobilized selectin chimeras was tested in the presence of a range of concentrations of different heparins. Control binding was based on measurements in the presence of buffer alone and background values were measured in 2.5 mmol/L EDTA. Each heparin concentration was tested in triplicate and the presented data is representative of results from multiple experiments. UFH, unfractionated heparin; TINZ, Tinzaparin; ENOX, Enoxaparin; DALT, Dalteparin; FOND, Fondaparinux.

(unfractionated heparin > Tinzaparin > Dalteparin = Enoxaparin >> Fondaparinux) was the same.

Pharmacokinetic studies in mice to normalize heparin dosing. Based on the above results, we chose to do further studies comparing unfractionated heparin, Tinzaparin, and Fondaparinux, thus encompassing the spectrum of selectin inhibition properties. Good documentation about the pharmacokinetics of these heparins in mice is not available in the literature. Indeed, most prior mouse studies have used high unfractionated heparin doses that are likely to achieve anticoagulant effects unacceptable in human clinical use (see Introduction). Before testing these heparins in metastasis assays, studies were done to normalize dosing, such that each administered heparin gave similar, clinically acceptable *in vivo* anti-Xa levels. Subcutaneous delivery is the preferred route of administration of low molecular weight heparins (46). Thus, subcutaneous heparin doses in mice were optimized to achieve clinically relevant anti-Xa levels. Therapeutic levels for patients treated with heparin for venous thromboembolism are ~1 anti–factor Xa unit/mL for low molecular weight heparins, typically measured at 3 to 4 hours after injection (35, 48). Thus, we systematically titrated doses of subcutaneous heparin to achieve approximately similar plasma anti-Xa levels in mice. We eventually determined single-dose injection amounts of 1× unfractionated heparin, Tinzaparin, and Fondaparinux that yielded mean anti-Xa levels within this range (as in humans, there is considerable variation among individuals in the effects of a single s.c. dose; Fig. 2A). Plasma anti-Xa levels were analyzed 30 minutes after subcutaneous delivery, which is when the tumor cells would be injected into the vasculature in the planned metastasis experiments. However, pharmacokinetic studies (data not shown) showed that Tinzaparin was actually cleared much faster in mice than in humans, in whom one daily dose is sufficient to maintain anticoagulation (35, 48, 52). Thus, it is impossible to precisely mimic the human situation in mice and use of the 1× dose could result in erroneously negative data. To partially overcome this limitation, we also increased the single heparin doses from 1× to 3× dosing, and analyzed the anti-Xa levels (Fig. 2B). Here, the initial peak level might be slightly higher than clinically acceptable, but practically relevant levels would be sustained a while longer. Both 1× and 3× heparin doses (see details in Materials and Methods) were used for the metastasis experiments, as approximating the range of concentrations that might be found in a patient on these drugs.

Carcinoma metastasis can be attenuated only by certain heparins. All subsequent *in vivo* studies utilized the "experimental" model of metastasis, in which tumor cells are injected i.v. Whereas this model has its limitations (53), it is the only way to study interactions between the tumor cells and blood

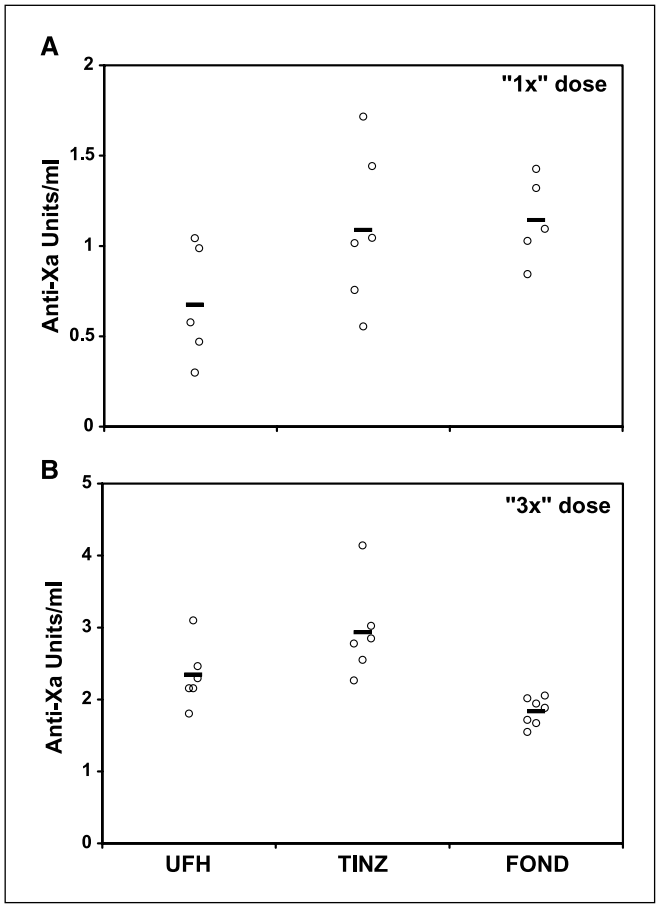

Fig. 2. Therapeutic range of anti-Xa units can be achieved with a single heparin dose. Anti-Xa levels were measured in plasma from multiple mice 30 minutes after each mouse received a single 1× (*A*) or 3× (*B*) subcutaneous dose of various heparins. Open circles, individual mice; horizontal bars, means. UFH, unfractionated heparin; TINZ, Tinzaparin; FOND, Fondaparinux.

cells within the first few hours of tumor cell entry into the vasculature in a controlled manner at a known time point (i.e., "spontaneous" metastasis experiments are unsuitable). Also, all our experiments utilized a single bolus injection of heparin before tumor cell injection. As this heparin dose is cleared within a few hours after injection, we are investigating the effect only during the first few hours of tumor cell interaction in the vasculature.

We previously showed that experimental metastasis of human and mouse colon carcinoma cells could be attenuated by i.v. injection of 100 units of unfractionated heparin 30 minutes before tumor cell injection (13, 14). Studies by others using 12.5 or 60 IU of unfractionated heparin before tumor cell injection also showed a decrease in metastasis of melanoma cells (15). While demonstrating the potential for heparin to reduce metastasis, these and most prior studies were done using heparin doses that are clinically unacceptable. To evaluate heparin treatment in a more clinically relevant setting, metastasis assays were done comparing unfractionated heparin, Tinzaparin, and Fondaparinux at 1× and 3× dosing. Mice were i.v. injected with syngeneic MC38GFP colon carcinoma cells known to carry selectin ligands (14) 30 minutes after subcutaneous dosing with the heparins at 1× or 3× dosing or with a PBS control. Results with 1× dosing showed a trend in reduction of metastasis that matched the observed *in vitro* selectin inhibition activity (i.e., unfractionated heparin > Tinzaparin >> Fondaparinux; Fig. 3A). However, these results were not statistically significant. Injection of 3× heparin gave almost complete attenuation of metastasis with unfractionated heparin and Tinzaparin, but still no significant difference between Fondaparinux and PBS (Fig. 3B). Notably, this dose of Fondaparinux gave plasma anti-Xa levels at or above the accepted range for clinical anticoagulation (Fig. 2B).

Heparin inhibition of melanoma metastasis is also dependent on selectin inhibitory activity. Whereas the results obtained with MC38GFP cells show the relationship between inhibition of colon carcinoma metastasis and the ability of the heparin to inhibit P-selectin and L-selectin, we wanted to see whether this phenomena was applicable to other models of cancer metastasis. Mice were injected i.v. with B16F1 melanoma cells 30 minutes following subcutaneous injection of 1× or 3× dosing of unfractionated heparin, Tinzaparin, or Fondaparinux (PBS as a control). Seventeen days following injection, the lungs were excised and evaluated for the presence of metastatic foci. In lieu of counting foci, lung weights were obtained and compared with the weight of lungs from mice not injected with tumor cells. This method has been used by others (54) and correlated quite well with the physical appearance of the lungs (sample photos are shown below quantification). In mice that received 1× heparin dosing, a statistically significant reduction in metastasis was observed in those that received unfractionated heparin and Tinzaparin (Fig. 4A). Again, Fondaparinux had no effect, with lungs appearing comparable with those of mice injected with PBS. When the amount of heparin was increased to 3× dosing, an even greater reduction in metastasis was observed with unfractionated heparin and Tinzaparin treatment, with lung weights similar to those of mice that did not receive tumor cells (Fig. 4B). Again, Fondaparinux had no effect on metastasis, even at the higher dosing. Thus, a single bolus of low-dose unfractionated heparin and Tinzaparin, given just before injection of melanoma cells, has the ability to reduce

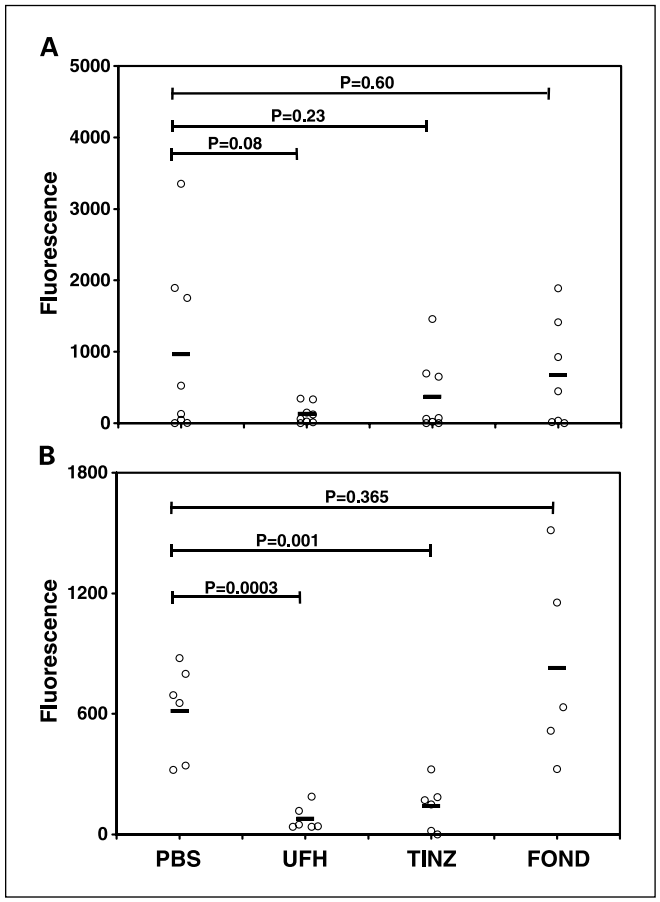

Fig. 3. Inhibition of metastasis of colon carcinoma cells is achieved at clinically tolerable levels of unfractionated heparin and Tinzaparin, with Fondaparinux having no effect. Mice were injected subcutaneously with 1× heparin or PBS as a control (*A*) or 3× heparin or PBS as a control (*B*), and 30 minutes later were injected i.v. with MC38GFP cells. After 27 days, mice were euthanized and metastasis was evaluated by quantifying the fluorescence of lung homogenate. Open circles, individual mice; horizontal bars, means. *P* values were determined by a Student's *t* test, assuming two-tailed, unequal distribution. UFH, unfractionated heparin; TINZ, Tinzaparin; FOND, Fondaparinux.

metastasis. This trend matches that observed with colon carcinoma cells, confirming the importance of selectin inhibition (and lack of importance of anticoagulant effect) across multiple tumor cell types.

Varying ability of low molecular weight heparins to inhibit selectins does not correlate with disaccharide composition. Heparins are complex polysaccharides with a polydisperse distribution of sulfation and epimerization patterns. It has been previously shown that sulfation patterns can affect the ability of chemically modified heparins to inhibit selectins (44, 55). Given different methods of preparation of the three low molecular weight heparin formulations (45), we hypothesized that distinct sulfation patterns might explain their differential ability to inhibit P-selectin and L-selectin. The structure of Fondaparinux is well known. We evaluated the disaccharide composition of two lots each of unfractionated heparin and of each of the three low molecular weight heparins as described in Materials and Methods. No significant differences in the percentage of each disaccharide were noted (data not shown). It is likely, therefore, that the differences in inhibition observed between the various low molecular weight heparins are not

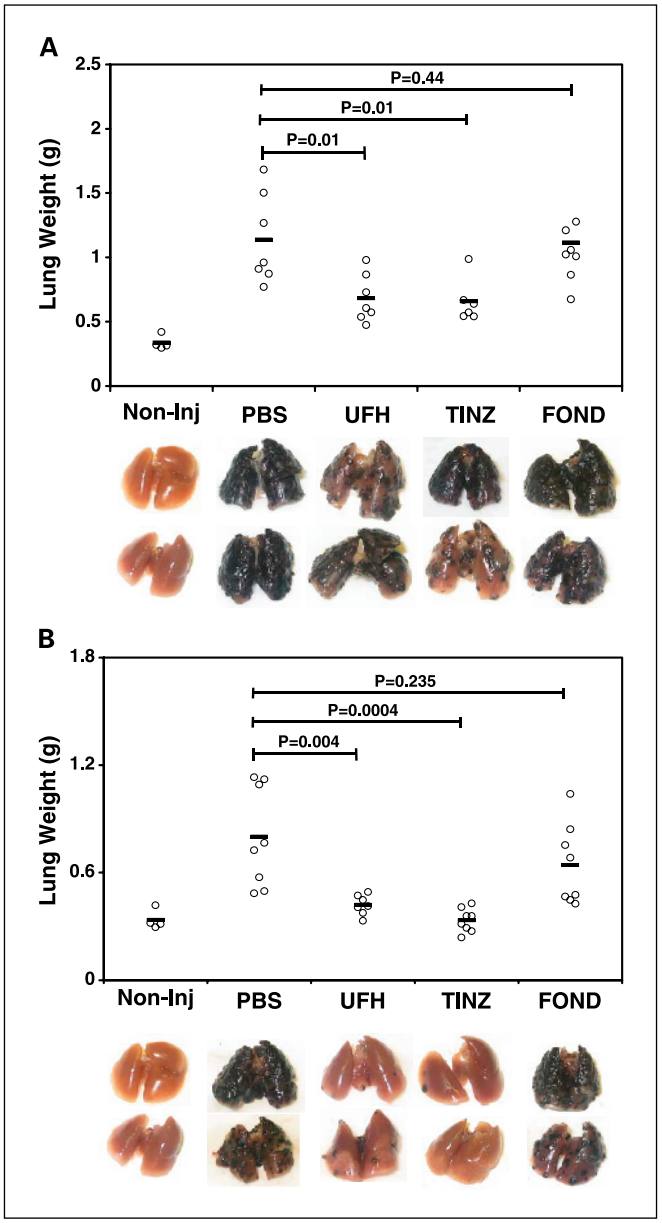

Fig. 4. Heparins with selectin inhibitory activity inhibit metastasis of melanoma cells. Mice were injected subcutaneously with 1× heparin or PBS as a control (*A*) or 3× heparin or PBS as a control (*B*), and 30 minutes later were injected i.v. with B16F1 cells. After 17 days, mice were euthanized, lungs perfused with formalin through the trachea, and then allowed to fix in formalin for a minimum of 24 hours. Metastasis was quantified by measuring lung weight, which correlated well with the physical appearance of the lungs, documented by photography (representative pictures are shown below quantification). Open circles, individual mice; horizontal bars, mean lung weights. *P* values were determined by a Student's *t* test, assuming two-tailed, unequal distribution. UFH, unfractionated heparin; TINZ, Tinzaparin; FOND, Fondaparinux.

due to major differences in basic sulfation patterns. Rather, it would have to be due to higher-order structure and/or overall length. In support of the latter possibility, our previous work showed that increasing length in the range of one to seven disaccharides correlated with increasing ability to inhibit P-selectin and L-selectin (40).

Size fractionation identifies heparins with potent selectin inhibitory properties relative to anticoagulant activity. The package inserts that accompany the heparin formulations indicate that Tinzaparin is likely to contain more high molecular weight heparin fragments than either Dalteparin or Enoxaparin. The amount of fragments of >8,000 Da is specified as 22% to 36% for Tinzaparin, 14% to 26% for Dalteparin, and 0% to 18% for Enoxaparin. To determine whether this potential difference in high molecular weight content was present in our samples, size exclusion HPLC analysis was done on all five heparins. The size profile of each heparin was determined by monitoring the UV absorbance at 206 nm (the profiles are jagged because of low sensitivity and the polydisperse nature of the heparins; Fig. 5A). As expected, each of the three low molecular weight heparins contained a noticeably smaller size range of heparin fragments than unfractionated heparin. Enoxaparin has a molecular weight profile lower than both Tinzaparin and Dalteparin. Whereas the average molecular weight seemed to be similar for Tinzaparin and Dalteparin, the profile of Tinzaparin was broader than that of Dalteparin. Thus, Tinzaparin contains a small amount of higher molecular weight molecules not present in Dalteparin (Fig. 5A).

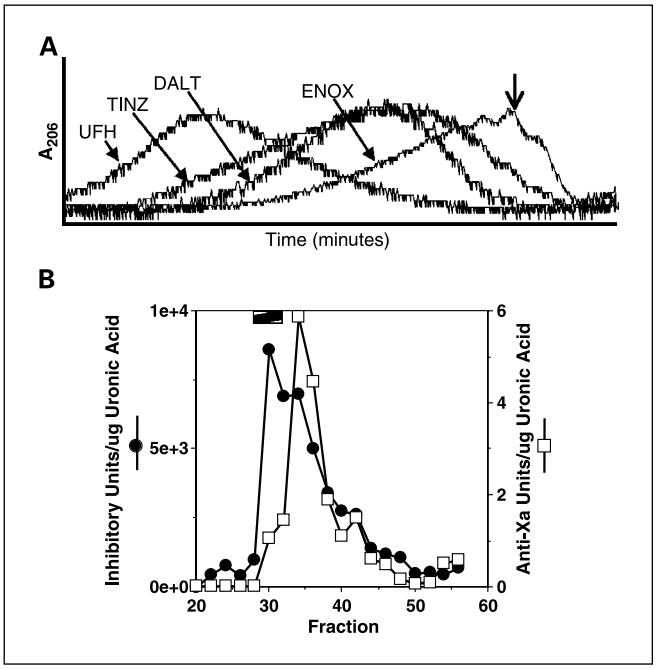

Fig. 5. Selectin inhibition by Tinzaparin is mediated mainly by high molecular weight fragments with relatively lower anti-Xa activity. *A,* aliquots of the five heparins (unfractionated heparin, the three low molecular weight heparins, and Fondaparinux) were run on an HPLC size exclusion system and their size profiles evaluated by tracking absorbance at 206 nm (the relevant part of the chromatogram shown is from t = 17.5-33.3 minutes). Open arrow, elution of the synthetic pentasaccharide Fondaparinux. *B,* an aliquot of Tinzaparin was run on the same HPLC system as in (*A*), and 0.5-minute fractions were collected post – UV detector. The total amount (μg) of uronic acid in each fraction was quantified using a carbazole assay. The ability of each fraction to inhibit binding of P-selectin to sLe^x was determined with appropriate dilutions so that all readings were in the linear range (~30-70% inhibition). One inhibitory unit is arbitrarily defined as 1% inhibition of P-selectin binding. The total number of anti-Xa units in each fraction was also determined in the linear range of that assay (if no activity was detected, the minimum detection limit of the assay was used). Total inhibitory units and total anti-Xa units were normalized to total uronic acid content. If no uronic acid was detected in a sample, the minimum detection limit of the assay was used for the calculation. The hatched box at the top of the graph designates fractions 28 to 32, which contain high P-selectin inhibitory activity and minimal anti-Xa activity, when normalized to uronic acid content. UFH, unfractionated heparin; TINZ, Tinzaparin; ENOX, Enoxaparin; DALT, Dalteparin.

To determine if this small population of larger fragments is disproportionately involved in P-selectin inhibition, fractions were collected following HPLC size separation of a larger aliquot of Tinzaparin. The total amount of heparin in each fraction was determined by measuring uronic acid content using a standard carbazole assay. Fractions were evaluated for their total number of P-selectin inhibitory units. A large amount of P-selectin inhibitory activity was noted in a small number of the highest molecular weight fractions (Fig. 5B). Indeed, this activity was seen even before uronic acid can be detected in the sample (data not shown), indicating that a relatively small amount of high molecular weight material has great P-selectin inhibitory activity. This result strongly supports our hypothesis that length is an important factor in determining inhibitory activity.

When evaluating the total number of anti-Xa units in the size fractionation profile of Tinzaparin (Fig. 5B), one can see that there is a shift between the peaks of P-selectin inhibition and anti-Xa. In fact, when these variables are normalized to the amount of uronic acid in each fraction, it can be seen that there is a small subset of fractions (fractions 28-32, denoted by the hatched box at the top of the graph) that contain a very high amount of P-selectin inhibitory activity and minimal anti-Xa activity (Fig. 5B). Thus, a commercially available heparin contains a subset of fragments that, at a given concentration, are capable of inhibiting P-selectin binding to its ligand while only minimally affecting the coagulation process.

Discussion

The close relationships of cancer and excessive systemic thrombosis are well-documented (25), and the need for anticoagulation in such situations is clear. Here, we address the converse issue, as to whether anticoagulation affects the spread of cancer. Numerous previous studies have shown unfractionated heparin inhibition of solid tumor metastasis in mice, and limited data suggest that the effect is likely to be relevant to humans as well. A basic assumption has, therefore, been that anticoagulation is the primary mechanism of its action in attenuating the metastatic process. As discussed in Introduction, heparins are complex mixtures of bioactive molecules with many effects potentially relevant to the overall biology of solid tumors. However, for the reasons mentioned in Introduction, we believe that the heparin effects relevant to the initial survival of tumor cells in the circulation are mainly due to inhibition of P-selectin and L-selectins, possibly along with blockade of intravascular fibrin formation via the fluid-phase coagulation pathway. Should heparin be given perioperatively as suggested, its other effects would benefit the patient during the time when tumor cells are not actively in the vasculature, as it has the potential to decrease primary tumor growth and invasion, as well as growth of established metastatic foci, due to inhibition of angiogenesis, heparinases, etc.

Almost all studies in rodents have used heparin at relatively high doses, and analysis of the various types of currently marketed heparins at clinically relevant doses had not been done. Here, we show, for the first time, that the ability of various heparins to inhibit P-selectin and L-selectin *in vitro* correlates with their ability to inhibit metastasis of two different types of syngeneic murine tumors. This reduction of metastasis is also shown to be independent of the anticoagulant activity of heparins, because Fondaparinux, an excellent anticoagulant, had no ability to inhibit metastasis at the same level of clinically tolerable anti-Xa activity, measured *in vivo*. In this regard, recent studies of metastasis inhibition with hirudin (a potent antithrombin) in mice (28) used a dose far above that recommended in humans (48) and caused anticoagulation levels sometimes beyond the upper limits of detection of their assay. Thus, while the previously reported effects of high-dose heparin and hirudin on fibrin formation supporting tumor metastasis are likely true, they may not be very relevant to the clinical situation in human patients. In support of this, we have also recently reported that the platelet- and leukocyte-mediated P-selectin and L-selectin–dependent microangiopathic coagulopathy of Trousseau syndrome can be induced by injecting tumor mucins into mice, even in the presence of hirudin (56).

Overall, while we do not discount a role for fibrin formation, our data indicate that selectin inhibition is the most practically

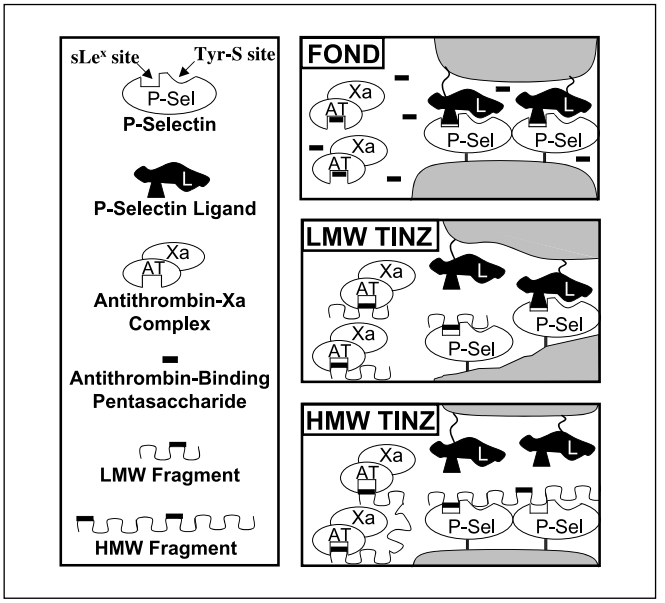

Fig. 6. Proposed explanation for selectin inhibitory activity being concentrated in higher molecular weight heparin fractions. P-selectin (presented by either activated platelets or endothelial cells) is known to have two binding pockets: one for the sialyl Lewis X moiety and another for the tyrosine sulfate – rich region of its native ligand PSGL-1, which is presented on leukocytes (58). The latter region of PSGL-1 is also rich in amino acids with carboxylate side chains. Other P-selectin or L-selectin ligands can be sulfated, sialylated mucins presented on endothelial cells or on carcinoma cells. Notably, these are also molecules presenting high densities of negatively charged sulfates and carboxylates. We hypothesize that heparins mimic these natural and pathologic ligands by virtue of their high density of sulfates and carboxylates (i.e., presenting a similar "clustered saccharide patch"; ref. 1). If the heparin chain is very short (as in Fondaparinux), it can only block one site at a time, making it a very poor inhibitor (*top*). A somewhat longer heparin chain could interact with both binding sites on P-selectin and have some inhibitory activity (*middle*). An even longer chain could block multiple P-selectin molecules and more dramatically affect the avidity of cell-to-cell interactions involving P-selectin ligands (*bottom*). In contrast, the antithrombin-factor Xa complex is a soluble one, and a single pentasaccharide (with the sequence identical to that found in Fondaparinux) is both necessary and sufficient to bind to antithrombin and catalyze the inactivation of Xa. Increasing the length of a heparin molecule would not change the outcome, unless there was more than one antithrombin-binding pentasaccharide in the sequence. However, unlike the case with the multivalent, multisite binding of P-selectin with its ligands in cell-to-cell interactions, the effect on antithrombin-Xa interactions would only be additive. The specificity of heparin structure for recognition by P-selectin is also not detailed in this model. However, previous work by us and others (see text) indicate a continuum of binding affinities, with 6-*O*-sulfation being necessary. FOND, Fondaparinux; LMW, low molecular weight; TINZ, Tinzaparin; HMW, high molecular weight.

important action of heparin affecting tumor metastasis at clinically relevant doses. The rank order of the ability of each heparin to inhibit P-selectin and L-selectin *in vitro* matched the effect on metastasis attenuation *in vivo*. Also, these studies used single boluses of heparin yielding clinically tolerable anti-Xa levels that are cleared from the system within a few hours. Thus, many of the other subsequent actions of heparin (e.g., angiogenesis inhibition, heparinase inhibition, etc.) are likely irrelevant to our current studies, as the single-dose heparin is not in the system long enough to influence these interactions. Moreover, these other actions of heparins are downstream of the selectin effect in our metastasis model, as tumor cells are introduced directly into the vasculature, where they interact first with P-selectin and L-selectin bearing blood and endothelial cells. In the clinical setting of continued heparin administration, they may or may not contribute to varying extents in different situations. It should be noted that, in the clinical setting, heparin would remain in the circulation for longer following each dose (because of its increased half-life in humans). Also, there would be a more extended duration of therapy. Thus, the dramatic effects seen in these single-injection studies would likely be even more pronounced in the clinical setting.

Our original model was that platelets and leukocytes support metastasis by interacting with selectin ligands expressed on the surface of tumor cells (10). However, the melanoma cell line used in these studies was previously shown to express low levels of sLe^x, a main component of selectin ligands (57), and experiments done in our laboratory indicated that recombinant P-selectin binds these cells minimally (data not shown). This presents the possibility that heparin inhibition of the melanoma cells might be due also to inhibition of endogenous selectin-ligand interactions (e.g., between PSGL-1 and P-selectin). This is supported by the recent finding that platelet aggregates around tumor cells can occur even when they do not carry P-selectin ligands (28). Interruption of these platelet aggregates by heparin inhibition of P-selectin and/or blockade of other effects of L-selectin may be sufficient to diminish metastasis. Therefore, the potential for heparin therapy may not necessarily be limited to patients whose tumor cells carry selectin ligands.

This work provides valuable information for designing a prospective clinical trial evaluating preoperative, perioperative, and postoperative heparin therapy in relation to surgery to remove a primary malignancy (10), which is a period of time in which malignant cells can enter the vasculature. It also shows the importance of choosing a heparin preparation known to be a potent inhibitor of P-selectin and L-selectin binding. The *in vitro* and *in vivo* data presented here would indicate that Tinzaparin would have more of an increase in metastasis-free survival than Dalteparin and Enoxaparin, and that Fondaparinux would have no effect on the outcome. Therefore, recent clinical trials demonstrating an improvement in patient survival with Dalteparin therapy (30, 49) might have seen an even bigger effect if Tinzaparin had been included in their studies. One might argue that perioperative heparin therapy might lead to adverse side effects, such as excessive anticoagulation and/or heparin-induced thrombocytopenia. However, there is an extensive history of use of these heparins in patients so the potential side effects are well documented. Moreover, we have identified a low molecular weight heparin, which traditionally carries fewer risks for harmful side effects, that is also capable of reducing metastasis via selectin inhibition. Additionally, our studies evaluating various fractions of Tinzaparin show that it should eventually be possible to isolate a subset of heparin fragments that allow administration of very low doses not affecting the coagulation state of a patient, but still having a significant ability to inhibit P-selectin. Finally, as anticoagulant therapy is frequently needed in cancer patients to treat thrombosis anyhow, we suggest that more attention should be paid in choosing the anticoagulant, as it might be possible to improve survival in a way that is independent of anticoagulation.

A brief discussion of the potential reasons for the differences in effects on anticoagulation and selectin inhibition is warranted (see Fig. 6 for details). The most likely reasons are due to the extended dual-site nature of the P-selectin lectin domain (58), and the multivalent avidity of selectin-ligand binding involving cell surfaces (1, 4–6). This stands in contrast to heparin-antithrombin binding, which involves only one pentasaccharide-binding site with a specific requirement for the precise structure found in Fondaparinux, which is also found scattered along the length of the longer heparin chains. These concepts are modeled in Fig. 6 and explained in the figure legend. Another possible (not mutually exclusive) explanation lies with the fact that as an anionic polysaccharide increases in length, many changes potentially occur in the middle of the chain, including changes in conformation and charge (59). Thus, extended heparin chains may have novel internal features that are preferred by P-selectin and L-selectin.

We and others have worked on designing new types of heparin to decrease anticoagulant activity yet retain other activities (42, 44, 55, 60, 61). However, such novel modified heparins will require complete preclinical and phase I to III clinical testing before they can eventually be approved for use in humans. Meanwhile, we have shown that no special modification is needed, and that an effective preparation could be isolated from a subset of fragments in currently Food and Drug Administration–approved forms of heparin.

Whereas this work addresses the importance of P-selectin and L-selectin inhibition by heparin in the reduction of metastasis, the findings are also of significance to the treatment of many other human diseases in which P-selectin and L-selectin have been shown to be important. These include inflammatory diseases, such as allergic dermatitis, asthma, atherosclerosis, and inflammatory bowel disease; diseases in which ischemia-reperfusion injury play a critical role, such as organ transplants, myocardial dysfunction following angioplasty of blocked coronary arteries, etc. (for general reviews, see refs. 7–9); and others, such as sickle cell disease (62). Thus, our work sends a cautionary note about the current clinical trend toward the use of smaller and smaller heparin fragments. Whereas pharmacokinetics may be better, side effects fewer, and anticoagulation equivalent, it is likely that hitherto unsuspected benefits of selectin inhibition in many diseases is in the process of being unwittingly discarded.

Acknowledgments

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References

1. Varki A. Selectin ligands. Proc Natl Acad Sci U S A 1994;91:7390–7.

2. Ley K, Tedder TF. Leukocyte interactions with vascular endothelium: new insights into selectin-mediated attachment and rolling. J Immunol 1995;155:525–8.

3. Kansas GS. Selectins and their ligands: current concepts and controversies. Blood 1996;88:3259–87.

4. McEver RP, Cummings RD. Role of PSGL-1 binding to selectins in leukocyte recruitment. J Clin Invest 1997; 100:485–92.

5. Lowe JB. Selectin ligands, leukocyte trafficking, and fucosyltransferase genes. Kidney Int 1997;51:1418–26.

6. Rosen SD. Ligands for L-selectin: homing, inflammation, and beyond. Annu Rev Immunol 2004;22: 129–56.

7. Bevilacqua MP, Nelson RM, Mannori G, Cecconi O. Endothelial-leukocyte adhesion molecules in human disease. Annu Rev Med 1994;45:361–78.

8. Lowe JB, Ward PA. Therapeutic inhibition of carbohydrate-protein interactions *in vivo*. J Clin Invest 1997; 99:822–6.

9. Ley K. The role of selectins in inflammation and disease. Trends Mol Med 2003;9:263–8.

10. Varki NM, Varki A. Heparin inhibition of selectin-mediated interactions during the hematogenous phase of carcinoma metastasis: rationale for clinical studies in humans. Semin Thromb Hemost 2002;28:53–66.

11. Kim YJ, Borsig L, Varki NM, Varki A. P-selectin deficiency attenuates tumor growth and metastasis. Proc Natl Acad Sci U S A 1998;95:9325–30.

12. Friederichs J, Zeller Y, Hafezi-Moghadam A, Gröne HJ, Ley K, Altevogt P. The CD24/P-selectin binding pathway initiates lung arrest of human A125 adenocarcinoma cells. Cancer Res 2000;60:6714–22.

13. Borsig L, Wong R, Feramisco J, Nadeau DR, Varki NM, Varki A. Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci U S A 2001;98:3352–7.

14. Borsig L, Wong R, Hynes RO, Varki NM, Varki A. Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc Natl Acad Sci U S A 2002;99:2193–8.

15. Ludwig RJ, Boehme B, Podda M, et al. Endothelial P-selectin as a target of heparin action in experimental melanoma lung metastasis. Cancer Res 2004;64: 2743–50.

16. Zacharski LR, Ornstein DL. Heparin and cancer. Thromb Haemost 1998;80:10–23.

17. Engelberg H. Actions of heparin that may affect the malignant process. Cancer 1999;85:257–72.

18. Hejna M, Raderer M, Zielinski CC. Inhibition of metastases by anticoagulants. J Nat Cancer Inst 1999;91:22–36.

19. Smorenburg SM, Van NCJF. The complex effects of heparins on cancer progression and metastasis in experimental studies. Pharmacol Rev 2001;53:93–105.

20. Kakkar AK, Hedges RA, Williamson RCN, Kakkar VV. Perioperative heparin therapy inhibits late death from metastatic cancer. Int J Oncol 1995;6:885–8.

21. Hettiarachchi RJK, Smorenburg SM, Ginsberg J, Levine M, Prins MH, Büller HR. Do heparins do more than just treat thrombosis? The influence of heparins on cancer spread. Thromb Haemost 1999;82:947–52.

22. Ornstein DL, Zacharski LR. The use of heparin for treating human malignancies. Haemostasis 1999;29 Suppl 1:48–60.

23. Smorenburg SM, Hettiarachchi RJK, Vink R, Büller HR. The effects of unfractionated heparin on survival in patients with malignancy—a systematic review. Thromb Haemost 1999;82:1600–4.

24. Zacharski LR, Ornstein DL, Mamourian AC. Low-molecular-weight heparin and cancer. Semin Thromb Hemost 2000;26 Suppl 1:69–77.

25. Rickles FR, Falanga A. Molecular basis for the relationship between thrombosis and cancer. Thromb Res 2001;102:V215–24.

26. Palumbo JS, Kombrinck KW, Drew AF, et al. Fibrinogen is an important determinant of the metastatic potential of circulating tumor cells. Blood 2000;96: 3302–9.

27. Francis JL, Amirkhosravi A. Effect of antihemostatic agents on experimental tumor dissemination. Semin Thromb Hemost 2002;28:29–38.

28. Im JH, Fu W, Wang H, et al. Coagulation facilitates tumor cell spreading in the pulmonary vasculature during early metastatic colony formation. Cancer Res 2004;64:8613–9.

29. Hu L, Lee M, Campbell W, Perez-Soler R, Karpatkin S. Role of endogenous thrombin in tumor implantation, seeding, and spontaneous metastasis. Blood 2004; 104:2746–51.

30. Lee AY, Rickles FR, Julian JA, et al. Randomized comparison of low molecular weight heparin and coumarin derivatives on the survival of patients with cancer and venous thromboembolism. J Clin Oncol 2005;23:2123–9.

31. Zacharski LR, Henderson WG, Rickles FR, et al. Effect of warfarin anticoagulation on survival in carcinoma of the lung, colon, head and neck, and prostate. Final report of VA Cooperative Study 75. Cancer 1984;53:2046–52.

32. Levine M, Hirsh J, Gent M, et al. Double-blind randomised trial of a very-low-dose warfarin for prevention of thromboembolism in stage IV breast cancer. Lancet 1994;343:886–9.

33. Rosenberg RD, Lam L. Correlation between structure and function of heparin. Proc Natl Acad Sci U S A 1979;76:1218–22.

34. Rosenberg RD. Biochemistry and pharmacology of low molecular weight heparin. Semin Hematol 1997; 34 Suppl 4:2–8.

35. Hirsh J, Raschke R. Heparin and low-molecular-weight heparin: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest 2004;126:188–203S.

36. Lindahl U, Kjellén L. Heparin or heparan sulfate—what is the difference? Thromb Haemost 1991;66: 44–8.

37. Skinner MP, Fournier DJ, Andrews RK, Gorman JJ, Chesterman CN, Berndt MC. Characterization of human platelet GMP-140 as a heparin-binding protein. Biochem Biophys Res Commun 1989;164:1373–9.

38. Nelson RM, Cecconi O, Roberts WG, Aruffo A, Linhardt RJ, Bevilacqua MP. Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. Blood 1993;82:3253–8.

39. Norgard-Sumnicht KE, Varki NM, Varki A. Calcium-dependent heparin-like ligands for L-selectin in nonlymphoid endothelial cells. Science 1993;261:480–3.

40. Koenig A, Norgard-Sumnicht KE, Linhardt R, Varki A. Differential interactions of heparin and heparan sulfate glycosaminoglycans with the selectins—implications for the use of unfractionated and low molecular weight heparins as therapeutic agents. J Clin Invest 1998;101:877–89.

41. Ma YQ, Geng JG. Heparan sulfate-like proteoglycans mediate adhesion of human malignant melanoma A375 cells to P-selectin under flow. J Immunol 2000; 165:558–65.

42. Xie X, Rivier AS, Zakrzewicz A, et al. Inhibition of selectin-mediated cell adhesion and prevention of acute inflammation by nonanticoagulant sulfated saccharides—studies with carboxyl-reduced and sulfated heparin and with trestatin A sulfate. J Biol Chem 2000;275:34818–25.

43. Folkman J, Shing Y. Control of angiogenesis by heparin and other sulfated polysaccharides. Adv Exp Med Biol 1992;313:355–64.

44. Wang L, Brown JR, Varki A, Esko JD. Heparin's anti-inflammatory effects require glucosamine 6-*O*-sulfation and are mediated by blockade of L- and P-selectins. J Clin Invest 2002;110:127–36.

45. Linhardt RJ, Gunay NS. Production and chemical processing of low molecular weight heparins. Semin Thromb Hemost 1999;25 Suppl 3:5–16.

46. Valentine KA, Hull RD, Pineo GF. Low-molecular-weight heparin therapy and mortality. Semin Thromb Hemost 1997;23:173–8.

47. Petitou M, Duchaussoy P, Herbert JM, et al. The synthetic pentasaccharide fondaparinux: first in the class of antithrombotic agents that selectively inhibit coagulation factor Xa. Semin Thromb Hemost 2002;28: 393–402.

48. Weitz JI, Hirsh J, Samama MM. New anticoagulant drugs: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest 2004;126: 265–86S.

49. Kakkar AK, Levine MN, Kadziola Z, et al. Low molecular weight heparin, therapy with dalteparin, and survival in advanced cancer: the fragmin advanced malignancy outcome study (FAMOUS). J Clin Oncol 2004;22:1944–8.

50. Toyoda H, Yamamoto H, Ogino N, Toida T, Imanari T. Rapid and sensitive analysis of disaccharide composition in heparin and heparan sulfate by reversed-phase ion-pair chromatography on a 2 micron porous silica gel column. J Chromatogr A 1999;830:197–201.

51. Filisetti-Cozzi TMCC, Carpita NC. Measurement of uronic acids without interference from neutral sugars. Anal Biochem 1991;197:157–62.

52. Cheer SM, Dunn CJ, Foster R. Tinzaparin sodium: a review of its pharmacology and clinical use in the prophylaxis and treatment of thromboembolic disease. Drugs 2004;64:1479–502.

53. Fidler IJ. New developments in *in vivo* models of neoplasia. Cancer Metastasis Rev 1991;10:191–2.

54. Yoshikawa N, Nakamura K, Yamaguchi Y, Kagota S, Shinozuka K, Kunitomo M. Suitable indices for evaluating the intensity of tumor metastasis in a mouse experimental metastatic model. In vivo 2001;15:485–8.

55. Wei M, Tai G, Gao Y, et al. Modified heparin inhibit P-selectin-mediated cell adhesion of human colon carcinoma cells to immobilized platelets under dynamic flow conditions. J Biol Chem 2004;279:29202–10.

56. Wahrenbrock M, Borsig L, Le D, Varki N, Varki A. Selectin-mucin interactions as a probable molecular explanation for the association of Trousseau syndrome with mucinous adenocarcinomas. J Clin Invest 2003; 112:853–62.

57. Ohyama C, Tsuboi S, Fukuda M. Dual roles of sialyl Lewis X oligosaccharides in tumor metastasis and rejection by natural killer cells. EMBO J 1999;18: 1516–25.

58. Somers WS, Tang J, Shaw GD, Camphausen RT. Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe(X) and PSGL-1. Cell 2000; 103:467–79.

59. Manzi AE, Higa HH, Diaz S, Varki A. Intramolecular self-cleavage of polysialic acid. J Biol Chem 1994; 269:23617–24.

60. Vlodavsky I, Ishai-Michaeli R, Mohsen M, et al. Modulation of neovascularization and metastasis by species of heparin. Adv Exp Med Biol 1992;313: 317–27.

61. Lapierre F, Holme K, Lam L, et al. Chemical modifications of heparin that diminish its anticoagulant but preserve its heparanase—inhibitory, angiostatic, anti-tumor and anti-metastatic properties. Glycobiology 1996;6:355–66.

62. Matsui NM, Varki A, Embury SH. Heparin inhibits the flow adhesion of sickle red blood cells to P-selectin. Blood 2002;100:3790–6.