

Heparin Inhibition of Selectin-Mediated Interactions during the Hematogenous Phase of Carcinoma Metastasis: Rationale for Clinical Studies in Humans

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ABSTRACT

Classic studies indicate that the formation of tumor cell-platelet complexes in the blood stream is important in facilitating the metastatic process. Metastasis in animal models can be inhibited by heparin, and retrospective analyses of heparin use in human cancer have shown promise. However, most follow-up human studies using vitamin K antagonists have failed, and conclusive proof for other previously proposed mechanisms of heparin action is lacking. Carcinoma progression and metastasis are associated with overexpression of sialylated fucosylated mucins. Structurally similar molecules happen to be natural ligands for vascular adhesion molecules called the selectins. Heparin also happens to be a good inhibitor of P-selectin, which is expressed on activated platelets or endothelial cells. We have found that heparin blocks P-selectin-mediated interactions of endogenous platelets with sialylated fucosylated mucins on circulating carcinoma cells and that this reduces tumor cell survival. The use of more specific and selective P-selectin inhibitors will some day help to dissect the relative importance of this mechanism of heparin action in cancer. Meanwhile, we suggest that the failure of vitamin K antagonists to improve cancer prognosis should be ignored and that heparin therapy should be immediately revisited under this new paradigm. Unlike the suggestions in most previous studies, we propose that heparin use should be reexplored specifically during the interval from initial visualization of a primary tumor until just after its definitive surgical removal. A suggested clinical trial is outlined.

KEYWORDS: Carcinomas, mucins, platelets, P-selectin, heparin

Objectives: Upon completion of this article, the reader should be able to (1) understand the role of mucins in tumor biology and (2) state the role of heparins in the hematogenous phase of cancer metastasis.

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There is a huge volume of literature about cancer metastasis, a process in which malignant cells from a primary tumor migrate to distant regions of the body via the lymphatics or the blood stream (reviewed in references 1–4). Although large numbers of malignant cells may enter the blood stream from a primary tumor, very few survive host defenses, arrest in the microvasculature of distant organs, and finally form metastatic tumor foci that are typically the cause of death. Here we correlate some seemingly disparate aspects of existing knowledge regarding hematogenous metastasis with each other and with recent findings in our own laboratory. Although many unanswered questions remain, we are able to connect much of this information together logically (Fig. 1) and to propose novel mechanistic connections that may have therapeutic potential in some human cancers. We conclude that heparin therapy for cancer should be reexamined and suggest the specific parameters for an initial clinical trial.

A. AN ATTEMPTED SYNTHESIS OF OLD AND NEW FINDINGS

A1. Platelet Interactions with Tumor Cells Are Involved in the Process of Metastasis

Circulating blood platelets are known to be involved in many physiological and pathological processes including hemostasis, thrombosis, inflammation, and wound healing. This typically involves receptor proteins on the platelet surface and/or the release of contents from granules within the platelet cytoplasm. Many classic studies have suggested a role for platelets in facilitating hematogenous tumor metastasis (reviewed in references 5–9). Thus, malignant cells from a primary tumor, having invaded through the matrix and basement membrane, gain access to the blood stream and form circulating complexes with platelets and leukocytes. The formation of these “microemboli” of malignant cells with platelets and other host cells has been proposed as a mechanism whereby the tumor cells are able to evade host defenses and eventually colonize distant organs to form metastatic foci.^{10–16} Indeed studies have shown that within a few minutes of intravenous injection, tumor cells are loosely arrested, singly or in small groups, in lung capillaries in association with a meshwork of platelets and fibrin. However, the detailed mechanism of formation of these platelet–tumor cell complexes and their precise role in facilitating metastasis have remained somewhat obscure.

A2. Lowering of Blood Platelet Counts Attenuates Tumor Metastasis in Mice

Clear evidence for the role of platelets came from observing the effects of experimentally reducing platelet counts. Early studies demonstrated that neuraminidase-

induced thrombocytopenia greatly reduced metastasis.⁶ Indeed, reduction in platelet counts inhibited metastasis of a number of tumors in experimental tumor systems.^{5,6,8,15} Several animal model studies have indicated that interference with platelet function *in vivo* can reduce tumor metastasis. It is also presumed that the interaction of malignant cells with the vascular endothelial surface is facilitated by platelets. However, the identity of the cell surface receptors involved in platelet–tumor cell interactions has remained inconclusive.

A3. Heparin Treatment Attenuates Metastasis in Experimental Animals

In apparently unrelated work, many studies showed a beneficial effect of the common anticoagulant heparin in animal models of metastasis (reviewed in references 17–21). This effect was most evident when malignant cells were introduced intravenously. However there were also some positive effects on the spread of some spontaneously metastasizing transplanted tumors. A recent exhaustive review of the effects of heparin in experimental metastasis concluded that there was a beneficial effect in most but not all such animal studies.²²

A4. Heparin May Improve Tumor Prognosis in Humans

Based on these animal studies, heparin was used along with standard chemotherapeutic agents in some humans with malignant disease, and apparent improvements in survival were noted.^{23,24} In more recent times, a positive effect of heparin has been noted in retrospective analyses of patients receiving heparin for prophylaxis or therapy of venous thromboembolism.^{22,25–30} The most impressive effect was noted in a retrospective reanalysis of one of the original randomized studies of perioperative low-dose heparin prophylaxis given to reduce postoperative morbidity from venous thromboembolism.²⁶ The subset of these patients who happened to have their surgery for cancer showed a significant reduction in 3-year mortality rates among those who also received low-dose heparin in the perioperative period.

A5. Vitamin K Antagonists Do Not Generally Improve Tumor Prognosis in Humans

Because heparin was originally developed as an anticoagulant, its antimetastatic effects were assumed to be mediated via blockade of blood coagulation. Indeed, there are several indications that the coagulation and fibrinolysis pathways are associated with angiogenesis and progression of cancer.^{17–20,28,31,32} Primarily because of their greater ease of use, most major clinical trials that followed used orally active vitamin K antagonists, which achieve anticoagulation by mechanisms very dif-

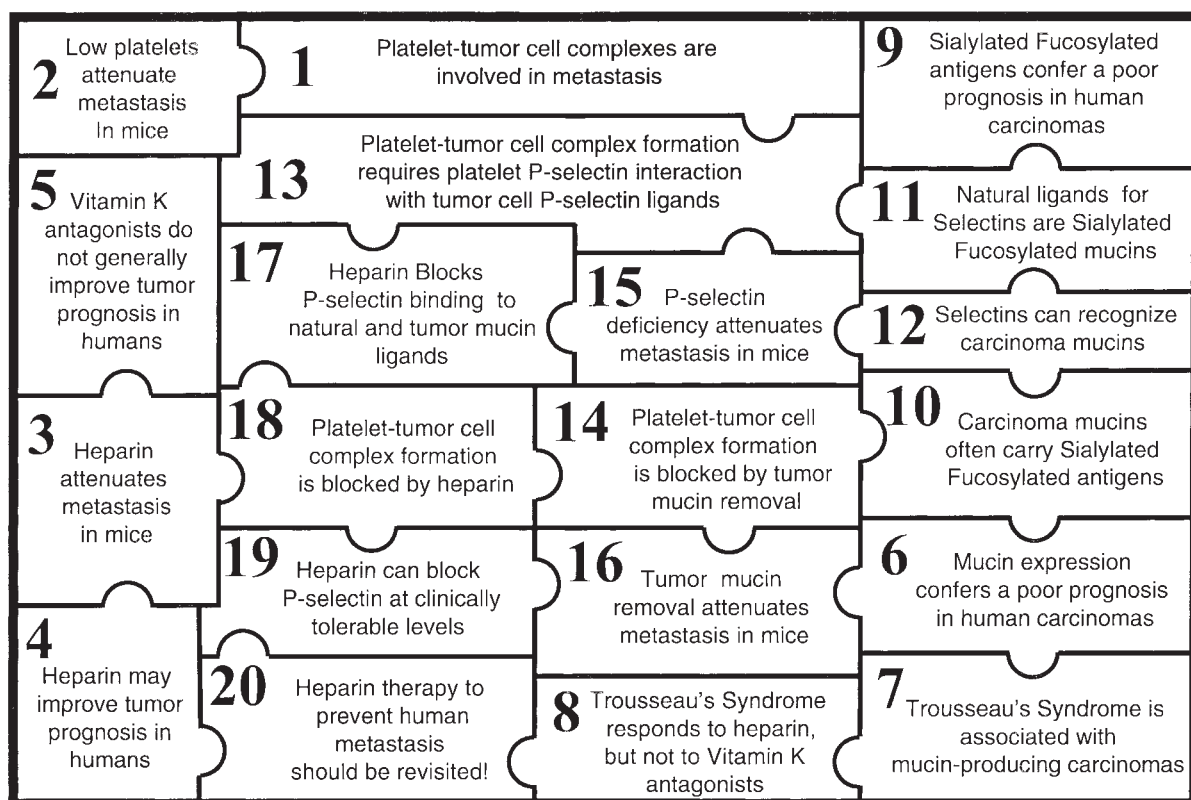


Figure 1 An attempted synthesis of old and new facts. See text for discussion. The numbers 1–20 correspond to sections A1–A20 of the text.

ferent from those of heparin (depressed production of vitamin K–dependent clotting factors).³³ However, beneficial effects of vitamin K antagonists were seen only in a few studies involving certain malignancies such as small cell carcinoma of the lung.³⁴ Despite this, the notion that anticoagulation might be an effective treatment for cancer has remained strong. Indeed, a retrospective analysis showed that the incidence of cancer was lower in the patients who had received a long-term dose of warfarin (Coumadin) for venous thromboembolism.³⁵ However, this study did not address whether such patients also received a larger cumulative dose of heparin.

A6. Mucin Expression Confers a Poor Prognosis in Human Carcinomas

Many epithelial cell surfaces are coated with mucins, which are high-molecular-weight rodlike molecules with numerous O-linked sugar chains (glycans) attached to the protein backbone.³⁶ Soluble forms of mucins are also secreted in large amounts by normal epithelial cells. As a general rule, such cell surface and secreted mucins are delivered vectorially toward the apical surface of the cells, facing the lumen of the organ in question. Cytopathologists have known for years that during progression to malignancy, the normal topology

and polarity of epithelial cells are markedly distorted. Altered glycosylation is also a very common feature of cancer cells (reviewed in references 37–42). Thus, cell surface and secreted mucins with aberrant glycosylation can become exposed to the blood stream in the setting of epithelial cancers.^{36,43} Despite the fact that mucin production is a “differentiated” phenotype, increased expression of (abnormal) cell surface and secreted mucins is known to be a prominent feature of progression and poor prognosis in many carcinomas.^{44–47}

A7. Trousseau's Syndrome Is Associated with Mucin-Producing Carcinomas

Idiopathic thromboembolism is sometimes the first sign of an occult malignant neoplasm. Trousseau's original description of superficial migratory thrombophlebitis was in patients with gastrointestinal carcinomas. What is now called Trousseau's syndrome represents a spectrum of abnormal processes resulting in many clinicopathological presentations, ranging from frank arterial and venous thromboses to deposition of platelet-rich microthrombi in the microvasculature to disseminated intravascular coagulation and microangiopathic hemolytic anemia.^{48–50} Nevertheless, it is of note that many of the classic cases involve patients with mucin-producing carcinomas.

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A8. Trousseau's Syndrome Responds to Heparin But Not to Vitamin K Antagonists

The risk of recurrent thrombotic events in patients with cancer who have developed some form of Trousseau's syndrome can be very high, even despite adequate anticoagulation. Heparin has been shown to be better than vitamin K antagonists both in treating the thrombotic problems and in preventing recurrence.⁴⁸⁻⁵² Thus, although very low dose warfarin prevents thromboembolism in patients with metastatic breast cancer who are receiving chemotherapy,⁵³ patients who already have cancer-related thrombosis often respond poorly to warfarin.⁵² Standard treatment of Trousseau's syndrome therefore consists of attempts at effective management of the underlying cancer and maintenance of continuous systemic heparin at a tolerable dose. As in the case of metastasis, the superior effects of heparin over vitamin K antagonists suggests that factors beyond the fluid phase coagulation pathway are involved.

A9. Carcinoma Mucins Often Carry Sialylated Fucosylated Antigens

The cell surface glycans on epithelial cells are modified at specific branch points by the action of several glycosyltransferases that add chain-terminating sialic acids and sometimes fucose residues in specific configurations. Some of these sialylated fucosylated structures such as sialyl Lewis A or sialyl Lewis X are expressed at high levels on the surface of some human tumor cells and/or are detectable in the sera of patients with various malignancies. Most of these epitopes turned out to be prominently expressed on the abnormal mucins derived from carcinoma cells. Indeed, although they were subsequently found on some normal cell types, sialyl Lewis X and A were originally recognized as tumor antigens recognized by specific monoclonal antibodies.^{54,55}

A10. Expression of Sialylated Fucosylated Antigens Confers a Bad Prognosis in Human Cancers

Many studies have shown that expression of such sialylated fucosylated groups on the cell surface of various human cancers is correlated with a poorer prognosis due to increased progression and metastasis.⁵⁶⁻⁶⁹ Increased levels of sialyl Lewis X antigen were also demonstrated within metastatic lesions of certain malignancies in comparison with their primary tumors. The subsequent discovery that selectins recognize sialylated fucosylated antigens suggested a possible explanation for this strong prognostic association.

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A11. Natural Ligands for the Selectins Are Sialylated Fucosylated Mucins

The selectin family of adhesion molecules comprises three structurally related membrane glycoproteins that are involved in the initial events of leukocyte adhesion to vascular endothelium and platelets in response to various stimuli.⁷⁰⁻⁷³ The N-terminal lectin and epidermal growth factor-like domains of the selectins are involved in recognizing sialylated fucosylated structures such as sialyl Lewis X and A, most often presented on mucin-like molecules whose polypeptide backbones are distinct from those of the epithelial mucins (Fig. 2). In the case of P- and L-selectin (but not E-selectin) there is also strong evidence that sulfate groups collaborate with sialylated fucosylated structures to generate the optimal ligands for recognition.⁷⁴⁻⁷⁶

A12. The Selectins Can Recognize and Bind to Some Carcinoma Mucins

On the basis of the preceding information, we and others hypothesized that selectins might recognize these aberrantly glycosylated carcinoma mucins bearing sialylated fucosylated (and sometimes sulfated) structures. Studies with cell lines⁷⁷⁻⁷⁹ and with purified carcinoma mucins and recombinant soluble selectins^{80,81} proved this to be true. We were also able to elicit all the possible selectin-based interactions shown in Figure 2, of carcinoma cells with platelets, leukocytes, and endothelium (all demonstrated *in vitro* and many *in vivo*).⁸⁰⁻⁸² Immunohistochemistry of sections from primary human colon carcinomas showed the heterogeneous expressions of calcium-dependent ligands for all three selectins in some but not all tumors.⁸¹ Thus, the expression of selectin ligands on malignant tumors provides an opportunity for selectin-based interactions in the blood stream with host platelets, leukocytes, and endothelial cells.

A13. Platelet-Tumor Cell Complex Formation Requires Platelet P-Selectin Interaction with Tumor Cell P-Selectin Ligands

In earlier studies, a model had been proposed for the extravasation of malignant cells from blood vessels wherein the tumor cells would adhere directly to E- or P-selectin expressed on endothelial cells.^{37,79,83} However, P-selectin is present not only in the Weibel-Palade bodies of endothelial cells but also in the alpha granules of platelets—and both pools are mobilized to the cell surface upon cellular activation, generating complex interactions involving leukocytes and endothelial cells.^{84,85} Given the past information about the role of platelets in facilitating metastasis, it seemed reasonable to focus specifically on the role of platelet P-selectin in the process.^{16,82,86} Indeed, we found that although thrombin-

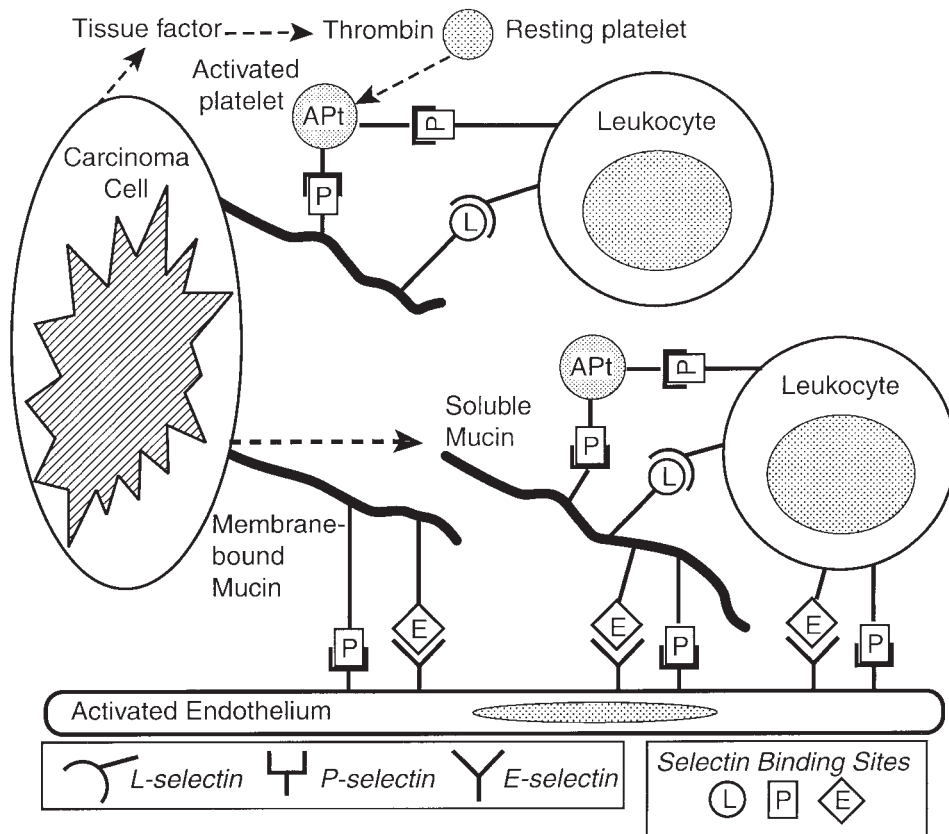


Figure 2 Interactions between carcinoma mucins and selectins. The cartoon depicts all possible pathological selectin-based interactions that could occur involving a carcinoma cell in the blood stream that expresses ligands for all three selectins. Also shown are the interactions of P- and E-selectin with natural ligands on leukocytes. All of these interactions have been demonstrated in vitro, and several in vivo. See text (sections A7–A18) for discussion.

stimulated mouse platelets adhere to human colon carcinoma cells bearing P-selectin ligands, the interaction is markedly diminished when the platelets used are from P-selectin-deficient mice.¹⁶ Similar differences were seen in vivo when fluorescently labeled P-selectin ligand-positive carcinoma cells were injected into wild-type and P-selectin-deficient mice.^{16,82} Immunohistochemical analysis of lung microvasculature showed that the fluorescent tumor cells were surrounded by a “cloak” of CD41-positive mouse platelets. There were fewer fluorescently labeled tumor cells arrested in the lungs of P-selectin-deficient mice, and the cloak of platelets around these cells was not as obvious.^{16,82}

A14. Platelet–Tumor Cell Complex Formation Can Be Reduced by Tumor Mucin Removal

To confirm that carcinoma cell surface mucins are the operational ligands for platelet P-selectin in these assays, tumor cells were pretreated with the mucin-specific enzyme *O*-sialoglycoprotease.⁸⁷ This highly selective protease can (partially) remove the tumor cell surface sialylated fucosylated mucin ligands while leaving other surface structures intact. Platelet binding to

such treated tumor cells was indeed reduced, to levels almost as low as those seen with platelets from P-selectin-deficient animals.^{16,82}

A15. P-Selectin Deficiency Attenuates Metastasis in Mice

To address the role of P-selectin in metastasis more directly, we generated P-selectin-deficient mice with a *Rag2*^{-/-} immunodeficient background, thus allowing the use of human carcinoma cells in experimental metastasis assays. When the mice were examined 4–6 weeks after the initial tumor cell injection, we observed that organ colonization was attenuated in P-selectin-deficient mice as compared with P-selectin-positive, control mice.^{16,82}

A16. Tumor Mucin Removal Attenuates Metastasis in Mice

Tumor cell surface mucins were removed with *O*-sialoglycoprotease just prior to the intravenous injection of cells into *Rag2*^{-/-} mice with or without P-selectin deficiency. When the lungs of the animals were examined

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30 minutes after tumor cell injection, there was a decrease in formation of platelet–tumor cell complexes and in the number of cells arrested in the lung microvasculature. When the lungs of another cohort were examined 4 weeks later, animals receiving *O*-sialoglycoprotease-treated cells showed diminished metastatic foci in comparison with those receiving untreated cells.¹⁶ Taken together, these data indicate that at least with the cell types we studied, the optimal formation of platelet–tumor cell aggregates requires platelet P-selectin, which interacts via tumor cell surface mucins.

A17. Heparin Blocks P-Selectin Binding to Natural and Tumor Mucin Ligands

Heparin is a complex mixture of glycosaminoglycans that does not have any obvious structural similarity to the natural or tumor mucin ligands for selectins. However, it can function as a ligand for P- and L-selectin.^{88–92} Heparin is similar to sialylated sulfated mucins in comprising dense clusters of glycans with multiple negatively charged carboxylates and sulfate esters. Perhaps because of this shared feature of a highly anionic surface, the binding of P- and L-selectin to their natural ligands is blocked well by heparin preparations that are currently in clinical use.^{16,90} We confirmed a similar blocking effect of heparin on in vitro interactions of recombinant soluble selectins with tumor mucin ligands and intact tumor cells.¹⁶ Likewise, the binding of wild-type P-selectin–positive mouse platelets to tumor cells was strongly inhibited by heparin.¹⁶

A18. Platelet–Tumor Cell Complex Formation Is Blocked by Heparin

P-selectin–positive and –negative mice were injected with varying intravenous doses of heparin at different time points and then intravenously injected with tumor cells. We observed that a single 100-unit dose of heparin could reversibly block the tumor cell–platelet interaction in the lung microvasculature for up to 4–5 hours after the injection. This was consistent with the timing of clearance of heparin from the mouse circulation, as measured by an anti-Xa assay.¹⁶ Such heparin- and tumor cell–injected mice were also examined 6 weeks later for metastatic foci. Although foci were easily seen in untreated P-selectin–positive mice, they were markedly reduced in the heparin-treated animals. There was a very limited synergism between the suppressive effects of P-selectin deficiency and heparin injection. Thus, a single dose of heparin given 30 minutes prior to tumor cell injection leads to only a short-term abrogation of P-selectin–dependent platelet–tumor cell interaction and yet has a dramatic long-term impact on the establishment of metastatic foci.

Thus, whether it was P-selectin deficiency, Is ___ mucin removal (*O*-sialoglycoprotease pretreatment), or
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there was inhibition not only of short-term tumor cell–platelet interactions in vivo but also of organ colonization (analyzed several weeks later).¹⁶ The absence of an obvious synergism between the three approaches suggests that they may all act via a common pathway. Three-dimensional reconstruction of the platelet–tumor cell complexes was done using deconvolutional microscopy and new types of volume-rendering software. Some of these images were further analyzed as “fly-by” movies, allowing more careful visualization of these complexes (see <http://www.pnas.org/cgi/content/full/98/6/3352/DC1>). These analyses confirmed that all three approaches result in fewer, more loosely packed platelets around individual tumor cells. Thus, at least with the tumor cell lines we studied, P-selectin–mediated tumor cell–platelet interactions are critical during the early phase of cancer metastasis, while the malignant cells are still in the blood stream.

A19. Heparin Can Block Human P-Selectin Binding at Clinically Tolerable Levels

To address the potential implications of this work in mice, we compared the effects of heparin in inhibiting tumor cell interactions with recombinant human and mouse P-selectin. We found that human P-selectin is even more sensitive to heparin blockade than mouse P-selectin.¹⁶ The IC₅₀ values obtained by using recombinant human P-selectin were within the currently accepted in vivo range for therapeutic use of heparin in clinical settings.

A20. Heparin Therapy to Prevent Human Metastasis Should Be Revisited

On the basis of all the foregoing considerations, we suggest that the general failure of vitamin K antagonists to improve cancer prognosis should be ignored and that heparin therapy for preventing tumor progression should be immediately revisited under this new paradigm.^{16,93} Unlike the authors of most previous studies, we conclude that heparin use should be explored specifically during the interval from initial visualization of a primary tumor until just after its definitive surgical removal (Figs. 3 and 4). The intention would be to prevent establishment of metastatic deposits by tumor cells that are circulating during this time period, by blocking the interaction of tumor cells with P-selectin on platelets.⁹³

B. QUESTIONS ARISING

B1. What About the Role of Leukocytes in the Complexes?

Heterotypic interactions of tumor cells with lymphocytes or polymorphonuclear leukocytes have been previously observed, and it has been suggested that these

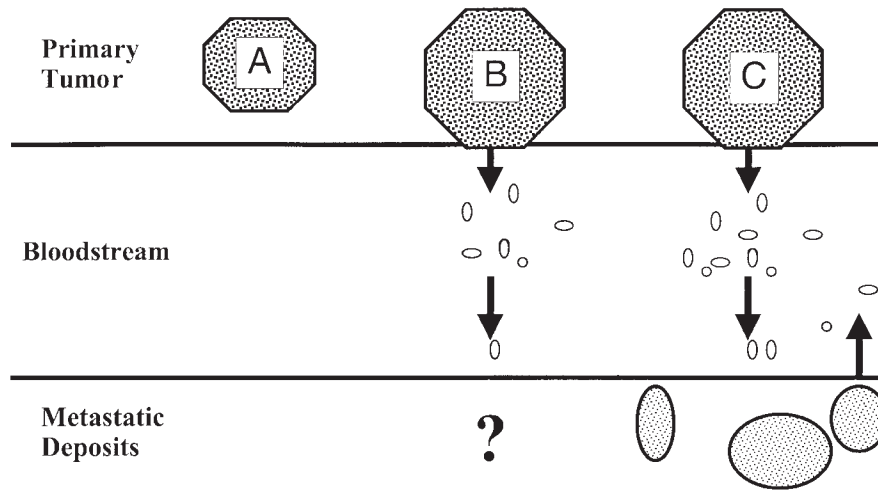


Figure 3 The window of opportunity for preventing metastasis. The situation (B) in the middle of the figure represents the only window of opportunity for blocking the formation of metastases, by interfering with survival of tumor cells in the bloodstream. See section C5 for discussion.

may also facilitate metastasis formation. In our studies we observed some leukocytes at the periphery of the dense platelet cloak surrounding the tumor cells in the vasculature of P-selectin-positive mice. However, following interference with formation of the platelet cloak by P-selectin deficiency, by tumor mucin removal, or by heparin, we detected a greater number of monocytes (macrophage precursors) directly associated with tumor cells in the lungs.¹⁶ It is possible that blocking of the

platelet “cloaking” of circulating tumor cells in the blood stream may allow effector cells to better associate with and eliminate the malignant cells. Indeed, the concept that monocytes/macrophages might help eliminate the formation of metastatic foci has been recognized for a long time.⁹⁴ In another tumor system, others have noted that platelet aggregation around tumor cells may allow them to evade destruction by host natural killer cells.¹⁴

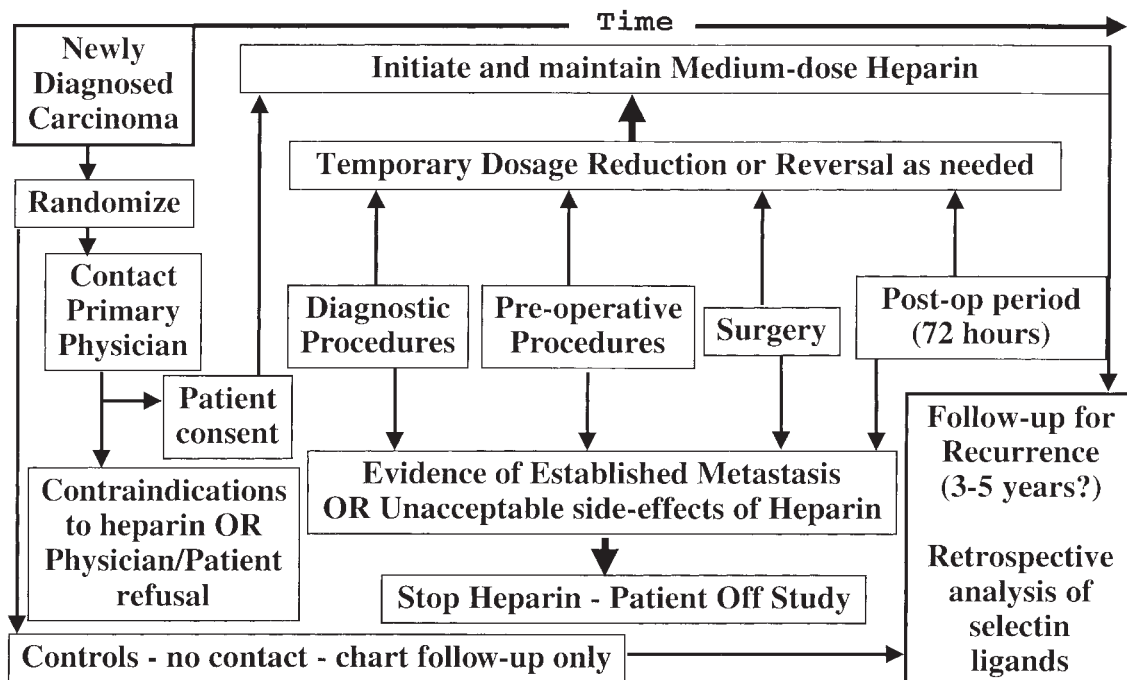


Figure 4 Proposal for a clinical trial of heparin prophylaxis in newly diagnosed carcinomas. Outline of a proposed clinical trial to study the effects of heparin in preventing metastasis of early-stage tumors. The gray boxes indicate the only possible time period during which it may be practically feasible to inhibit metastasis by interfering with selectin function. See text (sections C5 and C6) for discussion.

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B2. What About the Other Two Selectins?

Several authors have suggested that E-selectin on endothelial cells might facilitate their interactions with tumor cells.^{78,95-98} However, E-selectin expression requires induction of new synthesis via transcriptional activation of the E-selectin gene. Thus, we would not have observed this effect in our studies involving intravenous injections of tumor cells. Anyway, E-selectin recognition of tumor cells would not be blocked by heparin. However, heparin does inhibit L-selectin binding to mucins, and the potential role of L-selectin in metastasis is therefore currently under investigation. Indeed, our early results suggest that L-selectin can have an independent role in facilitating metastasis in these systems (Borsig et al, unpublished observations).

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B3. Why Not Use More Specific Inhibitors of P-Selectin?

As discussed earlier, heparin is probably a relatively nonspecific inhibitor of P-selectin. Moreover, heparin undoubtedly has many other biological effects that are independent of its effects on the selectins (see the following). More specific inhibitors of P-selectin such as the N-terminal glycosulfopeptide derived from PSGL-176,^{99,100} should indeed be studied in the future. However, the availability of large quantities of such molecules and their approval for clinical use are a long way off, and we suggest that heparin should therefore be used for a clinical trial in the immediate future.⁹³ If heparin is successful, it would eventually need to be compared head to head with a more specific P-selectin inhibitor when the latter becomes available. This approach would also help to dissect out any effects of heparin that are unrelated to P-selectin inhibition.

B4. Are These Results Applicable to All Carcinomas?

We and others have shown that ligands for the selectins are expressed on some primary human colon carcinomas *in vivo* and that ligands for all three may not be present simultaneously on the same tumor. We have started extending our studies to other primary human carcinomas. Our animal studies to date have involved only a few human and mouse carcinoma cell lines that express selectin ligands. However, as discussed earlier, the expression of sialylated fucosylated antigens denotes a poor prognosis in many human cancers. It is likely that some of these tumors use other mechanisms to generate interactions with platelets. There are also likely to be examples of malignant tumors that use completely different mechanisms to succeed in the metastatic pathway. It remains to be seen what proportion of naturally occurring carcinomas are dependent upon selectin ligands for successful metastatic progression.

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B5. Can Selectin Ligand Expression on Primary Tumors Provide a Prognostic Indicator and/or a Guide to Therapy?

Although the expression of sialyl Lewis X on primary tumors of many kinds is generally associated with a poor prognosis, there are examples of such tumors that still do not have P-selectin binding sites.¹⁰¹ Presumably these cells are missing some other critical component required for P-selectin binding, such as sulfate groups, or the proper mucin polypeptide backbone. Careful correlations of selectin-ligand expression on early-stage primary tumors with the ultimate prognosis are needed, specifically with regard to tumor recurrence and patient survival. If such correlations are found, then selectin ligand analysis of newly diagnosed tumors could become a useful prognostic indicator and/or a guide to therapy.

B6. Do Platelet-Tumor Cell Interactions Also Involve Other Adhesion Molecules?

The very nature of cancer and its genetic heterogeneity is such that every conceivable biological mechanism can and will be used by tumors to survive and succeed. Thus, it would not be at all surprising if some tumors use entirely different mechanisms to achieve the same purpose. Indeed, earlier studies have shown that other adhesion molecules may be involved in platelet-tumor cell interactions, including integrins and leucine-rich glycoproteins.^{8,12,15} However, their practical importance in generating interaction with tumor cells has not yet been defined. Another report described the suppression of experimental lung colonization of a mouse colon carcinoma by an anti-idiotypic monoclonal antibody recognizing an as yet unknown platelet surface molecule.¹³

B7. What About Other Biological Effects of Heparin?

The heparin that is currently approved for use as a clinical anticoagulant is actually a polydisperse mixture of variably modified glycosaminoglycan chains, only a minor portion of which actually interact with antithrombin and factor Xa to achieve anticoagulation.^{102,103} In addition to this, other overlapping or distinct subsets of heparin chains can interact with a large number of other bioactive molecules *in vivo*,^{104,105} including enzymes, cytokines, and cell adhesion molecules. Thus, previously suggested explanations for the heparin effect in cancer have included modulation of blood coagulation or growth factor function and the inhibition of angiogenesis or tumor heparanase action.^{18,106-108} There is no doubt that some or all of these mechanisms are operative for some tumors in some experimental systems. However, almost all of them would be occurring downstream of the initial blockade of the platelet-tumor cell interactions by heparin. Regardless

of the relative importance of the different mechanisms, it is a comforting fact that (with rare exceptions) heparin has not been reported to have a detrimental effect on tumor progression.

B8. Are Studies in Immunodeficient Mice Relevant to the Natural Immunocompetent State?

Our own studies were focused mainly on the early interactions of malignant cells with host cells in the vasculature, and we used an experimental metastasis assay system involving human tumors in immunodeficient animals. Given the well-known limitations of this approach, we are also currently studying the role of selectins in syngeneic and/or spontaneous metastasis models. Our early results suggest that the same principles are operative in these systems (Borsig et al, unpublished observations). Others have also studied diverse heparanase-inhibiting molecules such as sulfated polysaccharides and synthetic polyanionic molecules in syngeneic models such as B16-BL6 mouse melanoma cells¹⁰⁶ and the rat mammary adenocarcinoma 13762 MAT.¹⁰⁷ Many of these heparanase inhibitors also happen to block P-selectin (Borsig et al, unpublished).

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B9. Are Studies Using Intravenous Injections of Tumor Cells Relevant to “Natural” Metastasis?

Intravenous injection of tumor cells allows them to bypass the arduous requirement of invading through the basement membrane, which is a part of the normal cascade of events in metastatic progression. Although the direct injection of tumor cells into the blood stream is not completely “natural,” it did permit us to study the early interactions of tumor cells in the vasculature with host cells and thus allow the design of interventions at this particular stage of the metastatic process. As discussed subsequently, this step is the most practical one in which heparin therapy can be considered as a therapeutic option.

B10. What About the Genetic Heterogeneity of Tumors That Results from Genomic Instability?

It is well known that individual human carcinomas are highly heterogeneous from a genetic perspective, causing one to despair about ever finding a therapeutic intervention that can affect all the cells in a particular tumor. However, it is also well known that tumor cells in the blood stream are extremely vulnerable to a variety of nonspecific and specific host-defense mechanisms.^{1,2,4} Thus, the great majority of such circulating cells eventually die, explaining the common observation that most patients eventually die of just a handful of metastatic lesions, each of which presumably arose from

very few of the tumor cells that originally entered the circulation. A similar phenomenon is observed in experimental animal model systems, where several hundred thousand cells are injected and only a few metastatic foci are seen. Thus, a therapeutic intervention affecting the general survival of tumor cells in the circulation does not need to be completely effective against all the cells that originally entered the blood stream.

C. THERAPEUTIC CONSIDERATIONS

C1. What About Low Molecular Weight Heparins?

There is currently a major shift away from unfractionated heparin toward use of a variety of low molecular weight (LMW) heparins in clinical practice.^{103,109–112} Positive features of the latter include better and more consistent bioavailability, a more convenient dosing schedule, and the selective inhibition of activated factor Xa, all of which make them easier to manage clinically. In our original studies of selectin inhibition, we noted that the two LMW heparins we studied were less effective than unfractionated heparin.⁹⁰ However, these were only *in vitro* results, and it remains to be seen whether the *in vivo* antimetastatic effect follows the same pattern. It may well be that the better bioavailability and more consistent serum levels of LMW heparins will compensate for their relatively poor ability to inhibit the selectins. In this regard, retrospective analyses of large cohorts of patients treated with LMW heparins for other reasons have shown correlations with a better survival outcome for patients who happened to have cancer.^{20,30,51,113,114}

C2. What About the Heterogeneity of Heparin Preparations?

Heparin is isolated and purified from biological sources for its anticoagulant properties and has been in clinical use for many decades. However, although heparin preparations are well standardized for their anticoagulant potency, they are actually a very complex mixture of glycosaminoglycans, and it should not be surprising if there are some lot-to-lot variations in their ability to inhibit selectins when compared on an anticoagulant unit basis.^{88,90,92} Additional complexity arises from the fact that there are many different kinds of LMW heparins that might not be equivalent in their antiselectin effects. It is unknown whether or not such variability is sufficient to make a practical difference

C5. When to Treat with Heparin?

Many human tumors that appear to be localized at first clinical presentation are, in fact, metastatic or could be just about to become so. This accounts for the fact that

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the long-term surgical cure rate is not 100% for many common "early stage" malignancies. If a tumor already has obvious or occult metastases at the time of initial diagnosis (Fig. 3C), then heparin therapy is unlikely to succeed, at least via its mechanism of limiting survival of tumor cells in the blood stream. On the other hand, if the primary tumor is completely isolated and will be cured by surgery or radiation therapy (Fig. 3A), then the use of heparin is moot. In practical terms, there is *only one window of opportunity in which one can try to prevent the establishment of metastasis*—starting from the earliest possible time after the initial clinical or pathological diagnosis of malignancy and continuing through diagnostic and surgical handling of the case (Fig. 3B). It is possible that vascular release of tumor cells is facilitated by diagnostic or therapeutic manipulations during this time interval.¹¹⁵ We suggest that heparin at "clinically tolerable" doses could be administered during this interval, potentially preventing establishment of new metastatic deposits and blocking the interaction of circulating tumor cells with platelets via P-selectin and/or by other mechanisms. Given recent evidence of the continued intravascular survival of tumor cells for a few days after their entry into the blood stream,¹¹⁶ it seems reasonable to continue the treatment for a few days after the surgery.

C6. Proposal for a Clinical Trial to Test the Hypothesis

The use of more specific and selective P-selectin inhibitors will eventually help to dissect the relative roles of the different mechanisms of heparin action in malignancy. However, it will be a long while before such molecules become available for routine clinical use. Meanwhile, we suggest that the failure of vitamin K antagonists to improve cancer prognosis should be ignored and that heparin therapy be immediately revisited.⁹³ Unlike the authors of previous studies of heparin in cancer, we suggest that its use should be reexplored specifically during the time interval from initial diagnosis of a primary tumor until just after its definitive surgical removal (Fig. 4).

The following is a very simplified outline of the principles of the proposed clinical study. The study population would be adults with newly detected adenocarcinomas of the colon, lung, esophagus, stomach, pancreas, biliary tree, bladder, prostate, breast, head and neck, or ovary (picked up by endoscopy or by imaging methods) who have no overt evidence of metastatic disease. The patient would have to be considered for participation in this randomized clinical trial as soon as possible after a definitive diagnosis of malignancy in the primary lesion is reported by the pathologist (Fig. 4).

Is ___ Basic eligibility criteria would include 100% performance status, no concomitant or previous history of

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other malignancy, and no preexisting medical condition that would preclude the ability to give informed consent or to receive heparin. At the time of enrollment, patients must have normal coagulation and hematologic parameters, no evidence of major organ failure, and no history of bleeding disorder or ongoing major bleeding.

Regarding a control arm, it is difficult to justify the randomization of half the patients to receive saline injections instead of heparin. Moreover, the obvious effects of heparin on blood coagulation and the need to reverse the heparin effect temporarily in various circumstances (see later) will make it difficult to keep the study truly blinded. On the other hand, it is critically important to have a true control group for comparisons. A simple way to define a negative control population is simply to skip contacting every other eligible case (defined by the pathologist) at the outset. Thus, each even-numbered pathological diagnosis will trigger a contact for possible enrollment in the study, and odd-numbered ones would be simply recorded as such. The latter patients need never be aware that they represent the control arm of the study and can be simply tracked via hospital records for their final clinical outcome. If an even-numbered patient either refuses to participate or becomes ineligible for analysis, then the corresponding odd-numbered control case would be excluded from the final analysis.

Patients who are randomized to receive heparin would receive unfractionated porcine sodium heparin at a clinically tolerable dose that does not require frequent monitoring (e.g., 7500 units subcutaneously every 12 hours). Treatment would begin as soon as informed consent can be obtained and be continued for at least 3 days after definitive surgical removal of the primary lesion. The inclusion of an LMW heparin arm in the initial trial would allow a simultaneous comparison with unfractionated heparin. However, it would increase by a third the number of patients to be enrolled in order to see an effect.

During the treatment interval, all patients would undergo staging studies as indicated, along with monitoring of blood counts and chemistries. If evidence of existing metastatic disease is confirmed at any time before or during surgery, treatment would be stopped and the patient dropped from the study (the majority of initially enrolled patients may in fact be dropped at some point after initial diagnosis). Only the patients considered to have had a surgical cure would be continued on heparin, for 72 hours after completion of surgery. Protamine reversal and/or dosage reduction can be used during specific time periods if bleeding becomes a problem and/or if surgeons or interventional radiologists are concerned about the risk of bleeding in a particular patient. Treatment would be promptly stopped if platelet counts drop to less than 50% of the starting value and/or to <75,000/ L (suggesting the possibility of

heparin-induced thrombocytopenia—such patients would also be dropped from the study). Patients who have completed the full course of treatment would then be followed for the endpoints of tumor recurrence and survival for a period of 3–5 years. For each such case, the originally identified control case would be included for comparative analyses. All primary tumor samples would also be subjected to selectin ligand analysis so that one could determine retrospectively whether this parameter can predict the response to heparin.

Several practical issues have to be considered (Fig. 4). Because of the need to identify potential study candidates promptly after diagnosis, it is necessary to have one individual committed to screening all new reports in a given participating hospital as they are read out daily in the pathology department. As soon as candidates are identified by the initial pathology reports, their personal physicians need to be contacted promptly to obtain agreement to discuss the protocol with the patient. Protocol discussion and patient enrollment would have to be decided upon rapidly so that the first dose of heparin is started as soon as possible. Careful follow-up of enrolled patients will be needed to ensure that they are treated correctly, even when outside the hospital. The subcutaneous heparin will need to be continued throughout the “period of risk,” during part of which the patient may be out of the hospital (e.g., between diagnosis and surgery). This may require education of the patients concerning self-administration or other individualized approaches. Even though the doses suggested are only slightly higher than those used typically for prophylaxis of venous thrombosis, the surgeons or interventional radiologists involved would need to be aware of the situation and be able to make individual decisions regarding short-term stoppage of heparin and/or protamine reversal as needed. Some tumors of bladder and prostate may need to be excluded because of the location of the potential bleeding complications. With regard to the sample size needed, it is impossible to be certain at the outset. The study would have to be designed to detect a statistically significant difference, assuming that the recurrence rate in the control group of these early stage cancers at 5 years is only ~20–30%. An independent protocol monitoring committee will have to check periodically whether a clear positive or negative effect of the heparin therapy emerges.

C7. Practical Problems with Initiating the Proposed Clinical Trial

The logistical problems with the proposed study and the potential side effects of heparin can be managed. Although the number of patients to be studied will be a little uncertain, the extremely common occurrence of the relevant carcinomas should make it possible to

achieve rapid accrual. However, the actual duration of the study is likely to be long, and the results will not be known for many years. Nevertheless, even if the proportion of treated patients who benefit from this trial turns out to be low, the very large numbers at risk (hundreds of thousands per year) would make even a small percentage benefit worthwhile. Of course, even if the study is successful, heparin may eventually be replaced with more specific selectin inhibitors that are currently under development. Finally, the proposed study does not involve any new biopharmaceutical products and it is uncertain that any protectable intellectual property will emerge from the study. For many of these reasons, it may be hard to find the funding needed to conduct this study properly. If so, the potential of heparin as a simple intervention in many of the most common malignancies of humans will remain untapped.

ACKNOWLEDGMENTS

The authors recognize our collaborators and the many members of our laboratory (particularly Lubor Borsig) for many important contributions to the work described in this article. We also acknowledge Elaine Muchmore and Barbara Parker for very helpful discussions about the parameters for a potential clinical trial and Richard Hynes for his continuing encouragement and interest in our work. Work in the authors' laboratory was supported by NIH grant R01 CA38701.

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(Authors' note: space does not allow citation of all the relevant original studies in all of the topic areas covered in this review)

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