

# P-selectin, carcinoma metastasis and heparin: novel mechanistic connections with therapeutic implications

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## Abstract

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Metastasis is a multistep cascade initiated when malignant cells penetrate the tissue surrounding the primary tumor and enter the bloodstream. Classic studies indicated that blood platelets form complexes around tumor cells in the circulation and facilitate metastases. In other work, the anticoagulant drug heparin diminished metastasis in murine models, as well as in preliminary human studies. However, attempts to follow up the latter observation using vitamin K antagonists failed, indicating that the primary mechanism of heparin action was unrelated to its anticoagulant properties. Other studies showed that the overexpression of sialylated fucosylated glycans in human carcinomas is associated with a poor prognosis. We have now brought all these observations together into one mechanistic explanation, which has therapeutic implications. Carcinoma cells expressing sialylated fucosylated mucins can interact with platelets, leukocytes and endothelium via the selectin family of cell adhesion molecules. The initial organ colonization of intravenously injected carcinoma cells is attenuated in P-selectin-deficient mice, in mice receiving tumor cells pretreated with O-sialoglycoprotease (to selectively remove mucins from cell surfaces), or in mice receiving a single dose of heparin prior to tumor cell injection. In each case, we found that formation of a platelet coating on cancer cells was impeded, allowing increased access of leukocytes to the tumor cells. Several weeks later, all animals showed a decrease in the extent of established metastasis, indicating a long-lasting effect of the short-term intervention. The absence of obvious synergism amongst the three treatments suggests that they all act via a common pathway. Thus, a major mechanism of heparin action in cancer may be inhibition of P-selectin-mediated platelet coating of tumor cells during the initial phase of the metastatic process. We therefore suggest that heparin use in cancer be re-explored, specifically during the time interval between initial visualization of a primary tumor until just after definitive surgical removal.

### Key words

- Heparin
- Carcinomas
- P-selectin
- Mucin
- Metastasis

## **Introduction**

This review attempts to bring together a variety of previously known facts about the biology, prognosis and therapy of cancer, and correlates them with recent findings from our own laboratory. While many questions remain unanswered, we are able to logically connect together much of the available information, and thus propose some novel mechanistic connections that have therapeutic implications in humans.

## **Platelet interactions with tumor cells are involved in the process of metastasis**

Blood platelets are circulating anucleate cellular particles derived from bone marrow megakaryocytes that are involved in a variety of physiological and pathological processes such as hemostasis, thrombosis, inflammation and wound repair. They participate in such processes either via receptor proteins on their surface or by the release of contents stored within their cytoplasmic granules. Many classic studies (reviewed in Refs. 1-3) have suggested that blood platelets are also intimately involved in the process of cancer metastasis, whereby malignant cells derived from a primary tumor mass invade the surrounding tissue, enter the bloodstream, evade host defenses and eventually colonize distant organs (4-7). It appears that when cancer cells enter the bloodstream they rapidly form microemboli, which are multicellular complexes composed of tumor cells surrounded by platelets and leukocytes. These microemboli then arrest in the narrow capillaries within organs distant from the primary tumor. If this arrest occurs in conducive "soil", then the eventual extravasation and survival of the tumor cells results in established metastatic foci.

## **Lowering of blood platelet counts attenuates tumor metastasis in mice**

Clear evidence for the importance of

platelets in cancer metastasis came from studies where thrombocytopenia (a low circulating platelet count) was consistently associated with a decreased incidence of distant metastases. Early work demonstrated that neuraminidase (sialidase)-induced thrombocytopenia greatly reduced metastasis (8). A reduction in pulmonary metastases by thrombocytopenia induced by other methods was also reported (reviewed in Refs. 1-3). These studies concluded that specific cell surface receptors on tumor cells played a role in mediating the platelet-tumor cell interaction. Despite much subsequent work on the topic (for example, see Refs. 9,10), the identity of the cell surface receptors involved, and the precise mechanisms by which platelets play their role in contributing to cancer metastases remained obscure.

## **Heparin treatment attenuates metastasis in mice and may improve tumor prognosis in humans**

Several studies have shown that tumor metastasis in experimental animals can be inhibited by heparin (reviewed in Refs. 11-14). Prior hypotheses to explain this finding included the inhibitory effects of heparin on blood coagulation, alteration of growth factor action, suppression of angiogenesis, and the inhibition of heparanases required for vascular basement membrane penetration (12,15-19). A few clinical studies in humans with cancer also suggested a beneficial effect of heparin (17,20-22).

## **Vitamin K antagonists do not generally improve tumor prognosis in humans**

Given the initial assumption that the heparin effect was primarily due to its anticoagulant action, several large controlled studies in humans were carried out using more easily manageable oral anticoagulants (pri-

marily vitamin K antagonists such as Coumadin). However, most of these studies failed to show a benefit (13,23). A more recent trial reported that among patients with recurrent venous thromboembolism, the incidence of cancer was lower in those patients who had received a long-term dose of Coumadin (24). However, this study did not consider that the amount and duration of heparin therapy these patients received may also have been different. Overall, the mechanism of heparin action in cancer remained somewhat obscure.

### **Sialylated fucosylated antigens on cell surface mucins confer a poor prognosis in human carcinomas**

All cells have an outer coat made up of glycosylated molecules. Mucins are large rod-like glycoproteins with extensive O-linked glycosylation, typically found on epithelial cell surfaces. During progression to malignancy, the normal topology and polarity of epithelial cells changes markedly, and cell surface glycoconjugates are aberrantly expressed and displayed on the malignant cells (25-29). Increased expression and altered glycosylation of cell surface mucins are known to be prominent features of carcinoma progression. Sialyl Lewis A and sialyl Lewis X are sialylated fucosylated tumor-associated antigens that fall into this category. There is a general association between the expression of these antigens on tumor cells and poor prognosis due to tumor progression and metastasis (25,26,28-33).

E-, P-, and L-selectins are well-known vascular receptors for certain sialyl Lewis<sup>x/a</sup> antigens containing mucin-type glycoproteins found on leukocytes and endothelium (34-37). Thus, one explanation for poor prognosis of carcinomas carrying sialyl Lewis<sup>x/a</sup> antigens is that cell surface glycans can act as pathological ligands for all three members of the selectin family of cell adhesion molecules (38,39). As selectins can indeed me-

diate tumor cell interactions with platelets, leukocytes and endothelium *in vitro* (39-41), it is reasonable to suggest a role for these molecules in the metastatic spread of tumors. Earlier studies hypothesized a simple model whereby malignant cells would be recognized by E- or P-selectin on endothelial cells, thus permitting extravasation from the bloodstream into metastatic sites (33). However, L- and P-selectin (present on leukocytes and platelets) can also recognize the mucins on these tumor cells (40), thus predicting more complex interactions between selectin-positive carcinoma cells and host cells within the vasculature.

### **A role for P-selectin in platelet-tumor complex formation and facilitation of metastasis**

As discussed above, tumor cells in the bloodstream have frequently been observed to be associated with platelets, which appear to facilitate the metastatic process. To study the role of platelet P-selectin in tumor growth and metastasis, we generated P-selectin-deficient mice in a Rag2<sup>-/-</sup> immunodeficient background, allowing the use of human carcinoma cells in metastasis assays. The long-term organ colonization of intravenously injected carcinoma cells was attenuated in these mice. To study the mechanism of this effect, we injected fluorescently labeled carcinoma cells intravenously into P-selectin wild-type Rag2<sup>-/-</sup> animals, and determined their interactions with other cells in the vasculature *in vivo* (42). Frozen section immunohistochemistry analyses showed that the fluorescent tumor cells that were arrested in the lung vasculature were surrounded by a "cloak" of CD41-positive mouse platelets. In contrast, the P-selectin-deficient mice showed a much less obvious coating of platelets around these tumor cells (42). Thus, the efficient formation of platelet-tumor aggregates in this model system requires P-selectin expression by the platelets.

### **Platelet-tumor complex formation and metastasis can be attenuated by tumor mucin removal**

To confirm that carcinoma cell surface mucins are the operational ligands for platelet P-selectin *in vivo*, tumor cells were pre-treated with the mucin-specific enzyme, O-sialoglycoprotease, to remove the tumor cell surface sialylated fucosylated mucin ligands (the cells were viable after this treatment and could recover mucin expression during *in vitro* culture) (43). When the lungs of the animals injected with such treated cells were examined, there was a much lower capacity to form complexes with platelets compared to animals injected with sham-treated tumor cells. As with P-selectin deficiency, the lungs of animals examined 4 weeks later showed an attenuation of metastasis formation with the treated cells. Combinations of P-selectin deficiency and O-sialoglycoprotease treatment did not seem to be additive (43).

A single dose of heparin blocks P-selectin-based platelet-tumor cell complex formation and attenuates subsequent metastasis. We had previously found that clinical heparin preparations are excellent inhibitors of P- and L- (but not E-) selectin (44). To determine the ability of heparin to inhibit early platelet-tumor cell association, we used mice for tumor cell injections after they had received various doses of heparin at different time points. A single 100-unit dose of heparin given 30 min before the tumor cell injections could reversibly block the tumor cell-platelet interaction for up to 5 h. Three-dimensional reconstruction of the tumor cell-platelet complexes was done using deconvolutional microscopy and new types of volume-rendering software (43). These data confirmed that P-selectin deficiency, O-sialoglycoprotease treatment or heparin treatment all result in fewer, more loosely packed platelets around individual tumor cells. In addition we detected a greater number of monocytes (macrophage precursors) associ-

ated with tumor cells in each of these situations. Thus, a major effect of blocking platelet interactions with circulating tumor cells in the bloodstream may be to allow the access of immune effector cells to tumor cells.

Metastatic foci 6 weeks after tumor cell injection were found to be substantially reduced in the heparin-treated animals (43). Thus, a single injection of heparin can attenuate tumor metastases, probably via inhibition of P-selectin-based platelet interactions with sialylated fucosylated tumor cell surface mucins. As already indicated, P-selectin deficiency or O-sialoglycoprotease treatment also inhibits short-term tumor cell-platelet interactions *in vivo* as well as the degree of organ colonization analyzed several weeks later. The absence of an obvious synergism between the three approaches suggests that they may all act via a common pathway. Overall, it appears that the P-selectin-mediated tumor cell-platelet interaction is important only in the early phase of circulation in the bloodstream.

### **Heparin therapy to prevent human metastasis should be revisited**

To address the potential implications of this work in mice for the human situation, we compared the effects of heparin in inhibiting *in vitro* tumor cell interactions with recombinant human and mouse P-selectin. Human P-selectin was found to be even more sensitive to heparin blockade than mouse P-selectin, and the IC<sub>50</sub> value for human P-selectin is within the accepted range for the current therapeutic use of heparin in clinical settings (43). Thus, we suggest that the failure of vitamin K antagonists to improve cancer prognosis should be ignored, and studies of heparin therapy in cancer should be revisited taking into account this new paradigm. Unlike previous studies, we suggest that heparin use should be explored during the interval from initial visualization of a primary tumor until just after its definitive

surgical removal. We suggest that this approach could prevent establishment of metastatic deposits by tumor cells that are circulating during that time period, by blocking their interaction with platelets via P-selectin.

### Issues and questions arising

These findings have raised many additional issues and questions. While selectin binding sites can be detected on primary human carcinoma specimens (40), not all tumor samples are positive for P-selectin ligands. Thus, our findings are not likely to be applicable to all patients with carcinomas. In some cases, other adhesion molecules like integrins may be involved in the platelet-tumor cell interactions (45). Heparin itself is isolated and purified from biological sources for its anticoagulant properties, and has been in clinical use for many decades. However, while heparin preparations are well standardized for their anticoagulant potency, they are actually a complex mixture of glycosaminoglycans (46-48). Thus, it is possible that there will be some batch-to-batch variations in the inhibitory

potency of heparin for P-selectin. There is also a need to study low molecular weight heparins (49-52), which are easier to manage clinically and seem to have reduced side effects. In the long run, more specific inhibitors of P-selectin such as PSGL-1 (53-55) must be explored. These will also help to dissect out any additional effects of heparin that are unrelated to selectin inhibition.

The roles of the other two selectins also need to be explored further. Under the conditions we used for our studies, E-selectin would not have been expressed. Since heparin also inhibits L-selectin binding to tumor mucins, the role of L-selectin in metastasis is currently under investigation by our group. In particular, we are interested in the possible role of L-selectin-positive leukocytes in the tumor cell-blood cell complexes. One can also ask whether studies in immunodeficient mice are relevant to the situation in the immunocompetent state, and if studies using intravenous injections of tumor cells are relevant to "natural" metastasis. These issues are currently being explored using syngeneic and spontaneous models of murine tumor growth.

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