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Abstract

Vascular cell adhesion molecules, P- and L-selectins, facilitate metastasis of cancer cells in mice by mediating interactions with platelets, endothelium, and leukocytes. Heparanase is an endoglycosidase that degrades heparan sulfate of extracellular matrix, thereby promoting tumor invasion and metastasis. Heparin is known to efficiently attenuate metastasis in different tumor models. Here we identified modified, nonanticoagulant species of heparin that specifically inhibit selectin-mediated cell-cell interactions, heparanase enzymatic activity, or both. We show that selective inhibition of selectin interactions or heparanase with specific heparin derivatives in mouse models of MC-38 colon carcinoma and B16-BL6 melanoma attenuates metastasis. Selectin-specific heparin derivatives attenuated metastasis of MC-38 carcinoma, but heparanase-specific derivatives had no effect, in accordance with the virtual absence of heparanase activity in these cells. Heparin derivatives had no further effect on metastasis in mice deficient in P- and L-selectin, indicating that selectins are the primary targets of heparin antimetastatic activity. Selectin-specific and heparanase-specific derivatives attenuated metastasis of B16-BL6 melanomas to a similar extent. When mice were injected with a derivative containing both heparanase and selectin inhibitory activity, no additional attenuation of metastasis could be observed. Thus, selectin-specific heparin derivatives efficiently attenuated metastasis of both tumor cell types whereas inhibition of heparanase led to reduction of metastasis only in tumor cells producing heparanase.
P-selectin- and heparanase-dependent antimetastatic activity of non-anticoagulant heparins

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ABSTRACT Vascular cell adhesion molecules, P- and L-selectins, facilitate metastasis of cancer cells in mice by mediating interactions with platelets, endothelium, and leukocytes. Heparanase is an endoglycosidase that degrades heparan sulfate of extracellular matrix, thereby promoting tumor invasion and metastasis. Heparin is known to efficiently attenuate metastasis in different tumor models. Here we identified modified, nonanticoagulant species of heparin that specifically inhibit selectin-mediated cell-cell interactions, heparanase enzymatic activity, or both. We show that selective inhibition of selectin interactions or heparanase with specific heparin derivatives in mouse models of MC-38 colon carcinoma and B16-BL6 melanoma attenuates metastasis. Selectin-specific heparin derivatives attenuated metastasis of MC-38 carcinoma, but heparanase-specific derivatives had no effect, in accordance with the virtual absence of heparanase activity in these cells. Heparin derivatives had no further effect on metastasis in mice deficient in P- and L-selectin, indicating that selectins are the primary targets of heparin antimetastatic activity. Selectin-specific and heparanase-specific derivatives attenuated metastasis of B16-BL6 melanomas to a similar extent. When mice were injected with a derivative containing both heparanase and selectin inhibitory activity, no additional attenuation of metastasis could be observed. Thus, selectin-specific heparin derivatives efficiently attenuated metastasis of both tumor cell types whereas inhibition of heparanase led to reduction of metastasis only in tumor cells producing heparanase.—Hostettler, N., Naggi, A., Torri, G., Ishai-Michaeli, R., Casu, B., Vlodavsky, I., Borsig, L. P-selectin- and heparanase-dependent antimetastatic activity of non-anticoagulant heparins. FASEB J. 21, 3562–3572 (2007)

Key Words: thrombin generation · LMWH · selectin interactions · heparin derivatives

Heparin and low molecular weight heparin (LMWH) have been widely used as anticoagulants for more than 50 years. Cancer patients suffering from recurrent thrombosis are frequently treated with heparin and LMWH (1–9). Despite several clinical studies indicating an effect of heparin and LMWH on prolonging patients’ survival, the anti-neoplastic effect of heparin remains controversial (1–3). Although thrombotic complications associated with cancer have an adverse effect on prognosis in all cancer types, patients with such complications usually die from cancer rather than fatal thrombosis (10). Thus, the improved survival of cancer patients treated with heparin or LMWH is likely due to a direct effect on cancer progression. Based on these observations, several randomized-controlled trials were performed recently (5, 6, 8, 9). Although there was no improvement in survival in patients with an originally poor prognosis, a subgroup of patients with a better prognosis demonstrated a statistically significant increase in survival (5). Similarly, patients with advanced solid tumor malignancy treated with LMWH demonstrated an improvement in survival rate (6, 8, 9). Meanwhile, other studies showed that heparin and LMWH are not just mere anticoagulants, but rather complex glycosaminoglycans with several biological functions (11, 12). Among other biological activities, heparin and LMWH are known to affect thrombin generation (10), cause release of tissue factor pathway inhibitor (TFPI) from endothelial surfaces (13), regulate growth factor receptor binding and activity (14), affect angiogenesis (15), inhibit heparanase enzymatic activity (16), and reduce selectin-mediated interactions (17, 18). The hypothesis that heparin and LMWH directly influence cancer progression and metastasis was further supported by experimental studies in mouse models (for reviews, see refs. 12, 19, 20). Heparin and LMWH were shown to attenuate experimental metastasis in several mouse models of carcinomas and melanomas (16, 21–24). However, nonanticoagulant heparins could still efficiently inhibit metastasis; therefore, anticoagulation is just one activity of heparin in this process (16, 23, 25, 26). Heparin was also shown to affect metastasis through inhibition of heparanase activity (16, 27). The enhanced expression...
of heparanase in mammary adenocarcinomas, lymphomas, and melanomas is causally associated with increased invasiveness and metastasis (16, 27, 28). Heparin or LMWH treatment attenuated metastasis by inhibition of heparanase in experimental metastasis of human and rat mammary carcinoma and mouse melanoma (27, 29). We and others have reported that heparin attenuated metastasis in mouse models of carcinomas and melanomas primarily through inhibition of selectin interactions (17, 21, 22, 24). Selectins are vascular cell adhesion molecules promoting initial leukocyte-endothelial interactions. In addition, P- and L-selectin are implicated in pathological processes involving inflammation, reperfusion injury, and cancer (30, 31). The absence of P- and/or L-selectin led to attenuation of metastasis in mouse models, thus implicating both selectins in cancer metastasis (17, 21, 22, 32). Heparin is a potent inhibitor of P- and L-selectin (33). Heparin injection shortly before tumor cells was found to inhibit P-selectin-mediated platelet/tumor cell interactions; injection of heparin at later time inhibited metastasis in an L-selectin-dependent manner (21, 24, 32). Although there is general agreement on the potential of heparin to attenuate metastasis in mouse models and to improve prognosis of cancer patients, the underlying molecular mechanism of heparin action requires further clarification (10, 19, 34).

In the present study the elucidation of heparanase and selectin contribution to metastasis was analyzed using modified nonanticoagulant species of heparins. We previously observed that heparin primarily affected selectin-dependent interactions with carcinoma cells (17, 21) whereas the inhibition of heparanase was shown to attenuate metastasis in mouse melanoma (16, 27). Results from the current study show that, depending on the cell type, attenuation of metastasis could be achieved equally well by inhibition of selectin interactions or heparanase enzymatic activity.

MATERIALS AND METHODS

Cell lines and reagents

Mouse colon carcinoma cell line MC-38 stably expressing GFP MC-38GFP (21) and mouse melanoma cell line B16-BL6 (27) were grown in DMEM with 4.5 g/L of glucose supplemented with 10% FCS medium (Invitrogen, Carlsbad, CA, USA). Human colon carcinoma cells LS180 (ATCC, Manassas, VA, USA) were grown in αMEM media (Invitrogen) supplemented with 10% FCS (Invitrogen). All reagents were from Sigma (St. Louis, MO, USA) unless otherwise stated.

Mice

P-selectin-deficient mice (P-sel−/−) in a C57BL/6j background and control wild-type (wt) C57BL/6j mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). PL-selectin-double-deficient (PL-sel−/−) mice in a C57BL/6j background have been described (21).

Heparin derivatives

The reference heparin (H, mol wt 18,000) was a commercial preparation from pig mucosa (Laboratori Derivati Organici, Trino Vercellese, Italy). Detailed preparation and characterization of all heparin derivatives have been reported, including notations (35). The following derivatives were prepared: 1) two extensively 6-O-desulfated heparins, one of which (6Odes-H, mol wt 20,400) essentially retains the original content of 2-O-sulfate groups, and the other also partially (~15%) 2-O-desulfated (6Odes,p2Odes-H, mol wt 16,000); 2) two extensively 2-O-desulfated heparins, one retaining the original IdoA configuration (2Odes-H IdoA) and the other (2Odes-H GaLa), where the original residues were converted to L-galacturonic acids; 3) two partially 6-O-sulfated derivatives (3O6des-H and 4O6des-H); 4) N-acetylated heparins (5O-H, 6O-NaH, 7O-NaH, and 10O-Na-H); 5) a glycol-split heparin of the RO (reduced oxyheparins) type RO-H; 6) the RO, N-acetylated derivatives 5O-RO,H and 10O-RO,H; and 7) fluorescein-labeled RO,H and 10O-RO,H (prepared as reported for heparin) (36). All samples were recovered after desalting by freeze-drying and characterized by NMR spectroscopy.

Flow cytometry analysis

Selectin ligands expressed on MC-38 and B16-BL6 tumor cells were analyzed with mouse selectins as described previously (21). Briefly, cells were detached with PBS containing 2 mM EDTA for 5 min at 37°C, and washed with Hanks’ balanced salt solution (HBSS) prior to blocking with 0.5% BSA in HBSS. Mouse P-selectin chimera was preincubated with a biotinylated goat-anti-human IgG Ab for 1 h at room temperature (RT) and added to cells for 1 h at 4°C. After a wash, streptavidin-PE-Cy5 (Becton Dickinson, Mountain View, CA, USA) was added for another 20 min. Cells were washed with HBSS and resuspended in HBSS/BSA.

Determination of anti-Xa levels in mouse plasma

Mice were intravenously (i.v.) injected with 150 µg of heparin and its derivatives via the tail vein. Twenty minutes after injection, blood was collected by cardiac puncture into a syringe filled with 30 µL of 10 mM EDTA in PBS. Samples were centrifuged twice at 1000 g and the aliquoted plasma was stored at −80°C. Anti-Xa activity was determined as described previously (24). Briefly, 1.25 µL of plasma was added to 155 µL of 25 mM HEPES/159 mM NaCl (pH 7.5) together with human antithrombin III (3.3 µg/well; Enzyme Research Laboratories, South Bend, IN, USA) and human factor Xa (0.02 µg/well, Enzyme Research Laboratories). Samples were incubated with 25 µg of synthetic factor Xa chromogenic substrate (Diapharma, Columbus, OH, USA) for 15 min. The reaction was stopped by addition of 20% acetic acid and measured at 405 nm using a GENios ELISA reader (Tecan, Männedorf, Switzerland). Plasma anticoagulant activity of the heparin derivatives was calculated in anti-Xa units/ml in comparison to a standard curve. All measurements were done in triplicate and repeated three times with three independent mouse samples.

Determination of tissue factor pathway inhibitor (TFPI) in mouse plasma

Plasma samples were dialyzed against 20 mM Tris/Cl, pH 7.4. Plasma diluted 4-fold was separated on nondenaturing 8% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and blocked with 5% nonfat milk in TBS (block-
Mice were euthanized at 2 h and blood was collected by retro-orbital bleeding at different time points.

Heparin species were tested for their ability to inhibit heparanase, as described previously (16, 35). Briefly, sulfate-labeled ECM coating the surface of 35 mm culture dishes was incubated (4 h, 37°C, pH 6.0) with recombinant human heparanase (40 ng/ml) in the absence or presence of different amounts (0.2–5 μg/ml) of each heparin species. The proteoglycan fragments were separated by gel filtration and the radioactivity in each fraction was counted (16, 35).

Effects on FGF-2 displacement and mitogenic activity
Release of ECM-bound 125I-FGF-2 and stimulation of FGF-2 mitogenic activity were performed, as described (16, 35).

Selectin inhibitory activity
The ability of heparin and heparin derivatives to inhibit adhesion of LS180 cells to immobilized P- and L-selectin was examined as described previously (17). Briefly, ELISA plates (Nunc, Rochester, NY, USA) coated overnight with soluble protein A were blocked with 1% BSA in HBSS (HBSS/BSA) (Nunc, Rochester, NY, USA) coated overnight with soluble adhesion of LS180 cells to immobilized P- and L-selectin was performed, as described (16, 35). The FASEB Journal

Bioavailability of heparin derivatives in the circulation
Wild-type mice (C57BL/6J) were i.v. injected with 300 µg of FITC-conjugated heparin derivatives in 120 µl of PBS (either RO.H or 106NA-RO.H) via the tail vein. Blood samples were collected by retro-orbital bleeding at different time points. Mice were euthanized at 2 h and blood was collected by cardiac puncture. The anticoagulated blood was centrifuged at 2000 g for 10 min. Plasma (80 µl of the supernatant) was transferred to an ELISA plate and fluorescence was measured with a GENios ELISA reader (Tecan). Plasma from a mouse receiving PBS only was used as a control.

Platelet/tumor cell aggregation in vivo
Lungs were analyzed at various time points after i.v. injection of tumor cells by sectioning and staining as described previously (17). Frozen lung sections were incubated with anti-CD41 Ab (Becton Dickinson), followed by detection with goat anti-rat Ab conjugated with Alexa568 (Invitrogen). The extent of platelet/tumor cell association was quantified in 20 view fields (40× magnification) by immunofluorescence microscopy.

Experimental metastasis model
C57BL/6J mice were i.v. injected with 150 µg of heparin derivatives or PBS, followed by injection of 3 × 106 MC-38GFP colon carcinoma cells or B16-BL6 melanoma cells via the tail vein 10 min later. Mice injected with MC-38GFP cells were euthanized after 4 wk and metastatic foci were counted on dissected lungs. Lungs were homogenized and the GFP fluorescence was measured (21). Mice injected with B16-BL6 melanoma cells were euthanized after 14 days and the metastatic foci were counted. For the subcutaneous (s.c.) injection, 300 µg of heparin derivatives was applied 30 min before i.v. injection of B16-BL6 cells.

RESULTS
Selectin ligand expression and heparanase activity of MC-38 and B16-BL6 cells
To determine the role of heparin during metastasis, we analyzed two mouse cell lines, MC-38 colon carcinoma and B16-BL6 melanoma cells, in which reduction of metastasis has been shown to be achieved by inhibition of either selectin-mediated interactions or heparanase enzymatic activity, respectively. When selectin ligand expression was compared by flow cytometry, both MC-38 and B16-BL6 cells expressed P-selectin ligands (Fig. 1A). P-selectin binding to B16-BL6 cells was less efficient than to MC-38 cells. L-selectin ligands were detected on both cells, but no binding of E-selectin could be observed (Fig. 1A and ref. 21). Heparanase activity expressed by both cell lines was determined by analysis of sulfate-labeled material released from metabolically labeled ECM (27). B16-BL6 cells exhibited significant levels of heparanase activity, as detected by the large amount of low molecular weight material eluted in fractions 25–35 (Fig. 1B). Eluted labeled fragments were shown to be degradation products of heparan sulfate (HS), resistant to further digestion with papain and chondroitinase ABC, and susceptible to deamination by nitrous acid (16, 35). In contrast, MC-38 cells exhibited relatively little heparanase activity, and the major sulfate-labeled product was of high molecular weight eluted in fractions 5–10 (Fig. 1B). This material provides a readily accessible soluble substrate for the heparanase enzyme and is produced by proteolytic enzymes degrading the proteoglycan core protein and residing in the ECM and cell lysates (16). We have previously demonstrated that degradation of heparan sulfate proteoglycans in the ECM involves the sequential action of proteases and heparanase and that inhibition of heparanase is associated with a marked accumulation of soluble, nearly intact proteoglycans (16, 38). This was also the case for incubation of the ECM with B16-BL6 melanoma cells in the presence of RO.H or 106NA-RO.H (data not shown). Altogether, we compared MC-38 cells, expressing primarily selectin...
Chemically modified heparin derivatives inhibit selectins

A series of modified heparin derivatives of different degrees of sulfation, N-acetylation, and chain flexibility, including 2-O- and 6-O-desulfated, N-acetylated, and “reduced oxyheparins” (RO heparins), was prepared and analyzed for heparanase inhibitory activity (35). RO derivatives are a form of “glycol-split” heparins where the C(2)-C(3) bonds of all nonsulfated uronic acid residues are cleaved, with retention of the original chain length of heparins. Since glycol-split residues act as flexible joints along the polysaccharide chains (35), selected RO derivatives were inserted in this study because they enabled us to evaluate the effect of chain flexibility on the biological activities of interest. RO.H has been shown to inhibit experimental metastasis (26, 39) and block P-selectin-mediated acute inflammation (40). To identify heparin derivatives with selectin inhibitory activity, we tested 15 different compounds for their potential to inhibit adhesion of LS180 carcinoma cells to immobilized selectins (Supplemental Fig. 1).

The different heparin derivatives vary significantly in their ability to inhibit selectins as determined by their IC_{50} values (Table 1). Two heparin derivatives, 58% N-acetylated heparin (58NA-H) and reduced oxyheparin (RO.H), had better P-selectin inhibitory activity (IC_{50} 20–25 μg/ml) than unmodified, unfractonated heparin (H). In addition, RO.H was the only derivative that preserved L-selectin inhibitory potential comparable to heparin (IC_{50} values of 45 and 40 μg/ml, respectively). In contrast, 100% N-acetylated reduced oxyheparin (100NA-RO.H) was virtually inactive as an inhibitor of both P- and L-selectin (IC_{50}>250 μg/ml). In general, N-desulfation (N-acetylated derivative), 2-O-desulfation, and 50% glycol-splitting of heparin resulted in a marked reduction of selectin inhibitory activity (Table 1). The 6-O-sulfate was previously found to be critical for selectin binding (41), and its removal completely eliminated its recognition by selectins (Table 1). However, a partial 6O- or N-desulfation (39–70%), followed by N-acetylation, led to products still endowed with potent selectin inhibitory properties (IC_{50} values for P-selectin between 25 and 40 μg/ml), indicating that a certain de-O-sulfation did not interfere with P-selectin recognition but eliminated interactions with L-selectin. Based on the selectin binding properties, two heparin derivatives with a good inhibitory activity (58NA-H and RO.H) and one derivative with no selectin-inhibitory activity (100NA-RO.H) were selected for further study.

Heparin has a variety of biological activities that can help explain its effects on cancer metastasis and angiogenesis. Six of these activities were investigated in more detail: 1) anticoagulant activity; 2) potential to release tissue factor pathways inhibitor, TFPI; 3) inhibition of selectin-mediated interactions (discussed above); 4) inhibition of heparanase; 5) release of ECM bound fibroblast growth factor (FGF-2); and 6) stimulation of

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**Figure 1.** Characterization of selectin ligands and heparanase activity in mouse colon carcinoma MC-38 and melanoma B16-BL6 cell lines. A) Selectin ligands. Cells were probed with mouse P-selectin and L-selectin chimeras and analyzed by flow cytometry. Filled areas (gray): selectin-stained cells; boldface line: selectin staining in the presence of 5 mM EDTA; thin line represents control profiles with secondary antibody only. B) Heparanase activity. MC-38 cells and B16-BL6 melanoma cells were incubated with sulfate-labeled ECM. Degradation fragments released into the incubation medium were separated by gel filtration on Sepharose 6B as described in Materials and Methods. Fractions 25–35 corresponded to HS degradation fragments and material eluted in fractions 5–10 corresponded to undigested HS proteoglycans.
TABLE 1. IC_{50} values of heparin derivatives for inhibition of selectins

<table>
<thead>
<tr>
<th>Heparin and heparin derivatives</th>
<th>Inhibition of cell adhesion (IC_{50}, µg/ml)</th>
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<tbody>
<tr>
<td>H</td>
<td>P-selectin</td>
</tr>
<tr>
<td>H (H)</td>
<td>40</td>
</tr>
<tr>
<td>2-O-desulfated H (iduron. acid form) (2Odes-H IdoA)</td>
<td>&gt;250</td>
</tr>
<tr>
<td>2-O-desulfated H (galact. acid form) (2Odes-H GalA)</td>
<td>110</td>
</tr>
<tr>
<td>6-O-desulfated H (6Odes-H)</td>
<td>&gt;250</td>
</tr>
<tr>
<td>6-O-desul., partially 2-O-desulf. H (6Odes,p2Odes-H)</td>
<td>&gt;250</td>
</tr>
<tr>
<td>51% 6-O-desulfated H (^{14}6Odes-H)</td>
<td>30</td>
</tr>
<tr>
<td>42% 6-O-desulfated H (^{16}6Odes-H)</td>
<td>30</td>
</tr>
<tr>
<td>39% N-acetylated H (^{96}Odes-H)</td>
<td>25</td>
</tr>
<tr>
<td>50% N-acetylated H (^{90}NA-H)</td>
<td>35</td>
</tr>
<tr>
<td>58% N-acetylated H (^{86}NA-H)</td>
<td>25</td>
</tr>
<tr>
<td>70% N-acetylated H (^{70}NA-H)</td>
<td>40</td>
</tr>
<tr>
<td>100% N-acetylated H (^{100}NA-H)</td>
<td>175</td>
</tr>
<tr>
<td>50% N-acetylated, 25% glycol-split H (^{50}NA-RO.H)</td>
<td>27</td>
</tr>
<tr>
<td>100% N-acetylated, 25% glycol-split H (^{100}NA-RO.H)</td>
<td>&gt;250</td>
</tr>
<tr>
<td>25% Glycol-split H (RO.H)</td>
<td>20</td>
</tr>
</tbody>
</table>

Fitting 2 mitogenic activity (Table 2). We aimed to evaluate the contribution of selectin-mediated interactions and heparanase activity to the metastatic process. Heparin derivatives were previously evaluated for their potency to inhibit heparanase (35). Although ^{35}NA-H was a poor inhibitor of heparanase, RO.H and ^{100}NA-RO.H were both excellent inhibitors (Table 2 and 35). These heparin derivatives were tested for their anticoagulant activity in vivo. The anti-Xa activity was determined in plasma of mice i.v. injected with these heparin derivatives (Table 2). All three derivatives were found to be nonanticoagulant. N-Desulfation of heparin was previously described to eliminate the binding sites for antithrombin, thus consistently decreasing the anticoagulant properties (41). When these heparin derivatives were tested in normal mouse plasma ex vivo, the anti-Xa levels were about half the values measured in vivo (data not shown). These findings indicate that there could be other factors contributing to anticoagulation in vivo. Heparin is known to release tissue factor pathway inhibitor (TFPI) from the vascular endothelium, thereby increasing its plasma concentration severalfold (42–44). Whereas heparin injection increased TFPI concentration in the plasma of a mouse ~2-fold over basal levels, two of the derivatives (RO.H, ^{100}NA-RO.H) were less efficient than heparin (Table 2). The observed reduction in TFPI release was expected to be associated with the loss of sulfation, which is important for TFPI-heparin interactions (43). However, the TFPI release observed for RO.H is significantly lower than for H (Table 2) despite the same degree of sulfation. Heparin can efficiently release growth factors sequestered in the extracellular matrix (ECM), thus mobilizing the pool of molecules capable of potentiating tumor growth and metastasis (14, 16, 45). Compounds ^{35}NA-H and RO.H exhibited FGF-2-releasing activity comparable to heparin whereas ^{100}NA-RO.H showed limited efficacy (Table 2). Finally, stimulation of FGF-2 mitogenic activity by heparin derivatives showed that ^{100}NA-RO.H has a reduced potential, and the other two derivatives are similar to heparin. In conclusion, three heparin derivatives were identified that possess relatively little anticoagulant activity but are unique in their selectin and heparanase inhibitory activity: ^{35}NA-H as

TABLE 2. Biological activities of heparin derivatives

<table>
<thead>
<tr>
<th>Heparin derivatives</th>
<th>Anti-Xa activity (units/ml)^{a}</th>
<th>TFPI release activity (%)^b</th>
<th>Inhibition of selectin-cell adhesion (IC_{50}, µg/ml)</th>
<th>Heparanase inhibitory activity (%)^c</th>
<th>FGF-2 release from ECM (%)^d</th>
<th>Stimulation of FGF-2 mitogenic activity (%)^e</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>&gt;4</td>
<td>100</td>
<td>40</td>
<td>40</td>
<td>78.7</td>
<td>66.1</td>
</tr>
<tr>
<td>^{35}NA-H</td>
<td>0.1</td>
<td>98.2</td>
<td>25</td>
<td>125</td>
<td>40.9</td>
<td>83.4</td>
</tr>
<tr>
<td>RO.H</td>
<td>0.05</td>
<td>72.8</td>
<td>20</td>
<td>45</td>
<td>95.4</td>
<td>84.5</td>
</tr>
<tr>
<td>^{100}NA-RO.H</td>
<td>&lt;0.01</td>
<td>67.3</td>
<td>&gt;250</td>
<td>&gt;500</td>
<td>82.8</td>
<td>23.7</td>
</tr>
</tbody>
</table>

^a The anticoagulant activity of heparin and its derivatives was determined in plasma samples taken from mice 10 min after injection of 150 µg of the heparin derivative, as described in Materials and Methods. ^b Quantitation of TFPI release in the plasma of mice injected with 150 µg of a heparin derivative analyzed 10 min after the injection. Percentage of the activity of heparin. ^c Percent inhibition in the presence of a heparin derivative (1 µg/ml). ^d Percent release of ECM-bound FGF-2 in the presence of a heparin derivative (0.5 µg/ml). 100% = total ECM-bound FGF-2. ^e Percent stimulation of FGF-2-induced ^3H-histidine incorporation (BAF3 lymphoid cells) in the presence of a heparin derivative (0.5 µg/ml). 100% = ^3H-histidine incorporation in the presence of heparin.
an inhibitor of P- and L-selectin and a poor heparanase inhibitor; RO.H as a good inhibitor of P- and L-selectin as well as of heparanase activity; and 100NA-RO.H as a rather specific inhibitor of heparanase activity.

Pharmacokinetics of heparin derivatives in vivo

To determine an effective dose of heparin derivatives for the metastasis experiments, we first analyzed the bioavailability of the three heparin derivatives in vivo. Fluorescein (FITC)-conjugated heparin derivatives were i.v. injected and their plasma levels were determined. Derivatives 58NA-H and 100NA-RO.H had a similar clearance from blood circulation, as reported for unfractionated heparin (17) and observed for the same heparin derivatives under different conditions (s.c. injection at higher concentrations) in tumor-bearing mice (C. Pisano, Sigma Tau, Italy, personal communication). Heparin derivatives were rapidly removed from blood circulation with an average half-life of 30 min (Fig. 2A). Tumor cells carrying selectin ligands are known to form tumor cell emboli mediated primarily through platelet P-selectin (17, 46). To test heparin derivatives for their efficiency in inhibiting platelet/tumor cell interactions, wt mice (C57BL/6J) were i.v. injected with PBS or 150 μg of P-selectin-blocking heparin derivative, 58NA-H, followed by injection of MC-38GFP cells 5 min later. Mice were euthanized 30 min and 4 h postinjection, respectively. About 80% of tumor cells in the lung tissue of PBS-injected mice were associated with platelets (Fig. 2B). 58NA-H significantly reduced platelet/tumor cell emboli formation and was comparable to H. Furthermore, reduced platelet adhesion to tumor cells was observed after 4 h, but there was no difference in platelet/tumor cell interactions at 8 h postinjection (data not shown). These results indicate that the heparin derivative is biologically active for at least 4 h post-tumor injection despite its rapid clearing from the blood.

Selectin-specific heparin derivatives attenuate metastasis of MC-38 cells

To determine the ability of the selected nonanticoagulant heparin derivatives to attenuate metastasis, we i.v. injected wt mice with 150 μg of modified heparin, followed by injection of MC-38GFP cells 10 min later. Injection of unmodified heparin shortly before the tumor cells was previously shown to affect primarily P-selectin-mediated interactions (17, 22, 24). After 4 wk mice were euthanized and metastases were evaluated by counting of metastatic foci, followed by a detection of GFP fluorescence in the lung homogenate (Fig. 3). In mice receiving only PBS, lung tissues were displaced by metastases. A marked reduction of metastases formation was noted in mice receiving either 58NA-H or RO.H (Fig. 3A, B). Both the number of metastatic foci (Fig. 3B), reflecting the initiation of metastasis, and the GFP fluorescence of lung homogenates (Fig. 3A) reflecting the overall tumor burden were markedly reduced. The heparin derivatives have no effect on tumor cell proliferation or viability in vitro (data not shown). In contrast, the heparanase-specific inhibitor 100NA-RO.H had no significant effect on lung colonization of MC-38 cells. To test whether the observed attenuation of metastasis was due to P-selectin inhibition, we tested the effect of these heparin derivatives in P-selectin-deficient mice (P-sel−/−). Again, the heparanase-

Figure 2. Bioavailability of heparin derivatives in vivo. Mice were i.v. injected with FITC-conjugated heparin derivatives and analyzed as a function of time. A) Blood samples were drawn at different time points and the presence of heparin derivatives in the plasma was detected by fluorescence measurement. Plasma from a mouse injected with PBS was used as control. B) Platelet adhesion to i.v.-injected MC-38GFP cells was analyzed in mice receiving a heparin derivative and compared with PBS and heparin-injected mice. The number of platelet/positive tumor cells in the lungs of mice euthanized at 30 min and 4 h after injection were counted as described in Materials and Methods. The difference in platelet/tumor cell association between control-injected mouse (PBS) and heparin-injected mice was found to be statistically significant by an ANOVA test (P<0.01). C) Representative photomicrographs of platelet tumor cell adhesion in lungs of mice injected with either 58NA-H or PBS and euthanized 30 min later. Bar = 100 μm; inset bar = 50 μm.

Figure 3. Anti-metastatic activity of non-anticoagulant heparin.
specific inhibitor $^{100}$NA-RO.H did not affect metastasis. The number of metastatic foci was reduced by injection of the $^{58}$NA-H or RO.H derivatives (Fig. 3C). These derivatives effectively inhibited L-selectin binding but differed in other parameters (Table 2). We showed previously that heparin is an efficient inhibitor of L-selectin-mediated interactions that occur subsequent to P-selectin-mediated platelet/tumor cell interactions (32). To exclude any possible involvement of L-selectin inhibition, we tested the antimeatstatic effect of $^{58}$NA-H in mice deficient in both P- and L-selectin (PL-sel$^-/-$ mice). Notably, no further attenuation of metastasis could be detected (Fig. 3D). Taken together, these data show that in the MC-38 cell system $^{58}$NA-H affected primarily P-selectin-mediated interactions, and possibly also L-selectin. The complete inability of the heparanase-specific heparin derivative $^{100}$NA-RO.H to affect metastasis of MC-38 cells correlates with the lack of heparanase activity in these cells.

**Equal efficiency of selectin and heparanase inhibition to attenuate metastasis**

To compare the respective contribution of heparanase and selectin-mediated interactions with metastasis, we tested the effect of heparin and its derivatives on lung colonization of B16-BL6 melanoma cells producing both P-selectin ligands and heparanase (Fig. 1). Intravenous injection of mice with 150 µg of heparin or heparin derivatives, followed by injection of B16-BL6 cells, led to a significant attenuation of metastasis (Fig. 4A, B). The heparanase-specific derivative $^{100}$NA-RO.H reduced metastasis, albeit to a lesser extent than heparin (Fig. 4A). Notably, the selectin-specific inhibitor $^{58}$NA-H was as efficient an inhibitor of metastasis as $^{100}$NA-RO.H. To determine whether these two activities act synergistically during the initiation phase of experimental metastasis, we tested the heparin derivative RO.H, which exerts both selectin and heparanase inhibitory activities. However, the extent of metastases reduction achieved by RO.H was comparable to that of the other derivatives, indicating no synergy (Fig. 4A). Finally, we compared the efficacy of heparin derivatives delivered by either i.v. or s.c. injection. Despite limited data about pharmacokinetics of heparin in a mouse model, s.c. injection of unfractionated heparin was shown to attenuate metastasis (24, 26, 39). Mice were injected with 300 µg of selectin-specific $^{58}$NA-H and heparanase-specific $^{100}$NA-RO.H derivatives, followed by injection of B16-BL6 melanoma cells 30 min later (Fig. 4C). Attenuation of metastasis with an i.v. injection of 300 µg was comparable to the 150 µg dose for both derivatives (Fig. 4A). However, s.c. injection of $^{58}$NA-H further reduced metastasis compared with i.v. delivery. There was no difference in the antimetastatic effect of $^{100}$NA-RO.H regardless of the type of injection.

Taken together, inhibition of selectin-mediated interactions affects adhesion processes whereas targeting heparanase affects enzymatic degradation of the tumor cell microenvironment. The lack of synergy between these two mechanisms in the B16-BL6 melanoma model might indicate that both mechanisms occur concomitantly, affecting closely associated events in the metastatic process.
DISCUSSION

Numerous studies suggest that heparin and LMWH affect cancer progression in humans (10, 47). In addition, there is increasing experimental evidence suggesting that heparin is an inhibitor of metastasis in various mouse and rat models (19). Due to the known relationship between cancer and thrombosis, heparin and LMWH are commonly used primarily as anticoagulants in the treatment of cancer patients suffering from thrombosis (10, 20). The ability of heparin to affect the coagulation pathway led to the assumption that heparin reduces metastasis through its antithrombotic activity. Experimental evidence, however, indicated that heparin derivatives with no anticoagulant activity reduce metastasis in mice (16, 23, 25, 26). Here, we address the issue of other biological activities of heparin that could potentially affect metastasis focusing on the involvement of selectins and heparanase. The currently characterized heparin derivatives, with virtually no anticoagulant activity, allowed us to identify heparin preparations specific for the inhibition of selectins, 58NA-H, heparanase, 100NA-RO.H, or both activities, RO.H. The negligible anticoagulant activity of 58NA-H, RO.H, and 100NA-RO.H as expressed by their anti-Xa activity (Table 2) rules out any contribution of antithrombin-mediated activities to the antimetastatic activity of these heparin derivatives. On the other hand, anticoagulant properties can be contributed by release of TFPI, at least for 58NA-H and RO.H (Table 2). Such an activity is surprisingly high for 58NA-H, which is almost as active a TFPI releaser as heparin despite its consistently lower degree of sulfation due to replacement of more than half of the original N-SO$_3$ groups. It is possible that, for TFPI release, only half of N-sulfate groups on heparin are sufficient, similar to heparanase inhibition (35). The lower TFPI-releasing activity of RO.H compared with heparin (Table 2) suggests that other structural features, in addition to charge, affect its activity.

The selectin-specific heparin derivative 58NA-H was an efficient inhibitor of metastasis in two syngeneic murine models. The attenuation of metastasis observed in the MC-38 carcinoma model was comparable to the effect of unfractionated heparin (17). Unlike the almost complete reduction of metastatic foci lesions (5–6/foci/lung) achieved with MC-38 cells, only a partial (50%) inhibition of lung colonization was observed with B16-BL6 melanoma cells (~100 foci/lung). However, P-selectin binding to melanoma cells is low and correlates with the limited amount of selectin ligands on the cell surface (48). This is in contrast to abundant selectin ligand expression in most carcinomas (49). This opens up a possibility that heparin derivatives could also inhibit interactions of selectins with endogenous ligands (e.g., P-selectin binding to PSGL-1). This is supported by recent findings that endogenous selectin ligands, as determined by fucosyltransferase-7 activity, are required for metastatic progression (32). Despite the different nature of both tumor cells, targeted inhibition of P-selectin with 58NA-H markedly reduced metastasis, in agreement with previous observations obtained in P-selectin-deficient mice (21, 22). The fact that 58NA-H further attenuated lung colonization of MC-38 cells even in P-sel$^−$/− mice pointed toward an...
additional biological activity. No further effect could be observed in PL-selected−/− mice (Fig. 3D). It could be argued that the dramatic reduction of MC-38 metastasis in PL-selected−/− mice (just a few metastatic foci per lung) makes it virtually impossible to detect a further decrease. Yet a considerable amount of B16-BL6 metastatic foci was detected in PL-selected−/− mice (data not shown). If additional biological activities carried out by heparin derivatives would be involved, their further effect should have been detectable. Hence, these findings suggest that the potential of heparin to attenuate metastasis is mediated primarily through inhibition of selectin-mediated interactions.

Heparanase inhibition with 106NA-RO.H did not affect metastasis of MC-38 carcinoma cells in either wt or P-selected−/− mice (Fig. 3A, B). However, 106NA-RO.H could efficiently attenuate metastasis of B16-BL6 melanoma cells (Fig. 4A). Together with the finding of a virtual absence of heparanase expression in MC-38 cells, these data imply that tumor cell-derived heparanase is required for metastatic progression in melanoma (16, 50). Tumor cell invasiveness and extracellular matrix degradation require the expression of heparanase at specific sites in close vicinity of the cells (29). Extracellular proteases are produced by inflammatory cells, stromal cells, and/or the tumor cells themselves. Tumor cell emboli formation is associated with adhesion of platelets and leukocytes to tumor cells (51). Since platelets and leukocytes produce significant amounts of heparanase, their contribution to metastasis would be anticipated (52, 53). Our results suggest that the heparanase available from the metastatic microenvironment of the tumor cell does not contribute significantly to metastatic initiation. The inhibition of heparanase by modified heparins was shown previously to attenuate experimental metastasis only in a defined time period, either shortly before tumor cell injection or up to 3 h after the injection of tumor cells (16). Modified heparins capable of heparanase inhibition were also found to be good inhibitors of selectins (L. Borsig, unpublished observations, 1999). Taken together, these results indicate that heparanase activity expressed specifically by the tumor cells is essential for metastasis.

The use of experimental metastasis mouse models allowed us to study the effect of heparin in a time-dependent manner. Heparin derivatives were shown to stay biologically active in vivo for ~4 h after a single injection, as determined by reduction of platelet/tumor cell adhesion (Fig. 2). This narrow time frame for the action of heparin derivatives restricts their potential effects on the early step of metastatic colonization. Since selectins are known to initiate the first steps of cell-cell interactions (30, 31), the inhibition of selectin-mediated interactions probably belongs to one of the earliest actions associated with metastasis (17, 18). The availability of heparin or its derivatives during this short time frame makes any subsequent activities of heparin (e.g., inhibition of angiogenesis) unlikely. The observed inhibition of platelet-tumor cells aggregate formation after administration of 58NA-H signifies the involvement of P-selectin in this process (17, 46). Formation of platelet/tumor cell aggregates is associated with cancer progression, but the precise molecular mechanism remains to be determined (51). Platelets were shown not only to protect the tumor cells from the innate immune response and/or promote their retention in the vascular bed, but also to facilitate extravasation of tumor cells that have been attached to the vascular endothelium (54). Our observations arising from a direct comparison of selectin inhibitory and heparanase inhibitory heparin derivatives support the primary involvement of selectins during metastasis initiation. The efficiency of heparanase inhibition observed upon attenuation of B16-BL6 melanoma and breast carcinoma metastasis (16, 27, 50) demonstrates the relevance of heparanase in these tumor cells. Since no synergy between simultaneous inhibition of heparanase and selectins by the RO.H derivative could be detected, both mechanisms are likely to act in the same time period. When compared with any of the heparin derivatives, the higher efficiency of H to attenuate metastasis of B16-BL6 cells shows the different biology of melanomas and carcinomas while suggesting that other biological activities could be responsible for the observed additional efficiency in melanomas. Notably, s.c. delivery of 58NA-H derivative reduced metastasis to similar levels as H (Fig. 4). These findings indicate that the presumably prolonged delivery of 58NA-H affected other biological activities, possibly also L-selectin. Among the three investigated heparin derivatives, 58NA-H possesses biological activities quite similar to H, whereas 106NA-RO.H contains only heparanase inhibitory activity (Table 2). Nevertheless, a single treatment with these heparin derivatives leads to a considerable attenuation of metastasis, which underlines the importance of interfering with the initiation phase of this process.

The study of heparin derivatives during metastasis further delineates the underlying mechanism involved in this process. Even though a complete dissection of all individual biological activities of heparin is unlikely to be achieved, a better understanding of this process will help to design specific inhibitors targeting cancer progression. Further characterization of heparin biology in the context of cancer is indisputably necessary. Nevertheless, by modulating many biological functions (e.g., coagulation, heparanase, angiogenesis, selectins, growth factors, etc.), the effects of heparin and LMWH on cancer progression that have already been described would also make heparin therapy beneficial for cancer patients (18). The extensive experience with heparin and LMWH therapy for thrombotic indications provides a rationale for cancer treatment. Since there is no treatment currently available for metastasis, LMWH and its modified species could be used as the first feasible treatment of metastasis progression.

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