Inhibition of CXCR4-Mediated Breast Cancer Metastasis: A Potential Role for Heparinoids?

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Abstract Purpose: The pattern of breast cancer metastasis may be determined by interactions between CXCR4 on breast cancer cells and CXCL12 within normal tissues. Glycosaminoglycans bind chemokines for presentation to responsive cells. This study was designed to test the hypothesis that soluble heparinoid glycosaminoglycan molecules can disrupt the normal response to CXCL12, thereby reducing the metastasis of CXCR4-expressing cancer cells.

Experimental Design: Inhibition of the response of CXCR4-expressing Chinese hamster ovary cells to CXCL12 was assessed by measurement of calcium flux and chemotaxis. Radioligand binding was also assessed to quantify the potential of soluble heparinoids to prevent specific receptor ligation. The human breast cancer cell line MDA-MB-231 and a range of sublines were assessed for their sensitivity to heparinoid-mediated inhibition of chemotaxis. A model of hematogenous breast cancer metastasis was established, and the potential of clinically relevant doses of heparinoids to inhibit CXCL12 presentation and metastatic disease was assessed.

Results: Unfractionated heparin and the low-molecular-weight heparin tinzaparin inhibited receptor ligation and the response of CXCR4-expressing Chinese hamster ovary cells and human breast cancer cell lines to CXCL12. Heparin also removed CXCL12 from its normal site of expression on the surface of parenchymal cells in the murine lung. Both heparin and two clinically relevant dose regimens of tinzaparin reduced hematogenous metastatic spread of human breast cancer cells to the lung in a murine model.

Conclusions: Clinically relevant concentrations of tinzaparin inhibit the interaction between CXCL12 and CXCR4 and may be useful to prevent chemokine-driven breast cancer metastasis.

Breast cancer metastases are commonly found in regional lymph nodes, bone, liver, and the lungs (1). Evidence shows that the sites of metastasis are determined not only by the characteristics of the cancer cells but also by the microenvironment of the specific organ (2). It seems that breast cancer cells are able to metastasize to specific organs in a manner that is dependent on the ability of the organ to mediate tumor cell adhesion and extravasation and to support subsequent viability and proliferation.

Chemokines are members of a superfamily of chemotactic cytokines and were initially characterized because of their association with inflammatory responses. However, it is now known that they also play roles in homeostasis, cell proliferation, hematopoiesis (3), viral interactions, angiogenesis, and neovascularization (4). Four chemokine classes have been defined based on the location of the first two cysteine residues in the protein sequence (CXC, CC, C, and CX3C). CXCL12 (stromal cell–derived factor-1α) is a CXC chemokine and is the sole ligand for the receptor CXCR4, although CXCL12 can also signal through the orphan receptor RDC1 (CXCR7) on T lymphocytes (5). Chemokines activate members of the seven-transmembrane spanning G protein–coupled receptor family. On chemokine binding, dissociation of the heterotrimeric Gαiβγ proteins is induced, with the Gβγ subunits activating a range of intracellular signaling molecules, which in turn lead to the production of second messengers, including Ca2+ (6).

CXCR4 and its ligand CXCL12 are widely expressed by normal tissues and are important for embryonic development, lymphocyte trafficking, cell proliferation, and the mobilization of hematopoietic stem cells (7). Müller et al. (8) first showed a potential mechanism for site-specific metastasis, which is related to the CXC chemokine CXCL12. The CXCR4 receptor is up-regulated in primary breast cancers compared with normal breast tissue, whereas the CXCL12 ligand shows peak levels of mRNA expression in the common metastatic sites of breast cancer.

There is compelling evidence that CXCR4 is a key mediator of metastatic breast cancer. Immunohistochemical staining of primary breast tumors has revealed that normal breast epithelial cells do not express CXCR4, whereas between 5% and 73% of cancers do express this receptor (8–10). CXCR4
expression in the primary tumor has also been correlated with the degree of lymph node metastasis (9–11), bone metastasis (12), poor patient overall survival (13), and tumor grade (14). Although the expression pattern of CXCR4 did not have a significant correlation with hematogenous metastasis in a study by Kato et al. (9), CXCR4 expression has been linked to the ability of breast cancer cells to metastasize to the lungs (15).

The effect of CXCL12 on the proliferation of CXCR4-expressing breast cancer cells is still to be fully elucidated, although a few studies have shown a proliferative effect both in vitro and in vivo (16–18). Recent studies have also identified a link between HER2 and CXCR4 (13) and described a role for CXCL12 and CXCR4 in regulating the growth of estrogen receptor–positive breast cancer (16).

The importance of CXCL12/CXCR4 interactions in breast cancer growth is supported by the success of targeted anti-CXCR4 therapies in murine models of breast cancer. For example, Müller et al. showed that an anti-CXCR4 antibody produced a 61% to 68% reduction in the number of lung metastases (8). Administration of an anti-CXCL12 antibody to nude mice with s.c. implanted CXCR4-expressing breast cancer cells also led to a reduction in tumor growth and microvascular density (19). Silencing CXCR4 expression using small interfering RNA has also been a successful approach for decreasing both breast cancer growth and metastasis in multiple studies (13, 17, 18, 20). The CXCR4 antagonists TN14003 and AMD3100 have both shown some potential for reducing breast cancer metastases (18, 21).

Chemokines bind to glycosaminoglycan chains of proteoglycans present on the surface of epithelial cells and vascular endothelial cells and within the extracellular matrix. This interaction occurs between anionic glycosaminoglycan residues and basic amino acid domains on the chemokine (22). This interaction is now known to play a crucial role in the regulation of cell migration in vivo (23–25).

Heparan sulfate and heparin sulfate are chemically similar glycosaminoglycan species, with both macromolecules containing a repeating sequence of variably sulfated disaccharide units composed of glucosamine and glucuronic acid; heparin differs from heparan by being more heavily sulfated. Heparan sulfate seems to have a multitude of effects on chemokine function, including the inhibition of CXCL8-induced calcium flux in neutrophils (26). Importantly, heparinoids have been shown to inhibit CXCL12-induced T-cell migration and adhesion to extracellular matrix components (27).

The current study was designed to determine the potential of heparinoids, including unfractionated heparin and the low-molecular-weight heparin tinzaparin, to antagonize the effects of CXCL12 on breast cancer cells. Chinese hamster ovary (CHO) cells were transfected with the human CXCR4 receptor, and a series of in vitro assays was done to assess the potential of heparinoids to competitively inhibit CXCR4 activation. Investigations evaluated whether heparinoids could inhibit CXCL12 binding to its receptor, receptor activation, and subsequent chemotaxis of both transfected cells and CXCR4-expressing breast cancer cell lines. A final series of in vivo experiments investigated the effects of clinically relevant doses of heparinoids on hematologic metastasis of CXCR4-expressing breast cancer cells and their mechanism of action.

Materials and Methods

Cell culture. The MDA-MB-231 breast adenocarcinoma cell line, isolated from pleural effusions of a Caucasian patient with breast cancer, was obtained from the European Collection of Animal Cell Cultures (Porton Down, United Kingdom) and maintained in Leibovitz medium (Sigma, Poole, United Kingdom) supplemented with 10% FCS (Sigma), 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C in a humidified incubator. TMD-MDA-MB-231 (TMD-231) and LMD-MDA-MB-231 (LMD-231) cells were a gift from Dr. H. Nakashiti (Indiana Cancer Research Institute, Indianapolis, IN) and were grown as described previously (15). TMD-231 cells were isolated from the mammary fat pad of severe combined immunodeficient mice inoculated with MDA-MB-231, and LMD-231 cells were cultured from a lung metastasis of these mice; both cell lines expressed high levels of CXCR4 (15). Wild-type CHO cells (K1-CHO) were cultured in Ham’s F12 medium (Invitrogen, Paisley, United Kingdom) supplemented with 10% FCS, penicillin, and streptomycin in a humidified incubator with 5% CO2 at 37°C.

Construction of stable transfectants. The mammalian cell expression vector pcDNA3.1Zevo (Invitrogen), containing CXCR4 CDNA cloned from the Jurkat J16 cell line (gift from Prof. Kramer, German Cancer Research Center, Heidelberg, Germany), was transfected into wild-type, glycosaminoglycan-expressing K1-CHO cells using the SuperFect transfection reagent (QIAGEN, Crawley, United Kingdom). Stable cell lines were obtained by growing transfected cells at low density for 2 days followed by the addition of 300 µg/mL zeocin for selection over 3 weeks. Clones were picked and expanded before the analysis of CXCR4 expression.

Cell surface expression of CXCR4 and heparan sulfate by flow cytometry. The anti-CXCR4 antibodies (MAB173 and MAB172; R&D Systems, Abingdon, United Kingdom) used for this study were selected for optimal binding to a range of potential CXCR4 conformations based on the study by Baribaud et al. (28). These antibodies, the anti-heparan sulfate antibody (10E4; Seikagaku, Abingdon, United Kingdom), and appropriate isotype controls (R&D Systems and DAKO, Ely, United Kingdom, respectively) were all fully optimized before the collection of quantitative data. Cells were washed in PBS with 10% FCS and resuspended in 200 µL PBS with 10% FCS containing the appropriate antibody. Samples were incubated in the dark at 4°C for 30 min before washing in an equal volume of PBS with 10% FCS. Cells were then incubated with a FITC-conjugated goat anti-mouse secondary antibody (BD Pharmingen, Cowley, United Kingdom) in PBS with 10% FCS at 4°C for a further 30 min before washing and resuspension in 200 µL PBS with 10% FCS. Ten thousand events were analyzed by immunofluorescence flow cytometry (FACScan, BD Biosciences, Cowley, United Kingdom), and data plots were generated using FCS Express 2 software (De Novo Software, Thornhill, Ontario, Canada). The number of anti-CXCR4 antibody binding sites was determined (Quantum Simply Cellular kit, Sigma).

Calcium flux. Changes in intracellular Ca2+ concentrations in response to chemokine and heparinoids were analyzed using calcium-responsive dye fluorescence by flow cytometry. Indicator cells were washed in HBSS, resuspended at 1 × 10⁶/mL in supplemented HBSS (HBSS containing 1 mmol/L CaCl2, 1 mmol/L MgCl2, and 1% FCS), and incubated in 3 µmol/L Indo-1AM (Molecular Probes, Paisley, United Kingdom) at room temperature for 120 min before washing in supplemented HBSS. Calibration of each assay was done (29). Rmax was assessed using the calcium ionophore ionomycin (Sigma). Rmin values were determined following addition of the calcium-chelating agent EGTA. The sample was analyzed using the LSRII flow cytometer (Becton Dickinson, Cowley, United Kingdom) using a UV laser for excitation, with 440 and 530 nm fluorescence emissions recorded. Data plots were generated using the FlowJo software program (TreeStar, Inc., Ashland, OR). Stable cell lines were taken at 20°C for 120 s before the addition of chemokine up to a concentration of 200 nmol/L or heparinoid at a concentration up to...
Calculation of intracellular calcium concentrations was done using the equation \[ \text{[calcium (nmol/L)] = } K_d \times \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \], where \( K_d \) (844 nmol/L) is the dissociation constant of calcium bound to the fluorochrome (30) and \( R \) is the peak intracellular calcium flux in response to the additive (chemokine ± heparinoid).

**Radioligand binding assays.** Radioligand binding assays were done as described previously (31). Wild-type K1-CHO cells expressing the human CXCR4 receptor (K1-CXCR4) were seeded into 96-well flat-bottomed plates. \( ^{125}\text{I}} \)-CXCL12 (100 pmol/L) was added in the presence of the K1-CXCR4 cells at the presence of up to 80 \( \mu \text{g/mL} \) concentrations of heparin (Sigma) or tinzaparin (Leo Pharmaceuticals, Ballerup, Denmark). After washing, bound radioactivity was measured in a gamma scintillation counter (PerkinElmer, Beaconsfield, United Kingdom).

**Chemotaxis assay.** Transwell chamber chemotactic motility assays were carried out as described previously (31). All CHO cells were resuspended in chemotaxis buffer (Ham’s F12/0.1% bovine serum albumin) at \( 3 \times 10^5/\text{mL} \). Chamber assays were incubated for 6 h at 37°C with 5% CO\(_2\). Membranes were precoated with 2.5 \( \mu \text{g/mL} \) fibronectin (Sigma) for chemotaxis assays with MDA-MB-231 cells, which were incubated for 24 h in MEM with 0.1% bovine serum albumin. Assays were done in triplicate, with the migrant cells in 9 high-power fields being counted blindly per filter. Tinzaparin and heparin were added to the lower well of assays at concentrations up to 250 \( \mu \text{g/mL} \).

**Immunohistochemistry.** Immunohistochemistry of CHO cell blocks and mouse lungs were done using an immunoperoxidase-based staining method as described previously (8). Endogenous avidin and biotin were blocked (SP-2001; Vector Laboratories, Peterborough, United Kingdom) before anti-CXCR4 (1:100), anti-CXCL12 (1:20), or isotype-control antibodies were incubated with the sections overnight. Secondary antibody staining was done for 1 h using a rabbit anti-mouse biotinylated antibody (1:250; DAKO).

**In vivo metastasis study.** Ten-week-old female CB-17 severe combined immunodeficient mice (Charles River Laboratories, Margate, United Kingdom) were used in all experiments; all procedures were done in accordance with local Ethical Review Committee approval and UK Home Office license. The metastasis study was reproduced on three occasions; in all cases, seven animals were used in each drug treatment and PBS control group in accordance with statistical sample power calculations. No unexpected bleeding or weight loss was observed in any of the heparinoid treatment groups.

To assess the localization of heparan sulfate and CXCL12 domains within the lung, mice were injected s.c. with either 600 IU/kg heparin in 0.1 mL PBS or 0.1 mL PBS. Four hours following s.c. injections, the mice were killed humanely and the lungs from each mouse were snap frozen in isopentane before immunohistochemistry.

The effect of three different heparinoid treatment regimens on hematologic metastasis was assessed in each case by comparison of two treatment groups: one received a regimen of s.c. heparinoid in 0.1 mL PBS and the other group received a regimen of 0.1 mL PBS alone. Four hours after the first PBS or heparinoid injection, mice were injected with \( 10^5 \) LMD-231 breast carcinoma cells into the tail vein. Lungs were collected on day 28 and fixed for histopathology. Twenty coronal sections were cut from each mouse lung at intervals of 50 \( \mu \text{m} \) in depth. Lung sections were assessed blindly on \( \times 10 \) magnification (Leica...
Digital Module R, Knowlhill, United Kingdom); the area of metastasis was calculated using Leica Q-Win software. Both the mean number per high-power field and the area of the metastatic lesions were assessed blindly by two separate investigators (J.R.H. and H.E.).

**Statistical analysis.** Data values are expressed as mean ± SE. Statistical analysis was done using an unpaired two-tailed Student’s t test or using a one-way ANOVA when multiple data sets are being compared; *P* < 0.05 was considered statistically significant.

## Results

*Expression of CXCR4 receptor and heparan sulfate on transfectant and breast cancer cell lines.* Initial experiments showed that wild-type K1-CHO cells expressed no CXCR4 but expressed high levels of the 10E4 heparan sulfate epitope. K1-CXCR4 clones were selected after transfection (Fig. 1A); a clone that expressed CXCR4 at a level similar to the lymphoid Jurkat cell line was used in subsequent experiments.

Flow cytometric analyses with anti-CXCR4 antibodies MAB173 (data shown; Fig. 1) and MAB172 (data not shown) produced similar results and confirmed little CXCR4 expression on the metastatic primary breast cancer cell line MDA-MB-231. However, MDA-MB-231 cells isolated after their growth in the mammary fat pad (TMD-231) expressed 31% more CXCR4 compared with parental cells grown in culture (*P* < 0.05; Fig. 1B and D). Importantly, the LMD-231 cell line cultured from a lung metastasis of MDA-MB-231 cells expressed levels of CXCR4 that were 2.7 times higher than the parental cell line (*P* < 0.001; Fig. 1C and D). However, LMD-231 cells gradually lost expression of CXCR4 in culture (Fig. 1D), with no detectable expression remaining after 15 passages (*P* < 0.05; Fig. 1D). Flow cytometric analysis revealed that ~24% of LMD-231 cells were high expressers of CXCR4 compared with 14% in parental MDA-MB-231 cells. Both LMD-231 cells and TMD-231 cells showed strong cell surface heparan sulfate expression.

**Intracellular calcium flux in response to CXCL12.** Following confirmation that transfectant cells expressed the CXCR4 receptor, it was necessary to confirm the ability of the receptor to transduce intracellular signals in response to CXCL12 (Fig. 2A). K1-CXCR4 showed an intracellular calcium flux in response to CXCL12 (Fig. 2B). TMD-231 cells also showed significant levels of calcium flux in response to 12.5 nmol/L CXCL12 (data not shown; *P* < 0.05).

Although 25 nmol/L CXCL12 induced a significant calcium flux in K1-CXCR4 cells, this effect was inhibited by heparin at concentrations of 10 and 25 μg/mL (*P* < 0.05; Fig. 2C). Heparin alone had no effect on the concentration of intracellular calcium.

**Radioligand binding assays.** A series of radioligand binding assays was done to explore the ability of an increasing concentration of soluble heparinoids to competitively inhibit...
chemokine binding to cell surface glycosaminoglycan and CXCR4. Both heparin and tinzaparin significantly inhibited CXCL12 binding (P < 0.001; Fig. 2D) in a dose-dependent manner, but there were differences between these heparin species in their capacity to compete the chemokine. The inhibitory potential of heparin was greater than that of tinzaparin (P < 0.01), with 3 μg/mL heparin reducing CXCL12 binding by 63%, whereas a similar concentration of tinzaparin only reduced binding by 23%.

Investigation of the chemotactic potential of CXCR4 transfectants. Following confirmation that the transfectant cell line expressed the CXCR4 receptor and that the receptor transduced a CXCL12 signal, it was necessary to examine the functionality of these cells. It was found that K1-CXCR4 showed significant migration in response to CXCL12 (Fig. 3A); 12.5 nmol/L CXCL12 was the lowest concentration of chemokine to induce significant chemotaxis of K1-CXCR4 cells (P < 0.03).

Effect of heparinoids on chemotaxis of CXCR4-expressing transfectants. To assess the ability of heparinoids to prevent chemokine-mediated functions, an additional series of chemotaxis experiments was done using K1-CXCR4 cells. Heparin showed a trend toward inhibiting chemotaxis in a concentration-dependent manner, with significant inhibition being observed at a concentration of 250 μg/mL (P < 0.05; Fig. 3B). The low-molecular-weight heparin tinzaparin at concentrations of 25 to 250 μg/mL was able to inhibit the chemotactic effects of 12.5 nmol/L CXCL12 (P < 0.05) to the level of the negative controls (Fig. 3C). There was no demonstrable increase in the efficacy of tinzaparin at inhibiting chemotaxis at concentrations above 25 μg/mL. Protamine is a highly cationic molecule that is widely used as a heparin antagonist. Addition of protamine at a concentration of 0.5 mg/mL was able to fully abrogate the inhibitory effect of 1 mg/mL tinzaparin on CXCL12 chemotaxis (P < 0.01; data not shown).

Migration of breast cancer cells in response to CXCL12 and heparinoids. Flow cytometry revealed little CXCR4 on the parental MDA-MB-231 cell line. In accordance with these low expression levels, MDA-MB-231 cells failed to show a chemotactic response to CXCL12 (P > 0.5; data not shown). However, TMD-231 cells showed significant chemotaxis at concentrations of 5 to 25 nmol/L CXCL12 (P < 0.05; Fig. 4A). Early-passage (passage 4) LMD-231 cells also showed a significant chemotactic response toward CXCL12 (Fig. 4B) and were driven to invade Matrigel by this chemokine (data not shown). Importantly, tinzaparin significantly inhibited CXCL12-mediated chemotaxis of both TMD-231 and LMD-231 cells (Fig. 4C and D). Neither CXCL12 (up to 100 μg/mL) nor tinzaparin (up to 250 μg/mL) had an effect on the proliferation/apoptosis of these cells (P > 0.5; data not shown).

Investigation of the effects of systemic heparin treatment on CXCL12 expression in the mouse lung. Lungs from mice treated with heparin or PBS were examined for CXCL12 and glycosaminoglycan expression to investigate the possibility that systemic heparin therapy inhibits CXCL12 presentation by cell surface glycosaminoglycans. Both PBS-treated and heparin-treated mice showed similar patterns of heparan sulfate staining present on cell surfaces throughout the lung parenchyma, with strong staining on the endothelial surface of large vessels and on the bronchopneumial cell surface (Fig. 5A). The PBS-treated mice also showed strong CXCL12 expression in a pattern similar to that seen for heparan sulfate expression (Fig. 5B). In marked contrast, heparin-treated mice showed greatly reduced CXCL12 staining on the surface of bronchioepithelial cells and no luminal staining within the vessels (Fig. 5C).
**Inhibition of breast cancer metastasis using heparinoids.** Treatment for 28 days with 600 IU/kg/d heparin decreased the number of lung metastases by 87% \((P < 0.0001)\) compared with the PBS-treated control group (Fig. 6A). The number of lesions in the heparin-treated group was so small that comparison of lesion area with PBS-treated control animals did not reach statistical significance (Fig. 6A), although the trend was for smaller lesions in the heparin-treated animals. Cell lines were rederived from metastatic lesions in the lung of some PBS-treated control animals and showed expression of human CXCR4 at both mRNA and protein levels (data not shown).

A therapeutic dose of 175 IU/kg/d tinzaparin was also chosen for this study, as this is used in patients to treat thromboses. Peak anticoagulant activity occurred 4 h after injection, and at this time point, the anti-Xa activity in the mouse was 0.51; this lies within the pharmacologic therapeutic range (0.5-1.0). Treatment for 28 days with this therapeutic dose of tinzaparin led to a 23% reduction in the number of lung metastases \((P < 0.01)\) and an average 46% reduction in the area of lung metastases \((P < 0.0001)\) compared with PBS-treated mice (Fig. 6B, D, and E).

Patients undergoing surgery for breast cancer routinely receive a once daily prophylactic dose of tinzaparin in the perioperative period to prevent thromboembolism. Importantly, treatment with this prophylactic dose of 90 IU/kg/d tinzaparin given for 4 days starting at the time of cancer cell injection led to a 71% decrease in the number of metastases \((P < 0.05;\) Fig. 6C), although there was no significant difference identified in the average area of metastatic lesions between the treated and control groups.

**Discussion**

The stimulation of CXCR4 by CXCL12 has been shown to play an important role in enhancing motility as well as regulating adhesive and invasive changes during breast cancer.
Indeed, the metastatic selection of breast cancer cells seems to be directly related to their CXCR4 status, with cells derived in vivo from distant metastases and from s.c. infiltration in the mammary fat pad having significantly higher CXCR4 expression levels than the primary cancer cells (8, 15). Parental MDA-MB-231 cells expressing lower levels of CXCR4 failed to migrate in response to CXCL12. However, TMD-231 and LMD-231 cells showed significantly higher levels of CXCR4 expression and subsequent migration toward CXCL12. Interestingly, loss of CXCR4 expression by LMD-231 cells after 15 passes in culture led to a concomitant failure of chemotaxis.

Glycosaminoglycans are known to play important roles in several processes, including the presentation of a range of cytokines to their specific receptors, the maintenance of stable cytokine gradients required to direct vectorial cell migration, and the protection of vulnerable cytokine molecules from proteolytic degradation (34). However, radioligand binding data in the current study also confirm previous observations that heparin can compete efficiently with the high-affinity CXCR4 receptor for CXCL12 binding (35, 36) and extend this to show that the clinically relevant heparinoid tinzaparin can also prevent CXCL12 ligation. The blockade of receptor activation was validated by demonstration that soluble heparin molecules block the intracellular calcium flux normally induced by CXCL12 binding.

The heparin binding site of CXCL12 has been extensively characterized (37), and it is known that a minimum glycosaminoglycan chain length of 12 to 14 monosaccharide units is required for efficient binding. Radioligand assays in this study show that heparin and the low-molecular-weight heparinoid tinzaparin (average size, 27-38 monosaccharide units) both act as potent competitors of CXCL12.

Heparin and tinzaparin inhibited CXCL12-induced migration of transfectant cells, the data suggesting that this inhibition may be concentration dependent. The observation that heparin prevented CXCL12-induced calcium signaling may suggest that calcium signaling is involved in the migratory function of these cells. However, this conclusion cannot be confirmed until all the downstream CXCR4 signaling pathways affected by heparin have been investigated fully.

The concentration of heparinoids required to inhibit CXCR4-mediated function was often high; for example, 25 to 250 μg/mL of heparinoid were required to significantly inhibit CXCL12-driven chemotaxis. However, given that the micro-environmental concentration of heparan sulfate on a cell surface is typically between 100 and 200 μg/mL (38), it is perhaps not surprising that relatively large concentrations of heparinoids are required to compete CXCL12 from the cell.

Immunohistochemistry revealed that CXCL12 seems to be colocalized with 10E4 heparan sulfate domains on murine endothelial and bronchoepithelial cell surfaces. This suggests that CXCL12 is bound to cell surface glycosaminoglycans in vivo and that this interaction is of functional importance. Immunohistochemistry also showed that CXCL12 expression is greatly reduced on the bronchoepithelial and endothelial cell surfaces after a single high dose of s.c. heparin therapy. Presentation of CXCL12 by glycosaminoglycans on the endothelial cell surface may be important for the adhesion and subsequent migration of CXCR4-expressing breast cancer cells. A potential mechanism by which heparin may prevent CXCR4-mediated breast cancer metastasis is by competitive displacement of CXCL12 from the endothelial surface. Hence, glycosaminoglycan-mediated displacement of CXCL12 from these sites could provide a potential route to antimetastatic therapy.

Treatment with heparin significantly decreased the number of hematologic lung metastases following intravascular
administration of breast cancer cells. Administration of tinzaparin for 4 days also inhibited the number of lung metastases seen at 28 days, suggesting an action during the earliest stages of hematogenous spread of the disease. Long-term tinzaparin therapy had an additional effect on tumor growth, with the observed decrease in cancer area potentially being caused by a direct effect of the heparinoids on cancer cell proliferation or apoptosis, although no such effects were observed in vitro.

Importantly, it has been shown recently that CXCL12 can bind another cancer cell–associated receptor, CXCR7 (39). Activation of this receptor on breast cancer cells induces neither calcium mobilization nor migration but does increase cancer cell survival and tumor development in vivo (39). Heparinoids have the potential to inhibit the activity of a wide range of factors that can enhance cancer growth (40). This include not only the chemokines but also vascular endothelial growth factor, tissue factor, fibroblast growth factors, IFN-γ, histamine, complement, proteases, and heparanases (41). The potential to inhibit simultaneously this multiplicity of tumor growth–promoting cytokines may explain the reduction in metastatic lesion area observed in animals that received daily injections of tinzaparin.

In conclusion, this study shows the potential of heparinoids to competitively bind chemokine presented by endogenous glycosaminoglycans and to inhibit the functional effects of CXCL12 on CXCR4-expressing cancer cells. Heparinoids compete for the binding of CXCL12 in vivo and prevent the binding of CXCL12 to CXCR4-expressing cancer cells, thereby preventing hematologic metastasis. Heparinoids may also inhibit tumor growth by altering the balance of protumorigenic and antitumorigenic factors in the metastatic site. At the time of cancer surgery, cancer cells are liberated into the vasculature (42, 43). Short-term prophylactic doses of tinzaparin at the time of tumor cell release into the vasculature are able to successfully prevent the seeding of and subsequent growth of distant metastases. Hence, the administration of tinzaparin at the time of cancer surgery might have benefits in addition to the prevention of thromboembolic disease.

**References**


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