NON-THEMATIC REVIEW

Malignant melanoma as a target malignancy for the study of the anti-metastatic properties of the heparins

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Abstract The outlook for metastatic melanoma to the brain is dismal. New therapeutic avenues are therefore needed. The anti-metastatic mechanisms that may underpin the effects of low molecular weight heparins (LMWHs) in *in vitro* and preclinical melanoma models warrant translating to a clinical setting. This review outlines a rationale that supports our proposal that metastatic melanoma to the brain is a clinical setting in which to study the anti-metastatic potential of LMWHs. Prevention or delay of brain metastases in melanoma is a clinically relevant and measurable target. Studies to explore the effect of anti-coagulants on cancer survival are underway in other malignancies such as lung, pancreas, ovary, breast, and stomach cancer. However, no study to our knowledge has a methodology that could produce clinical evidence in support of a mechanism for whatever benefit may be seen. The setting we propose would allow translation of the molecular knowledge of the metastatic pathways mediated by platelets and the selectins—all potential targets of heparin—in a "time to appearance" of brain metastases endpoint. Since brain metastases are so common and they have a singularly adverse impact on survival, the "biological neuroprotection" model we propose in metastatic melanoma could provide the translational evidence to support the benefit of LMWHs in melanoma. More significantly, this would open the door to a wider "anti-metastatic" approach that could have much greater impact in patients with minimal disease being treated in adjuvant settings for the more common malignancies such as breast and colon cancer.

Keywords Low molecular weight heparins · Metastatic melanoma · Selectin · Anti-metastatic

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1 Introduction

The benefits from using anticoagulants in the treatment of patients with cancer may arise from two clinically different but biologically intertwined routes: direct effects (treatment or prevention) on thrombotic complications of malignancy and postulated anti-metastatic cancer effects.

From early laboratory and preclinical studies, melanoma may be a malignancy that would benefit from anticoagulant agents. Unlike pancreatic cancer [1], melanoma is not highly thrombogenic, and the clinical benefit suggested is unlikely to be solely due to the prevention of venous thromboembolism [2]. Preclinical experiments in the 1980s of anticoagulants in cancer treatment led to some poorly designed underpowered clinical trials which suggested improved survival in small cell lung cancer (SCLC) and melanoma. These two malignancies have three things in common: a relatively low incidence of thrombosis [2, 3], neuroectodermal origin, and early metastasis, often to the

brain. These two malignancies, however, are very different in terms of sensitivity to conventional treatments. The exquisite radiosensitivity of SCLC has led to survival gains through the neuroprotective strategy of prophylactic cranial irradiation [4] whereas melanoma is relatively radioresistant. However, if a method of neuroprotection can be found for melanoma, similar survival benefits to those in SCLC may result.

These almost unique clinical properties of malignant melanoma—a tendency for indiscriminate and multiple metastases and lethal consequences of brain metastases, coupled to the lack of confounding parameters of thrombophilia or a neuroprotective strategy—suggest melanoma as a model malignancy to study the anti-metastatic potential of low molecular weight heparins (LMWHs). This paper outlines the rationale supporting this proposal.

2 Brain metastases in melanoma

The high mortality in melanoma patients relates to the high incidence of brain metastases; the median survival of unselected melanoma patients with brain metastases is only 2–4 months [5]. Melanoma cells appear to have substantial neurotropism; it is the fourth most common primary site to metastasize to the brain [6]. Melanoma accounts for 10% of all brain metastases and has the second highest incident proportion percentage [7]; up to 13% of patients with regional disease (AJCC stage III) [8], 18–46% of stage IV patients [6, 8], with a prevalence of up to 75% at autopsy [8–11].

It was hoped that two cytotoxic agents that cross the blood–brain barrier (BBB) (temozolomide and/or fotemustine) would improve survival. Unfortunately, neither drug provided any statistical advantage compared with the current gold standard, dacarbazine (DTIC) [12, 13]. This probably reflects the poor overall chemosensitivity of melanoma, although fotemustine [12] did fare somewhat better than dacarbazine in demonstrating a delay in the onset of brain metastases; the median time to brain metastases was 22.7 months (95% confidence interval (CI), 9.62–23.33) in the fotemustine arm versus 7.2 months (95% CI, 6.28–11.70) in the DTIC arm (P=.059). This just failed to translate into an overall survival advantage; 7.3 months (95% CI, 6.01–8.84) in the fotemustine arm versus 5.6 months (95% CI, 5.03–6.54) in the DTIC arm (P=.067 in the intent-to-treat population) which may reflect an underpowered study. Nevertheless, it strongly suggests that a survival benefit could accompany better CNS control of melanoma metastases and that a strategy of "biological interference" with the metastatic process to the brain may result in survival benefit for patients with advanced melanoma.

3 Elements of neuroprotective strategies

The data from the above mentioned phase III trial [12] show that 20% of patients had brain metastases at study entry and 20% developed them over the assessment period of around 6 months. It is unlikely that all the brain metastases that develop post-randomization result from pre-implanted melanoma cells. It is more likely that a proportion of these have resulted from an ongoing process of melanoma cells shed from other metastases continuously arriving in the microcirculation of the brain—slowing down, attaching to endothelium, and finally, successfully implanting.

A neuroprotection model of "biological interference" would assume that BBB invasion by melanoma cells is a stochastic event, i.e., occurs randomly on a background of a predictable continuous process, thus requiring continuous prophylaxis. This model can be broken down into three components:

(1) Cytoreduction—i.e., reducing the burden of BBB implanted cells.
(2) Inactivation of the "metastatic niche"—i.e., interfering with the mechanisms driving invasion.
(3) Prevent implantation—i.e., interfering with the process of endothelial cellular attachment.

This model differs to that of SCLC mostly because the conventional modalities of chemotherapy and radiotherapy achieve all the above stated components through the exquisite sensitivity of SCLC to these treatments. Thus, cytoreduction and inactivation are achieved by the chemotherapy and the radiotherapy while further implantation is reduced by the systemic response to chemotherapy; the efficacy of this strategy is mostly seen in patients who have achieved complete or consolidated partial response with radiotherapy [4].

In melanoma, this is not possible as we have no agents that can deliver this "neuroprotective" component through significant cancer cell kill. However, if a strategy of continual "inactivation" and "interference" with the metastatic process can be developed to complement a cytotoxic induction phase, however modest, one may produce a survival benefit.

4 Coagulation pathways and the metastatic process

Cancer cells are continuously shed into the circulation either from the primary or from established metastases. However, the metastatic process is inefficient and cell survival in the circulation is low due to a combination of shear forces, phagocytosis, and obstruction within capillary beds. Only 1.5% of nonhematogenous cells injected into the bloodstream survive for more than 24 h [14].

The complex biological pathways of the metastatic process can, simplistically, be categorized as coagulation-dependent and coagulation-independent. These insights have existed for more than 30 years [15]. Although there is some evidence that some of the coagulation factors may also be involved in coagulation-independent promotion of metastasis (such as tissue factor [16]), it appears that the successful negotiation of the intravascular phase of the cancer cell journey is heavily dependent on mechanical and signaling interactions that would promote thrombosis in the physiological state. There is unequivocal evidence of the interactions between disseminating tumor cells and blood cells, mainly platelets [17–19]. Initial support for an active role of platelets in metastasis was obtained from a mouse model in which experimental thrombocytopenia led to attenuation of metastasis [19]. The significance of the tumor cell microemboli formation was suggested when the intravenous injection of tumor cells led to a rapid association with platelets, whereas in the absence of this interaction, tumor cells were cleared by natural killer (NK) cells [20]. The intravenous tumor cell injection coincided with a temporal reduction of peripheral platelet counts. Interference with platelet–tumor cell interactions resulted in the attenuation of metastasis, further supporting the protective role of microemboli formation for tumor cell survival [21, 22]. These "transit" metastatic emboli include leucocytes and function as vehicles that shield the cancer cell(s) from shear forces and immunosurveillance (NK cells) [23], maintain viability of the cancer cell(s) through signaling [24, 25], promote retention in capillaries, and provide an *in situ* readily available repository of signaling molecules that facilitate colonization of distant organs [26, 27] when the microembolic phase is initiated. Propagation–prolongation of this process through maintenance of the micro thrombosis-related microenvironment (activation of cytokines and coagulation factors) promote establishment of the "metastatic niche" (Fig. 1).

4.1 Coagulation factors and melanoma metastases

Coagulation factors, particularly thrombin and tissue factor (TF), play an important role in melanoma metastasis [28–31]. Thrombin stimulates platelets, induces tumor angiogenesis, regulates tumor cell adhesion to platelets and endothelial cells, and promotes tumor growth and metastasis [28–30]. Thrombin responses in melanoma cell motility and metastasis depend on proteinase-activated receptor-1 (PAR1) [32]; PAR1 is also required in the development of TF-mediated metastasis [33]. The PAR1 expression is significantly higher in metastatic melanoma cells, which however have undetectable levels of endogenous activator protein 2 (AP-2) [30, 32]. The loss of transcription factor AP-2 is a crucial event in the development of malignant melanoma [34] and results in overexpression of PAR1 that regulates the metastatic phenotype of melanoma [35] by induction of cell adhesion molecules, matrix-degrading proteases, stimulating the secretion of angiogenic and invasive factors [30, 36–38]. PAR1 and thrombin could be potential targets for antitumor and anti-metastasis therapy for melanoma patients [30, 35, 37]. The data contribute to a mechanistic explanation of the preclinical data that have persistently pointed to potential anti-metastatic properties of anticoagulants [30, 39] via effects on the coagulation cascade.

5 Endothelial accesses and the metastatic process

The arrest of cancer cells in small vessels is an important step in metastasis. Although simple mechanical entrapment has been proposed [40, 41], more complex processes are involved [26, 39, 42–44] including the alteration of cell surface glycosylation, which is a common feature of carcinoma progression and metastasis; in particular, high expression of sialylated fucosylated glycans such as sialyl Lewisx/a. A further significant role in these processes is played by integrins [45] and although very recently, anti-metastatic effects of heparins in melanoma have been postulated to be mediated in certain cases though interaction of heparin with integrins [46, 47], the complexity, diversity, and the non-uniform impact of heparins [48] on this pathway put a detailed discussion beyond the scope of this review.

5.1 Selectins and cell "docking"

Selectins are a family of mammalian vascular adhesion molecules: P(latelet)-selectin, E(ndothelial)-selectin, and L(eucocyte)-selectin [49]. Both E- and P-selectins are inducible membrane proteins expressed on activated endothelial cells or platelets following various proinflammatory cytokine stimulations such as TNF and thrombin [50]. Selectins could interact with those sialyl Lewisx/a-containing cell surface glycoconjugates, normally found on mucin-type glycoproteins of leukocytes and endothelium, and mediate tethering, rolling, and adhesion of cells [49, 51, 52]. Thus, "deceleration" to achieve surface purchase is achieved through cellular glycocalyx and selectin-mediated interactions inducing "rolling", in a manner that is similar to that in the regulated recruitment of leukocytes to tissue sites of damage and inflammation.

P-selectin has been demonstrated to mediate crucial cancer cell interactions with platelets and endothelial cells [39, 49, 51, 53]. The expression of E- and P-selectin on endothelial cells initiates tethering and rolling of the cancer cells and formation of weak adhesions to the endothelial cells within the capillary bed [21]. Rolling is now thought

Fig. 1 Potential role of heparin–selectin interaction in melanoma metastasis. a Platelet–tumor cell microemboli formations are primarily mediated by P-selectin and platelet aggregation. L-selectin mediates the recruitment of leukocytes to tumor cells. These "transit" metastatic emboli include leucocytes and function as vehicles that shield the tumor cell from shear force and immunosurveillance (NK cells). The expression of E- and P-selectin on endothelial cells initiates tethering and rolling of tumor cells. This weak adhesion to the endothelium promotes establishment of the "metastatic niche" usually associated with more "definitive" binding to intercellular cell adhesion molecules. b Heparins bind to selectins and inhibit their function
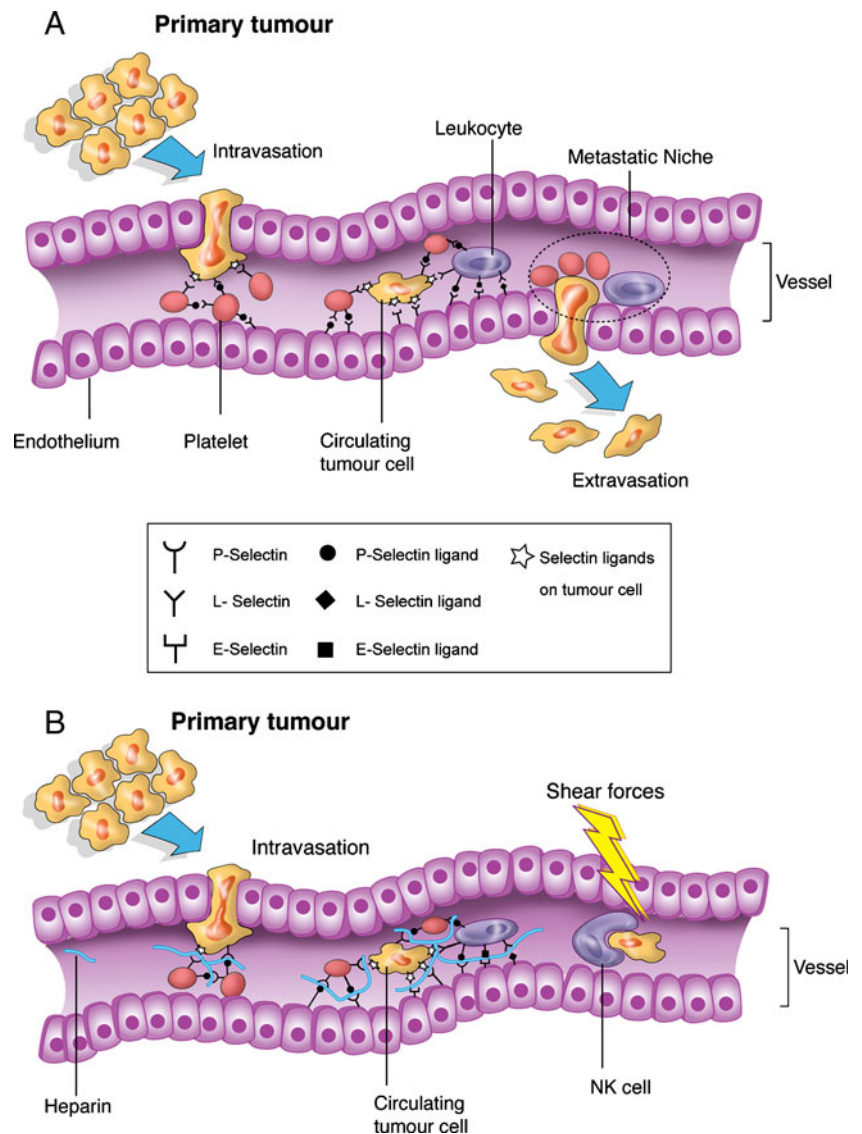
to be a major primary deceleration mechanism used by melanoma cells prior to more definitive integrin-related anchoring to the endothelium [54].

6 Heparins and melanoma metastases

6.1 The *in vitro* and preclinical evidence

The effect of the heparins on the melanoma metastatic process has been repeatedly demonstrated since in both experimental and spontaneous metastatic models (Table 1). The emergence of the role of selectins regulating this "docking" process of the cancer cell has pinpointed that at least in the experimental tumor cell models the total effect of heparin on the platelet–tumor cloak can be accounted for by its interactions with P-selectin molecules [39, 55]. Moreover, these investigators and others [54, 56] have

demonstrated that the interactions of the cancer cell with P- and E-selectins on endothelium are equally important for adhesion and similarly a major therapeutic target of the heparins [55, 57] (Fig. 1).

6.2 The proposed mechanism of the heparin–selectin interaction

P-selectin binds a large range of heparin sulfate and heparin fragments [57]. L-selectin may require a more specific sequence for recognition. Although binding to Sialyl-Lewis X motif on mucin-like molecules is thought to be a shared ligand between the selectins, this interaction is weak [42, 58], and there is no clear superior single structural motif. The suggested mechanism is that the recognition involves a "clustered anionic patch", rather than a linear-defined oligosaccharide sequence. The most convincing high affinity interaction discovered is that of P-selectin glyco-

Table 1 Preclinical and *in vitro* evidence of molecular targets of heparins in malignant melanoma

Molecular target	Model	Type of Heparin	Mechanisms
Endothelial P-selectin	B16F10 injected mice; murine B16F10, human NW624 and NW1539 cells	UFH	Heparin not only inhibits endothelial P-selectin-mediated melanoma cell rolling on microvasculature, but also attenuates melanoma metastasis formation *in vivo* [61].
P-selectin	Human A375 cells	Modified heparin	Heparin can block P-selectin-mediated A375 human melanoma cell adhesion [67, 68].
P-selectin	B16 injected mice; murine B16, human NW624 and NW1539 cells	UFH; LMWHs (nadroparin, enoxaparin); synthetic pentasaccharide fondaparinux	UFH and LMWHs inhibit P-selectin me-diated rolling of melanoma cells *in vitro* and *in vivo* [56].
P-selectin	B16-BL6 injected mice; murine B16-BL6 cell	Heparin derivatives	Modified non-anticoagulant species of heparin specifically inhibit selectin-mediated cell–cell interactions, hepara-nase enzymatic activity, or both [69].
Selectins	B16F10 injected mice; murine B16F10 cell	Orally absorbable heparin derivative (LHD)	Orally active LHD could have anti-metastatic effect via inhibition of cell–cell interaction between melanoma cells and platelets or HUVECs by interrupt-ing selectin-mediated interactions [54].
Integrin α4β1 (VLA-4)	Murine B16F10, human MV3 cells	UFH; LMWH (tinzaparin), a fraction of tinzaparin, fondaparinux.	Heparin is shown to interfere with the VLA-4/VCAM-1 interaction and inhibit integrin VLA-4-mediated interactions [46, 70].
Integrin αIIbβ3	B16F10 or A375 injected mice; murine B16F10 and Human A375 cells	Modified heparins	Modified heparins can inhibit melanoma tumor cell–platelet interaction mediated by platelet integrin αIIbβ3 [47].
PAR1and CD24	B16 and K1735 injected mice; murine B16 and K1735 cells	LMWH (nadroparin)	LMWH inhibits thrombin-dependent PAR1 activation, thus, further inhibiting the platelet or endothelial cell activation and L- and P- selectin-mediated cell–cell adhesion; cancer cell express CD24 takes part in metastasis as a ligand of P-selectin. LMWH inhibits P-selectin/CD24 interaction prevent binding of platelets to cancer cells [71].

UFH unfractionated sodium heparin, *LMWHs* low molecular weight heparins

protein ligand-1 (PSGL-1) with P-selectin. This sialomucin polypeptide is expressed on all leucocytes but becomes specialized for selectin recognition on neutrophils and monocytes. The most interesting biophysical aspect of interaction of these molecules is that it involves novel "catch bonds" which help to explain the rollin g behavior shown by leucocytes coming into contact with endothelium [59, 60].

Unrelated glycans (e.g., the heparins) can also act as ligands for L- and P-selectins and the clinically used molecules (highly sulfated) are potent inhibitors, causing complete inhibition of both L- and P-selectins *in vitro* assay within recommended clinical anticoagulation target levels, with unfractionated heparin being the most potent (Fig. 1). Ludwig et al. [56, 61], employing the murine B16 melanoma lung metastasis model, revealed that heparin

treatment inhibited lung colonization (by >80%) if given prior to, but not after intravenous inoculation of melanoma cells. They showed that heparin inhibits melanoma rolling in a P-selectin-dependent fashion. Whereas in P- selectin$^{-/-}$ mice rolling remained unaffected by heparin, in wild-type mice heparin treatment reduced rolling of three different melanoma cell lines (Mel1539, Mel624, and B16F10) in a dose-dependent manner. Together, these data support the assumption that both platelet and endothelial P-selectin are potential targets of heparin action in melanoma metastasis formation. These effects have been reproduced using an orally administered heparin (a chemical conjugate of LMWH and deoxycholic acid) in a murine melanoma lung metastases model [54]. The LMWHs have similar effects but IC$_{50}$ values of these compounds against selectin–PSGL-1

interactions were closer to the recommended target range for therapeutic anticoagulation. This difference between unfractionated and LMW heparins is likely to be due to the reduced efficacy of smaller-sized fragments interacting with the "clustered anionic patch". Nevertheless, even these agents, dosed at therapeutic (rather than prophylactic) levels, are highly effective selectin inhibitors [56, 61].

6.3 The clinical evidence

There has been a recent resurgence in the interest of the potential effect of anticoagulants in cancer. Unfortunately, the studies reported to date [62, 63], although hinting at potential survival benefits from the use of LMWH, are difficult to interpret as the populations studied are usually mixed in terms of histology type and stage of cancer. It is difficult to determine whether the effect is through prevention of thrombosis (e.g., in mucin-producing adenocarcinomas) or through potential non-anticoagulant-mediated anti-malignancy effects. Therefore, it is interesting to consider the historical studies which, while smaller and less robust, focused on specific cancers.

At least two studies in SCLC demonstrated a survival benefit for those receiving unfractionated sodium heparin (UFH) or LMWH [3, 64]. The thrombosis incidence in the control groups was negligible but the benefit was significant. This was difficult to explain and although it was attributed to a better response rate at least in one study, we suggest that other mechanisms relating to modification of the natural history of this disease (such as reducing incidence or delaying onset of brain metastases) may have played a role. There are no data from these trials to support or refute this hypothesis.

The data suggesting clinical role and effect of anticoagulants on melanoma have been previously summarized [65, 66]. All the studies reviewed pointed to survival benefit with anticoagulants although warfarin was the only anticoagulant used in these early studies. Mechanisms suggested were those of thrombin inhibition and possibly beneficial effects on TF generation.

We therefore suggest that heparin or LMWH strategies employed for their capacity to interfere with the metastatic process have reasonable data in support and that the model of prevention or delay of melanoma brain metastases could be one that could elicit this benefit in a clinically measurable sense (brain metastases-free interval) that could also result in a patient relevant benefit in terms of survival.

7 Summary

The anti-metastatic mechanisms that may underpin the effects of heparin–LMWH in *in vitro* and preclinical

melanoma models warrant testing in the clinical setting as prevention or delay of brain metastases in melanoma appears to be a clinically relevant and measurable target. Studies to explore the effect of anticoagulants on cancer survival are underway in other malignancies such as lung, pancreas, ovary, breast, and stomach cancer. However, no study to our knowledge has a methodology that could produce clinical evidence in support of a mechanism for whatever benefit may be seen. The "biological neuro-protection" model we propose in metastatic melanoma could provide this evidence, supporting the benefit of LMWH in melanoma, but more significantly, opening the door to a wider "anti-metastatic" approach that could have much greater impact in patients with minimal disease being treated in adjuvant settings for the more common malignancies such as breast and colon cancer.

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