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Heparins attenuate cancer metastasis

Are selectins the link?

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Abstract

Heparin is often used to prevent or treat thromboembolism in cancer patients. Clinical and experimental evidence suggest that heparin also has anti-cancer activities. Experimental evidence consistently supports the ability of heparin to attenuate metastasis. The potential anti-metastatic effects of heparin include the inhibition of cell-cell interactions or heparanase and modulation of growth factors and anticoagulant activity. Heparin inhibits selectin-mediated interactions of tumor cells with leukocytes, platelets and endothelial cells, which are likely to mediate the initial steps of hematogenous metastasis. Prospective clinical trials can be designed based on the insights obtained from experimental studies.
I Cancer-Thrombosis-Heparin

Thromboembolic events are frequent complications in cancer patients and represent a major reason of morbidity and mortality (1-3). The hypercoagulable state often observed in cancer patients is either activated by the tumor itself or due to anticancer treatment-related factors. Several molecular pathways leading to this procoagulant state were identified and are thoroughly reviewed elsewhere (e.g. see reviews 4, 5), Tissue factor (TF), a main inducer of the coagulation cascade, is upregulated either by oncogenic activation or the loss of tumor suppressors in tumor cells and in activated endothelial cells within the tumor stroma (6, 7). Recently, a novel mechanism was described, which links the MET oncogene to an increased expression of COX-2 and PAI-1, thereby inducing a procoagulant state (8). PAI-1 and TF are also upregulated in hypoxic areas of tumors, thus suggesting a connection between the HIF pathway and hemostasis (9). Moreover, mucins of adenocarcinomas were shown to activate the hemostatic system via selectin-mediated interactions between leukocytes and platelets (10). Standard anticoagulant therapy of cancer patients which experience thromboembolism or receive chemotherapy are mostly treated with heparin or oral vitamin K antagonist. Low-molecular weight heparin therapy was shown to be associated with an improved survival of cancer patients (1, 11-14).

Heparin is a natural glycosaminoglycan molecule and consists of N- and O-sulfated alternating galactosamine/glucosamine and glucoronic/iudoronic acid carbohydrate moieties (15). Unfractionated heparin (UFH) is isolated from porcine intestine and represents a very heterogeneous mixture of polymers containing of about 200-300 monosaccharides. Depolymerisation and fractionation of UFH results in the generation of low molecular weight heparin (LMWH) with more constrained biological activity (15). Importantly, the characterization of the antithrombin binding
pentasaccharide sequence led to the identification of the heparin active site (15). Many activities of heparin are known to affect the biology of tumor cells, but only a few of them are relevant in vivo (16-19). In this review, we will discuss the in vivo activities of heparin on tumor progression, with a special emphasis on the inhibition of selectin-mediated cell-cell interactions and heparanase activity during hematogenous dissemination.

II Heparin as cancer treatment: Impact on patient survival

Heparin is commonly used for the prevention or treatment of venous thromboembolism. Its effect on tumor progression in cancer patients, however, remains controversial. The positive effect of heparin on survival of cancer patients was evaluated in several clinical studies and excellently reviewed elsewhere (e.g. see reviews 13, 14).

Several prospective, randomized and placebo-controlled studies were specifically planned to analyze heparin as an anti-cancer drug. One such multicenter trial involving 138 patients with small lung cancer demonstrated a significant reduction of mortality and increased complete response rate in the group treated with UFH subcutaneously over a period of 5 weeks (20). In another study by Altinbas and colleagues, small lung cancer patients (n=84) were treated with dalteparin or placebo in parallel to normal chemotherapy regimens (21). The group treated with chemotherapy and dalteparin had a significantly prolonged median survival. However, no significant differences were observed in advanced cancer patients treated with nadroparin for 2 years (n=138), (22). Although a trend towards an increased survival in the nadroparin group could be determined. In the FAMOUS study of Kakkar and associates 374 patients with advanced malignancies (Stage III, IV) were treated with dalteparin over a period of 1 year. After 3 years, the dalteparin
group showed a higher survival rate, albeit not significantly (p=0.19). Interestingly, a not *a priori* defined subgroup analysis of patients with an initial better prognosis resulted in a significantly improved survival of the dalteparin treated patients (23). The MALT study was conducted to test the effect of a 6 week treatment with nadroparin on the survival of patients with advanced cancer of various origins (n=302) (24). Nadroparin treatment led to an improvement of survival by 12% after one year and 10% after two years (p=0.021). These subgroup analyses of patients with better prognosis in the FAMOUS and the MALT studies make heparin an interesting candidate to be tested during early stages of cancer. Meta-analysis of all patients, including those in above mentioned studies, demonstrated a significant improvement of the survival in the LMWH treated group (13, 25).

The CLOT study was designed to compare the effect of LMWH or oral anticoagulation on the prevention of recurrent venous thromboembolism in cancer patients with acute venous thrombosis or pulmonary embolism (26, 27). A treatment with nadroparin for six months was significantly more effective than oral anticoagulation (26). A posthoc analysis in a subgroup of patients without metastases resulted in the detection of an increased survival in the dalteparin treated arm of the study (27). In contrast to heparin treatment, other studies using coumarin derivatives in cancer patients failed to show any decrease in mortality (13, 14).

Based on these promising results together with observations from animal models, heparin seems to directly affect cancer progression. Meanwhile, animal models help to study and to validate the activity of heparin during metastasis.

### III Heparin attenuates metastasis in mouse models

Several animal models were used to test heparins as a potential anti-cancer treatment. Most of the available experimental data have been obtained in
experimental metastasis models, where tumor cells are inoculated directly into the blood circulation. Despite several limitations, this approach allows the analysis of the cellular events between tumor cells and their microenvironment during the hematogenous phase of metastatic colonization. This experimental setting enables the evaluation of heparin treatment according to the timely defined molecular events and thereby makes it possible to narrow down the potential mechanism. However, several aspects of metastasis including lymphatic dissemination, local tumor invasion and intravasation can be better examined in spontaneous metastasis models. For more than 40 years, UFH was repeatedly shown to reduce experimental and spontaneous metastasis in rodents (for review see (17, 19). Because UFH consists of highly heterogeneous polysaccharide chains with a wide variety of biological activities, the determination of the molecular mechanisms responsible for the anti-metastatic effect was difficult (17, 28). A first hint was derived from spontaneous metastasis models that showed that the primary tumor size was only minimally affected, while metastasis was clearly reduced (17, 29). This observation led to the conclusion that metastasis rather than tumor growth is inhibited by heparin treatment. Many mouse studies analyzing the effect of heparin on metastasis have been reported (for review see (17, 19). In these studies, different tumor cells, including human and mouse carcinoma and melanoma cells were used, and a variety of heparins, including UFH, various low molecular weight heparins (LMWHs) and chemically modified heparins were evaluated. While the method of application as well as the time period of heparin treatment varied among these studies, the anti-metastatic effect of heparin treatment was observed mostly in situations when heparin was applied when tumor cells were still in circulation. In general, a bolus injection of heparin or LMWH prior to tumor cell application was shown to attenuate metastasis of colon, lung and breast carcinoma, as well as melanoma (28, 30-34).
all these studies the applied concentration of heparins exceeded the therapeutical doses. Nevertheless, recent studies have confirmed that also clinically relevant doses of UFH and LMWHs efficiently attenuated experimental metastasis (35, 36).

IV “Anti-cancer” activity of heparin

Heparin is a complex mixture of glycosaminoglycans containing a variety of biological activities. Although clinical preparations of heparin are enriched for its ability to inhibit coagulation, heparin also contains activity to: block P- and L-selectins, alter integrin binding, affect activity of growth factors and cytokines, inhibit heparanase and angiogenesis, modulate the activity of proteases and thereby the components of extracellular matrix (15, 17, 18, 37-40). While any of these activities could potentially affect cancer progression, all the experimental evidence strongly suggests that heparin is likely affecting the early steps of metastasis. Hematogenous metastasis consists of cascade of events in which metastatic tumor cells enter the blood circulation, evade immune responses, adhere to the endothelium of distant organs and extravasate. The observation that a single injection of heparin at the time of tumor cell injection attenuates metastasis (30, 31) indicates that selectins, which can mediate the initial cell-cell interactions among tumor cells, platelets, leukocytes and endothelium, contribute by initiating the process of metastasis. Furthermore, no benefit of prolonged heparin treatment over the single heparin injection could be observed (41). Considering the relatively short half-life (4-6 h) of heparin in circulation, several other activities of heparin (e.g. effects on angiogenesis or growth inhibition) are not likely to affect the early steps of metastasis.

V Anticoagulant activity of heparin and metastasis
The use of heparin in cancer management indicated effects seemingly unrelated to its anticoagulant properties (37, 42, 43). Heparin effect on metastasis was also demonstrated in animal models and confirmed to be additional to its anticoagulant activity (17, 44). In addition the capacity of heparin to release TFPI from the vasculature was shown to affect metastasis (41). Non-anticoagulant heparin derivatives were shown to attenuate experimental metastasis, thus confirming that the effect on the coagulation system is rather limited with respect to the other anti-metastatic properties of heparin (29, 30, 34, 45-48). Experimental studies have shown that the use of an anti-thrombin inhibitor, hirudin, reduces metastasis (49, 50). However, in both these studies the amount used of hirudin resulted in an excessive anticoagulation, which is not compatible with clinical applications. Finally, the pentasaccharide fondaparinux, which specifically inhibits antithrombin did not reduce metastasis in mice at clinically tolerable levels (35, 36).

VI Heparin inhibits heparanase

A critical event in cancer progression is the invasion of cancer cells into the surrounding tissue, which is associated with their capacity to degrade the various components of extracellular matrix, including collagen, fibronectin and heparan sulfate proteoglycans. Cancer cells produce several hydrolytic enzymes such as matrix metalloproteinases and heparanase. Heparanase have an endoglycosidase activity and cleave heparan sulfates. Elevated heparanase expression is associated with cancer progression of several carcinomas including colon, liver, pancreas, bladder, breast and prostate carcinoma as well as leukemia and multiple myeloma (51). Silencing of the heparanase gene led to decreased angiogenesis and reduced metastasis of tumor cells, directly linking heparanase activity to cancer progression (52). Several studies provided evidence that heparin inhibits heparanase activity in
vitro, and the inhibition of heparanase activity by heparin correlated with attenuation of metastasis (30, 53, 54). Recently it was analyzed whether non-anticoagulant heparin treatment affects primarily heparanase activity or inhibits selectin-mediated interactions and thereby contributes to reduction of metastasis (45). Heparin derivatives showing strong heparanase inhibitory activity significantly reduced the metastatic capacity of tumor cells expressing this enzyme. However, P-selectin specific heparin derivatives were functional inhibitors of metastasis regardless of heparanase expression in the corresponding tumor cells.

**VII Cell-cell interactions during metastasis**

Circulating tumor cells are known to interact with platelets, leukocytes and endothelial cells. The creation of tumor cell emboli consisting of platelets and leukocytes was demonstrated to facilitate metastasis (31, 55-59). Elimination of platelets resulted in attenuation of metastasis (56). Furthermore, any interference in platelet-tumor cell interactions led to enhanced elimination of tumor cells by NK cells or enhanced association of CD11b positive cells, suggesting that the platelet thrombus mechanistically “protects” tumor cells from immune response (31, 57, 60). While the exact molecular mechanism of platelet-tumor cell interactions remains to be identified, several lines of evidence suggest that platelets P-selectin is one of the mediators in this process (31, 61, 62).

Although the presence of leukocytes in tumor cell emboli is widely recognized, their role in the process of tumor cell emboli formation remains largely unknown. In contrast, the contribution of leukocytes to cancer at sites of primary tumors is well described (63, 64). Recent studies provided evidence that the leukocyte recruitment to the metastatic environment of tumor cells is critical for cancer progression (32, 65). The absence of leukocyte L-selectin led to attenuation of metastasis, thereby directly
involving leukocytes in this process. Further analysis has revealed the association of granulocytes and monocytes/macrophages with tumor cells in the vasculature (65).

**VIII Selectins - vascular cell adhesion molecules**

Selectins are vascular cell adhesion molecules involved in interactions of leukocytes, platelets and endothelium within the circulation. The physiological functions of selectins are well described in processes like inflammation, immune response and hemostasis (66). Selectins mediate the initial contacts of leukocytes with the vascular endothelium, which are supported by rapid and reversible interactions with selectin ligands - carbohydrates. The family of selectins consists of three members: P-, L- and E-selectin (66). P-selectin is present in the storage granules of platelets (α-granules) and the endothelium (Weibel-Palade bodies), thus permitting rapid exposure on cell surfaces upon activation. L-selectin is constitutively expressed on almost all leukocyte subpopulations. In contrast, E-selectin expression on the surface of endothelial cells requires de novo transcription, thus occurring several hours after stimulation (66). The physiological selectin ligands consist of various oligosaccharide structures such as mucins, glycosaminoglycans and sulfated glycolipids (67). The lectin domain of selectins recognizes sialylated, fucosylated lactosamine oligosaccharides, which present the terminal structure commonly known as sialyl Lewis x (sLe^x) (66, 67).

**IX P- and L-selectins facilitate metastasis**

Selectins are also involved in various pathophysiological processes including cancer progression (68). Hematogenous metastasis is the major route of systemic spread for carcinomas. Epithelial cells are covered with mucins that line the lumen of inner organs and are secreted on the apical side of epithelium. Mucins are high-molecular
weight molecules containing a protein core heavily modified with O-linked glycan structures (69, 70). Carcinoma cells show altered cell surface glycosylation with enhanced presence of sLe\(^x\) or sialyl-Tn oligosaccharide structures on mucins (69-71). The positive correlation between sLe\(^x\) expression and poor prognosis, due to metastasis, has been demonstrated in colon, gastric, lung, prostate and breast cancers (72-79). The entrance of carcinoma cells, carrying abundant selectin ligands, into the blood circulation makes them potential partners for selectin-mediated interactions with platelet, leukocytes and endothelium. In turn, the constant presence of L-selectin on leukocytes together with the rapid expression of P-selectin on platelets and endothelium makes selectins the potential mediators for early interactions during metastasis (19).

The evidence that P- and L-selectin contribute to metastasis has been recently confirmed in different animal models (31, 33, 65). Although the absence of P- or L-selectin significantly attenuated metastasis of carcinoma cells, there was virtually no metastasis detected in the P- and L-selectin double deficient mice, strongly suggesting a synergistic effect of both selectins in this process (32). P-selectin deficiency reduced metastasis of carcinomas as well as melanomas (31-33). The absence of P-selectin led to a reduction of platelet-tumor cell interactions and tumor seeding to the lung vasculature (31). Similarly, the removal of selectin ligands on tumor cells also caused decreased platelet-tumor cell emboli formation and attenuation of metastasis (31, 32, 60). This P-selectin-mediated formation of platelet-tumor cell emboli seems to protect tumor cells from elimination by NK cells (57). Platelet aggregates were detected also by tumor cells not carrying P-selectin ligands, thus it is possible that P-selectin mediates also aggregations of platelets (50).

In addition, the endothelial P-selectin expression was demonstrated to contribute to metastasis (33). Lethal irradiation of P-selectin deficient mice followed by bone
marrow reconstitution with wild type cells resulted in mice expressing P-selectin only in platelets. A significant reduction of metastasis was observed in such chimeric mice, indicating that endothelial activation of endothelium and associated P-selectin expression contributes to metastasis.

The potential involvement of L-selectin in metastasis was tested in L-selectin deficient mice (32, 65). Metastatic progression was dependent on L-selectin, because the absence of L-selectin resulted in attenuation of metastasis. L-selectin-mediated recruitment of leukocytes to vascular tumor cells was associated with an enhanced expression of L-selectin ligands surrounding the tumor embolus (65). Furthermore, intravenous injection of a function blocking L-selectin antibody following tumor cell inoculation resulted in attenuation of metastasis directly indicating that leukocytes contribute to the initial steps of metastasis. In conclusion leukocytes may facilitate metastasis by potentiating tumor cell extravasation as was previously observed in experiments both in vitro and in vivo (80-83).

X Heparin attenuates metastasis by inhibition of selectin interactions

Heparin affects several processes in the metastatic cascade (Figure 1). To address the role of heparin in the process of tumor cell invasion and migration into the blood vessels, spontaneous metastatic models, that release metastatic tumor cells into the circulation over time are necessary. However, the widely used experimental metastasis model makes it possible to obtain insight into the molecular mechanisms underlying the initiation phase of metastasis. In particular, the direct injection of tumor cells into the blood circulation leads to immediate interaction between tumor cells and blood cells, implicating selectins as likely candidates facilitating the early steps of hematogenous metastasis. Since the applied heparin is cleared from the circulation
within a few hours (31), the other effects of heparin (e.g. effect on angiogenesis) are unlikely to participate in this process within this time frame.

Heparin was shown to efficiently bind to P- and L-selectin (84, 85). Despite no structural similarity between heparin and natural selectin ligands, the selectin inhibitory effect of heparin is likely explained by clusters of negatively charged sulfates and carboxylates recognized by selectins (84). To delineate the antimetastatic activity of heparin with respect to selectin inhibition, P- and/or L-selectin deficient mice were analyzed (31-33, 45, 65). While the absence of either P- or L-selectin significantly attenuated metastasis, heparin injection shortly before the tumor cell inoculation - “early heparin” further reduced metastasis in L-selectin deficient mice (32). This early administration of heparin efficiently inhibited platelet-tumor cell emboli formation, which are P-selectin dependent (31). However, “early heparin” administration did not affect metastasis in P-selectin deficient mice (31-33).

The presence of heparin in circulation at a point in time when tumor cells are still intravascular was shown to attenuate metastasis of tumor cells derived from various tissues (30, 31, 33, 35, 65). Indeed, UFH treatment was shown to prevent tumor cells interactions with platelets, leukocytes and endothelial cells, thereby reducing tumor cell survival within the metastatic microenvironment (31-33, 65). Since attenuation of metastasis was achieved also with heparin derivatives without anti-coagulant activity and also since heparin showed no effect in P-selectin deficient mice, early heparin treatment likely affects primarily P-selectin mediated interactions during early steps of metastasis.

The additional reduction of metastasis achieved by early heparin treatment in the absence of L-selectin indicates that the L-selectin-dependent involvement of leukocytes is an event subsequent to the initial P-selectin-mediated platelet aggregation. This is in agreement with the observation that a temporal inhibition of L-
selectin several hours after the tumor cell injection resulted in attenuation of metastasis comparable to the one seen in L-selectin deficient mice (65). Similarly, heparin injection several hours (6-12 h) after the tumor cell injection – “late heparin” led to an attenuation of metastasis in P-selectin deficient mice, but had no effect on metastasis in L-selectin deficient mice (65). Finally, the evidence that heparin affects P- and L-selectin and thereby attenuates metastasis was obtained in mice deficient both in P- and L-selectin (PL-sel-/-). Treatment of PL-sel-/- mice either with “early” or “late” heparin had no effect on metastasis observed in these mice (32). Interestingly, repeated injection of high doses of UFH further reduced metastasis in PL-sel-/- mice suggesting that heparin at higher doses may have supplementary activities to the selectin inhibition (86). Other recent studies with different LMWH and modified heparins demonstrated that their anti-metastatic effect mainly depends on their selectin inhibitory capacity (35, 36, 45). Altogether, these findings indicate that heparin attenuates metastasis primarily by inhibition of P- and L-selectin interactions of tumor cells with the blood cells.

**XI Heparin as an antimetastatic treatment**

Hematogenous metastasis of solid tumors is directly or indirectly responsible for most cancer-related deaths, yet there is no specific anti-metastatic therapy available (87, 88). In addition, several studies demonstrated that circulating tumor cells are detectable in the circulation of cancer patients (89), while the number of circulating tumor cells correlated with poor prognosis (90). Hence tumor cells are present in the circulation, where these cells are vulnerable to potential therapies.

Several prospective clinical trials indicate that LMWH affects cancer progression, and this effect cannot be solely ascribed to prevention of thrombo-embolism (23, 24, 27). Recently a rationale for a clinical study on heparin treatment of cancer has been
proposed (18). To achieve an effective inhibition of P- and L-selectin by heparin treatment during the time period from the initial detection of the primary tumor all the way through the time of the surgical removal has been suggested. During this period tumor cells are in vasculature, thus heparin treatment would be most beneficial to the patients. Based on the encouraging results obtained from clinical trials thus far, two prospective clinical trials have been recently initiated.

References


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Figure Legend:
Figure 1 Potential mechanisms of heparin implicated in its anti-metastatic activity.

A. Heparin inhibits the angiogenesis within the primary tumor by interfering with cytokine signaling and reducing thrombin dependent PAR activation, thereby attenuating the intravasation of tumor cells. After the intravasation, tumor cells rapidly interact with platelets which protect them from NK cell dependent clearing. These interactions are mainly P-selectin-mediated and are efficiently inhibited by heparin. B. At distant sites, heparin suppresses the cell-cell interactions via blocking selectin- and integrin-mediated interactions. The inhibitory activity of heparin on platelet activation and the coagulation cascade is also mediated via antithrombin and release of TFPI from endothelial cells. In addition, the homing of tumor cells and the recruitment of leukocytes contributing to metastasis is impeded by heparin treatment through its blocking effect on chemokine signaling. All these activities hinder the arrest of tumor cells in the microvasculature and the generation of a permissive microenvironment which promotes the formation of metastatic foci. C. Heparin further inhibits the heparanase activity which leads to a decreased invasion and extravasation. Heparanase inhibition can additionally reduce the release of matrix bound cytokines. A direct effect on cytokine signaling also reduces the proliferation of metastatic tumor cells.
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Figure 1

- Angiogenesis
- Heparanase activity
- Platelet aggregation and tumor embolus formation

A

- Cell-cell interactions (integrins, selectins)
- Coagulation and platelet activation
- Leukocyte recruitment

B

- Heparanase activity
- Cytokine induced proliferation

C

- Tumor cell
- Leukocyte
- Platelet
- Endothelial cell