Low-molecular-weight heparins and angiogenesis

Review article

KLAS NORRBY

Department of Pathology, Sahlgrenska Academy, Göteborg University, Sweden


The involvement of the vascular system in malignancy encompasses not only angiogenesis, but also systemic hypercoagulability and a pro-thrombotic state, and there is increasing evidence that pathways of blood coagulation and angiogenesis are reciprocally linked. In fact, cancer patients often display hypercoagulability resulting in markedly increased thromboembolism, which requires anti-coagulant treatment using heparins, for example. Clinical trials reveal that treatment with various low-molecular-weight heparins (LMWHs) improves the survival time in cancer patients receiving chemotherapy compared with those receiving unfractionated standard heparin (UFH) or no heparin treatment, as well as in cancer patients receiving LMWH as thrombosis prophylaxis during primary surgery. This anti-tumor effect of the heparins appears to be unrelated to their anti-coagulant activity, but the mechanisms involved are not fully understood. Tumor growth and spread are dependent on angiogenesis and it is noteworthy that the most potent endogenous pro- and anti-angiogenic factors are heparin-binding proteins that may be affected by systemic treatment with heparins. Heparin and other glycosaminoglycans play a role in vascular endothelial cell function, as they are able to modulate the activities of angiogenic growth factors by facilitating the interaction with their receptor and promoting receptor activation. To date, preclinical studies have demonstrated that only LMWH fragments produced by the heparinase digestion of UFH, i.e. tinzaparin, exert anti-angiogenic effects in any type of tissue in vivo. These effects are fragment-mass-specific and angiogenesis-type-specific. Data on the effect of various LMWHs and UFH on endothelial cell capillary tube formation and proliferation in vitro are also presented. We hope that this paper will stimulate and facilitate future research designed to elucidate whether the anti-angiogenic or anti-tumor effects of commercial LMWHs in their own right are agent specific and whether anti-angiogenic properties increase the anti-tumor properties of the LMWHs in the clinic.

Key words: Low-molecular-weight heparin; heparin molecular mass; heparin; tinzaparin; dalteparin; angiogenesis; anti-angiogenesis; heparin-binding growth factors; angiogenic growth factors; anti-angiogenic growth factors; endothelial cells.

Klas Norrby, Department of Pathology, Sahlgrenska Academy at Göteborg University, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden. e-mail: klas.norrby@pathology.gu.se

A causal link may exist between genetic tumor progression, angiogenesis and cancer coagulopathy (1–3), and tissue factor (TF) is apparently an important common denominator in these processes. In fact, TF appears to be a target of at least three of the most common genetic alterations in human malignancy, namely p53, K-ras and EGFR, suggesting that these genetic alterations play a causative role in cancer hypercoagulability. Moreover, pathways of blood coagulation and angiogenesis are reciprocally linked (4, 5). Clinical data show that a hypercoagulable state per se may predispose to cancer (6, 7). One explanation is that before patients develop a clinical cancer, tiny tumors exist predisposing the individual to a hypercoagulable state. Alternatively, individuals with an underly-
ing predisposition to hypercoagulability are more likely to develop cancers.

Patients with active malignancy are prone to develop venous thromboembolic disease. Factors that are thought to contribute to the increased risk of venous thrombosis in these patients include pro-coagulant effects of cancer cells themselves or effects caused by their interaction with the host environment, activation of platelets, chemotherapeutic drugs, certain other systemic cancer therapies, surgery and immobility. Surgery, which is first-line therapy for many cancer patients, is known to activate the hemostatic system and is a particularly high risk for venous thromboembolism (VTE). The spectrum of thromboembolic manifestations in cancer patients includes deep vein thrombosis and pulmonary embolism, as well as disseminated coagulation and abnormalities in the clotting system in the absence of clinical manifestations (8). VTE is a significant cause of mortality in cancer patients and the main objective of thrombosis prophylaxis in these patients is to reduce the risk of fatal pulmonary embolism. At present, anti-coagulant therapy is the main clinical application of heparins.

Commercial low-molecular-weight heparins (LMWHs) have been validated in both the prevention and treatment of thromboembolic disease in cancer patients and have improved survival time in retrospective and prospective trials. This is believed to relate to factors such as direct anti-neoplastic effects, the modulation of the immune system, the suppression of the effect of coagulation proteases in the tumor environment (which may change the tumor phenotype) and anti-angiogenic effects. Tumour growth is angiogenesis dependent and the anti-angiogenic effects in vivo of discrete fractions of the LMWH tinzaparin have been demonstrated in preclinical studies. This raises the question of whether it would possible to enhance the anti-tumor effect of LMWHs in patients by using anti-coagulant LMWHs exhibiting potent anti-angiogenic efficacy.

FEATURES OF HEPARIN

Heparin is found exclusively in the secretory granules of mast cells (MCs) and for commercial reasons it is commonly extracted from porcine intestinal mucosa. Standard, unfractionated heparin (UFH) is a heterogeneous mixture of glycosaminoglycans that bind to antithrombin via a pentasaccharide, catalyzing the inactivation of thrombin, resulting in the suppression of fibrin formation and other clotting factors. UFH also binds various proteins, endothelial cells (ECs), platelets and other circulating cells, leading to somewhat unpredictable pharmacokinetic and pharmacodynamic properties (9). Only heparins inducing anti-coagulant activity possess a pentasaccharide sequence that interacts with a serine protease inhibitor (antithrombin) involved in attenuating blood coagulation. Commonly, only one-third of the heparin chains in UFH preparations contain this sequence (10). Heparin binds to and neutralizes platelet factor-4 (Table 1), which is synthesized exclusively in megakaryocytes and stored in platelet alpha granules and this is also regarded as an important component of the anti-coagulant effect of heparin (11).

Most of the known pro- and anti-angiogenic endogenous factors are heparin-binding proteins (Table 1) that also bind to soluble heparan sulfate proteoglycans (HSPGs) that are constitutively synthesized by ECs and are also harbored in the extracellular matrix (ECM). Heparan sulfates are a complex and heterogeneous family of macromolecules composed of linear polysac-
TABLE 1. Major heparin-binding pro- and anti-angiogenic endogenous proteins

<table>
<thead>
<tr>
<th>Proangiogenic</th>
<th>Anti-angiogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiogenin, a ribonuclease with angiogenic activity</td>
<td>Angiostatin, fragment derived from plasminogen</td>
</tr>
<tr>
<td>Fibroblast growth factors (FGFs)(^a)</td>
<td>Bovine apo-lactoferrin(^c)</td>
</tr>
<tr>
<td>Heparin-binding epidermal growth factor (EGF)-like growth factor</td>
<td>Endostatin, fragment derived from collagen XVIII(^f)</td>
</tr>
<tr>
<td>Hepatocyte growth factor (HGF), also denoted scatter factor</td>
<td>Interferon-gamma-inducible protein-10 (IP-10)</td>
</tr>
<tr>
<td>Human apo-lactoferrin(^b)</td>
<td>Interleukin-10 (IL-10)</td>
</tr>
<tr>
<td>Interleukin-1-alpha (IL-1 alpha)</td>
<td>Macrophage inflammatory protein-1 (MIP-1)</td>
</tr>
<tr>
<td>Interleukin-1-beta (IL-1 beta)</td>
<td>Platelet factor-4 (PF4)(^g)</td>
</tr>
<tr>
<td>Interleukin-8 (IL-8)(^c)</td>
<td>Thrombospondins I and II (TSP-I and TSP-II)</td>
</tr>
<tr>
<td>Platelet growth factor (PIGF)</td>
<td>Tissue factor pathway inhibitor (TFPI)</td>
</tr>
<tr>
<td>Tissue factor (TF)</td>
<td></td>
</tr>
<tr>
<td>Transforming growth factor-beta (TGF-beta)</td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor-alpha (TNF-alpha)</td>
<td></td>
</tr>
<tr>
<td>Vascular endothelial growth factor-A (VEGF-A)(^d)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Heparin fragments containing fewer than 10 sugar units have been found to inhibit the biological activity of bFGF (151).
\(^b\) Enhances specifically VEGF-A-mediated angiogenesis in the rat mesentery assay (152).
\(^c\) Formed by cultured ECs exposed to cross-linked fibrin (153), as well as by many other cell types.
\(^d\) Heparin fragments with fewer than 18 sugar units inhibit the binding of VEGF to its receptors on ECs (121).
\(^e\) Suppresses specifically VEGF-A-mediated angiogenesis in the rat mesentery assay (108).
\(^f\) Integrins serve as cell surface receptors for endostatin (154, 155).
\(^g\) See (86).

**charide chains endowed at the cell surface in most tissues.** The main biochemical difference between heparin and heparan sulfate is that heparan sulfate is less sulfated and more acetylated than heparin. Soluble heparins compete with heparan sulfates to bind heparin-binding proteins and may cause the release of these proteins from the ECM. Heparin and other glycosaminoglycans (heparan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate and hyaluronic acid) play a role in EC function, as they are able to modify the activities of angiogenic growth factors by facilitating the interaction with their receptor and promoting receptor activation. For example, heparin potentiates the binding of bFGF, a prototypic heparin-binding growth factor, to its surface receptor and protects it from proteolytic degradation and suppresses its diffusion.

UFH is a highly sulfated and anionic polysaccharide consisting of a repeating disaccharide structure. It is a heterogeneous, polydispersed mixture of sulfated polysaccharides ranging in fragment weight from 3- to 30-kDa (Fig. 1), with an average of approximately 15-kDa (9). In addition, there are variations in the distribution and position of sulfate ester groups and sulfated amino groups within repeating disaccharides. Heparin, by its anionic glycosaminoglycan chains, interacts through electrostatic bonds with a vast array of circulating and cell-surface proteins and receptors. Cell-surface proteins of this kind bound to heparin tend to be internalized by receptor-mediated endocytosis in many types of cell, including ECs, leading to gene transcription. The heparin-binding proteins include growth factors, extracellular matrix (ECM) proteins, chemokines and enzymes (Table 1).

Therapeutic levels of heparin in the circulatory system trigger complex processes, causing the systemic release of heparin-binding angiogenic growth factors such as basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) lodged on ECs and in the ECM (12, 13). Heparins also induce anti-oxidant activity (10, 14, 15) that can significantly influence angiogenesis (16–18).

**Fig. 1.** Typical molecular weight distributions of UFH and commercial LMWHs in general (MW= 5.5-kDa), as well as a tinzaparin fraction with an MW of 2.5-kDa, illustrate that the molecular weight distribution in any of these heparin preparations is wide.
TABLE 2. Relationship between molecular weight and number of heparin oligosaccharides

<table>
<thead>
<tr>
<th>Heparin oligosaccharides</th>
<th>Molecular weight, kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>2.4</td>
</tr>
<tr>
<td>12</td>
<td>3.6</td>
</tr>
<tr>
<td>16</td>
<td>4.8</td>
</tr>
<tr>
<td>18</td>
<td>5.4</td>
</tr>
<tr>
<td>24</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Modified from (27).

FEATURES OF LOW-MOLECULAR-WEIGHT HEPARINS

The commercially available LMWHs are produced by the depolymerization of heparin derived from porcine mucosa and display weights ranging from about 2- to 9-kDa (Fig. 1), depending on the manufacturing process (9). The relationship between molecular weight and the number of heparin oligosaccharides is shown in Table 2. Most of the LMWHs are isolated from UFH by chemical means, followed by gel filtration chromatography, differential precipitation with ethanol, partial depolymerization (nitrous acid, benzylation followed by alkaline hydrolysis, isoamyl nitrate digestion, peroxidative cleavage, beta elimination or nitrous acid digestion and so on). Uniquely, tinzaparin is produced by enzymatic hydrolysis with heparinase, as shown in Table 3. The mean molecular weight (MW) of tinzaparin is 6.5-kDa. Regulatory agencies, such as the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMEA), regard each of the LMWHs as distinct pharmacological agents that should not be regarded as interchangeable (19). The LMWHs that are approved for use in Europe, Canada and the U.S., and which are discussed in this paper, are shown in Table 3.

The interactions of the LMWHs with anti-thrombin and heparin cofactor II, attenuating coagulation, are clearly not the only determinants of their biological actions. The release of tissue factor pathway inhibitor (TFPI), the regulation of cytokines, nitric oxide and eicosanoids contribute to their individuality; TFPI is the natural inhibitor of TF. However, the pharmacologic differences in these agents do not result in clinically important differences in the outcomes of anti-coagulation therapy in patients with VTE or in those at risk of VTE (20). Apparently because of this, the LMWHs have often been regarded and discussed in the literature as closely related entities, which has contributed to the conundrum that exists regarding many of the non-anti-coagulation actions, including angiogenesis modulation, of discrete LMWHs.

The LMWHs differ from UFH in that they display less variation in fragment size, a lower level of sulfation (a lower degree of charge density) and a higher ratio of anti-factor Xa to anti-factor IIa activity, greater bioavailability (when assessed in terms of anti-Xa) and a longer half-life. LMWHs are also thought to feature a more predictable anti-coagulant response when administered subcutaneously (s.c.) in fixed doses. Anti-thrombin activity has been proposed as the major action of UFH and LMWHs (21) and the pharmacokinetics of UFH and LMWHs in man are similar with respect to anti-thrombin activity, however (21). The administration of s.c. tinzaparin produces more predictable anti-thrombin levels than UFH, or the LMWHs dalteparin and enoxaparin (21). LMWHs demonstrate s.c. bioavailability approaching 100% at low doses (9). The s.c. administration of LMWHs is now universally accepted as the treatment of choice for the postsurgical prophylaxis of deep vein thrombosis and pulmonary embolism (22–24).

TABLE 3. A list of LMWHs that are approved in Europe, Canada and the US and that are referred to in this paper, and the structural changes brought about by the method of preparation

<table>
<thead>
<tr>
<th>INN Designation</th>
<th>Method of Preparation</th>
<th>Chemical change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Certoparin</td>
<td>Isoamyl nitrate digestion</td>
<td>Formation of anhydromannose (5 member ring)</td>
</tr>
<tr>
<td>Dalteparin</td>
<td>Nitrous acid depolymerization</td>
<td>Formation of anhydromannose (5 member ring)</td>
</tr>
<tr>
<td>Enoxaparin</td>
<td>Benzylation, alkaline hydrolysis</td>
<td>Introduction of double bond at the end grouping*</td>
</tr>
<tr>
<td>Reviparin</td>
<td>Nitrous acid digestion</td>
<td>Formation of anhydromannose (5 member ring)</td>
</tr>
<tr>
<td>Tinzaparin</td>
<td>Heparinase digestion</td>
<td>Induction of double bond at the end groups</td>
</tr>
</tbody>
</table>

Modified from (27 & 33).

* Differentiated from tinzaparin by the presence of anhydro sugars at the reducing end (19).
The recommended optimum anti-thrombotic interventions for secondary prophylaxis against VTE in patients with cancer include treatment with either dalteparin or tinzaparin (25).

**HEPARINS BIND TO CELLS AND PROTEINS AND RELEASE HEPARIN-BINDING PROTEINS**

As heparins enter the circulation, they bind to a large variety of heparin-binding proteins, any circulating cell, including tumor cells, platelets and ECs (26, 27) (see Fig. 2). Heparins of higher-molecular-weight fractions bond more readily than LMW fractions to cells and proteins. The molecular mass is thus an important feature for the interaction of heparins with the binding sites on the EC surface, including putative 45- and 47-kDa protein receptors (28–30). Heparin binds to ECs in a saturable and specific manner. The binding of heparin to a variety of cell types results in specific changes in cell function. ECs exposed to heparin display various changes, including the increased synthesis of a peculiar heparan sulfate that may relate to the anti-thrombotic activity of heparin (28) and the reduction of endothelin-1 gene transcription (31), which leads to vasodilation. Tables 1 and 4 list a selection of heparin-binding proteins. Heparin-binding growth factors can bond to heparan sulfate proteoglycans present on the surface of ECs and other cells, as well as in the ECM. Soluble heparins compete with heparan sulfate for the binding of these growth factors and other heparin-binding proteins, releasing these proteins from the cell surface and the ECM (32).

LMWHs exhibit major differences in their ability to release various mediators from the vascular lining (33). LMWHs may thus release fibrinolytic activators such as tissue plasminogen activator and anti-platelet substances, including prostacyclin, and may also inhibit the TF-mediated activation of platelets and macrophages and the TF-mediated interaction with vascular endothelium (33). Heparins also release TFPI, which displays higher cellular distribution in ECs, where it is associated with cell surface HSPG, than in other cell types (22). TFPI acts as a polydomain inhibitor of the coagulation process (33).

**CANCER, ANGIOGENESIS AND THE CLINICAL BENEFIT OF ANTI-ANGIOGENESIS THERAPY**

The growth and spread of cancer are angiogenesis dependent (34, 35), possibly with minor exceptions (36). As proposed by Folkman back in 1971 (37), this opens a new and promising avenue for anti-tumor therapy. While tumor cells are genomically unstable and mutagenic, which often results in the development of drug resistance and poor clinical outcome, the ECs in tumor vessels are normal, angiogenically activated, genomically stable and therefore much less prone to develop drug resistance. Tumor angiogenesis is caused by: (i) the secretion of...
pro-angiogenic factors by the normoxic neoplastic cells; (ii) hypoxia following tumor expansion, which induces hypoxia-regulated genes including VEGF in all cell types within the tumor; (iii) the production of pro-angiogenic factors from non-neoplastic cells in their interaction with the neoplastic cells; and (iv) the release of heparin-binding pro-angiogenic factors from the ECM in the tumor tissue or its close surroundings. VEGF-A is a key angiogenic factor in hypoxic normal tissue (38, 39), as well as in most tumors (40–42), and also appears to be a mediator of angiogenic pathways related to other angiogenic factors (41). In addition to VEGF-A, cells in many tumors can produce other heparin-binding potent pro-angiogenic factors such as bFGF, IL-8, angiogenin and HGF (Table 1).

In 2004, bevacizumab, Avastin®, an antibody against VEGF, was approved by the U.S. FDA for the first-line treatment of metastatic colorectal cancer in combination with conventional chemotherapy. This was based on a study in which bevacizumab was administered in combination with chemotherapy (irinotecan, fluorouracil and leucovorin) showing a significantly improved survival compared with patients not receiving bevacizumab (43). Avastin is the first approved agent to target tumor angiogenesis (44). Avastin also significantly improves survival time in other major human tumor types, including lung and breast cancer.

As recently shown, so-called continuous or metronomic low-dose chemotherapy may inhibit tumor angiogenesis (45–47) in mice and affects VEGF-A-mediated angiogenesis in a drug-specific and dose-dependent way in rats (18). Certain metronomically administered cytotoxics exert anti-angiogenic effects in preclinical models and also appear to suppress tumor growth and spread in the clinic compared with conventional chemotherapy using the same agents (48). Chemotherapy is conventionally given at high doses with typical intervals of 2–4 weeks and probably does not accomplish an overall anti-angiogenic effect. Combinations of anti-angiogenic therapies usually strengthen the anti-angiogenic response. As a result, anti-angiogenic heparins when used as an anti-coagulant may be of anti-tumor importance, even when specific inhibitors of various pro-angiogenic growth factors and their receptors are developed on a broader scale and combined with metronomic and/or conventional chemotherapy to induce what has recently been denoted “chemo-switch” (49).

HEPARINS AND EXPERIMENTAL TUMOR GROWTH

Heparin exhibits many activities affecting tumor growth and spread, which appear to have little to do with anti-coagulation (15). Heparin may modify gene expression and induce apoptosis, differentiation and drug resistance (50). While primary tumor growth depends on its proliferation potential, activity of the immune system and ability of tumor angiogenesis, the anti-coagulant drugs used in the clinic can influence all these aspects (50). Heparins of various molecular weights, including LMWHs, UFH and chemically modified non-anti-coagulant heparins, have, for instance, been shown to inhibit tumor growth and modify or inhibit metastasis formation in animal studies (10, 51–56). It has been claimed that these agents are more effective in inhibiting stages of the metastatic cascade than in their influence on primary tumors (50). Blood-borne carcinomas interact with platelets and leukocytes, thereby forming cell emboli, which arrest in capillaries of distant tissues. By binding to adhesive proteins such as fibronectin, vitronectin and laminin (Table 4), heparins modulate integrin-mediated cellular adhesion, thus affecting the migration and invasion of ECs, tumor cells and other cells (57). Heparins can thereby affect the migration, recruitment and proliferation of pericytes, which play a significant role in angiogenesis by stabilizing blood vessels.

A variety of mechanisms have been proposed to explain the effects of heparin on tumors. The effects of UFH and LMWH administration in a vast number of experimental animal models of malignancy were recently reviewed in (15, 53). All the animal studies published between 1960 and 1999 that report effects of heparins on the growth of s.c. implanted tumors, spontaneous metastasis or experimentally induced metastasis are reviewed in (57). Because of the wide variety
of activities of heparins in experimental systems, it was concluded at that time, e.g. in 2001, that the ultimate effect of heparin treatment on cancer progression in man is uncertain (57). The mechanisms of the anti-tumor effect of heparins have been attributed primarily to a large variety of features. They include: (i) the inhibition of tumor angiogenesis; (ii) the enhancement of immune attack on tumors; (iii) a direct effect on tumor cells, including inhibiting expression of some oncogenes, including \( c\)-\( myc \) and \( c\)-\( fos \); (iv) altered enzymatic activity, including the inhibition of tumor cell heparanase (which degrades heparan sulfate and heparan sulfate proteoglycans) that mediates invasion and metastasis and the inhibition of matrix-degrading enzymes; (v) anti-oxidant effects; (vi) the modification of growth factor activity; (vii) the inhibition of blood coagulation activation; (viii) the inhibition of tumor cell migration; (ix) the inhibition of tumor cell adhesion to ECs; (x) the inhibition of multidrug resistance; and (xi) the suppression of coagulation proteases in the tumor environment (4, 10, 51, 53, 57–60).

**HEPARINS, AND LMWHs IN PARTICULAR, IMPROVE SURVIVAL TIME IN CANCER PATIENTS**

The main objective of thromboprophylaxis in cancer patients is to reduce the risk of fatal pulmonary embolism. As noted, angiogenesis is a prerequisite for tumor growth (34, 35) and VEGF-A is a key angiogenic factor in most tumors (40–42). In addition to VEGF-A, cells in many tumors can produce other heparin-binding pro-angiogenic factors as mentioned above (Table 1). As a consequence of anti-coagulant treatment, given in combination with standard conventional chemotherapy or during primary surgery, several retrospective studies report that the long-term survival time in cancer patients is significantly improved by treatment with UFH (13) and even more so in cancer patients receiving various LMWHs as compared with those receiving UFH (50, 61–71). As yet, however, there are only a few prospective studies in this field (67, 69, 72). Interestingly, it is thought that the anti-tumor influence displayed in terms of improved survival time and death rates in these studies is influenced by a biologic effect in addition to anti-thrombotic activity. It is, furthermore, noteworthy that heparin is a reversing agent of multidrug resistance. Heparins may therefore be regarded as potential multidrug-resistance modulators (73) when administered on a long-term basis together with chemotherapeutics. Recent studies have, moreover, shown that one important benefit that the hemostatic system affords to tumor cells is to impede the natural killer (NK)-mediated elimination of tumor cells. Adjunct therapies designed to limit cell-associated pro-coagulant function could thus be effective in reducing the risk of metastases in cancer patients (74). Notably, NK-cell activation has been found both in vitro and \textit{ex vivo} in peripheral blood mononuclear cells from healthy donors and in lung cancer patients after treatment with LMWHs, independent of their anti-coagulant activity (75). The role of anti-angiogenic effects exerted by LMWHs as a basis for improved survival time in cancer patients has, however, recently attracted increasing interest as one of several possible mechanisms, including prevention of fatal thromboembolic disease, inhibition of coagulation proteases and direct anti-neoplastic effects (50, 60, 67, 69–71).

In a prospective, randomized, double-blind trial, in which the LMWH certoparin (Table 3) was compared with UFH in patients receiving thrombosis prophylaxis during primary breast and pelvic surgery, long-term survival in the LMWH group was significantly improved (\( p<0.007 \)) but only after 650 days (72). The effect on survival is remarkable, as the UFH and

**TABLE 4. Heparin-binding enzymes and extracellular matrix proteins**

<table>
<thead>
<tr>
<th>Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin G</td>
</tr>
<tr>
<td>Elastase</td>
</tr>
<tr>
<td>Heparin cofactor II</td>
</tr>
<tr>
<td>Lipases</td>
</tr>
<tr>
<td>Mucus protease inhibitor</td>
</tr>
<tr>
<td>Protease inhibitors</td>
</tr>
<tr>
<td>Proteases</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ECM proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagens</td>
</tr>
<tr>
<td>Fibronectin</td>
</tr>
<tr>
<td>Laminin</td>
</tr>
<tr>
<td>Thrombospondin</td>
</tr>
<tr>
<td>Vitronectin</td>
</tr>
</tbody>
</table>

Modified from (10).
NORRBY

LMWH treatment period was only 7 days post-operatively and the first injection of UFH or LMWH was given 2 h before surgery. With a half-life of about 6 h, certoparin is no longer detectable in the patient’s blood 24 h after the final injection. It is therefore difficult to believe that 7 days of heparin anti-coagulant treatment can influence the natural history of a cancer. It is more likely that the surgery and the immediate post-operative period involved an increased risk of tumor cells being disseminated and lodged in the microcirculation in a way that would allow these tumor cells to survive and produce long-term effects (72). This purported chain of events was apparently more strongly impeded by certoparin than by UFH.

VENOUS THROMBOEMBOLISM IN CANCER PATIENTS

Tumor cells possess the capacity to interact with every part of the hemostatic system. The frequency of thromboembolic events varies, however, in different tumor types, with cancers in the pancreas, brain and ovaries most commonly implicated. Moreover, the coagulation system is further augmented by chemotherapy, radiotherapy or surgery because of vascular damage (15). Hypercoagulability in cancer patients is thought primarily to relate to: (i) tumor-derived TF; TF on tumor cells is reported to regulate the angiogenic balance of the tumor microenvironment and this influence depends on factors that are specifically present only under in vivo conditions (3); (ii) tumor-derived thrombin generation; (iii) tumor-mediated monocyte activation and monocyte TF production; (iv) tumor-derived pro-coagulants; (v) tumor-mediated platelet activation; and (iv) tumor-mediated EC activation (4). Notably, VEGF-A, the main pro-angiogenic factor in tumors, induces TF expression in ECs causing pro-coagulation activity (76, 77).

THROMBOSIS-MEDIATED ANGIOGENESIS

Clearly, the involvement of the vascular system in malignancy comprises not only angiogenesis but also systemic hypercoagulability (78, 79). Thrombin, the final enzyme produced by the blood coagulation cascade in intravascular thrombosis, performs a pro-malignant role in tumor adhesion, growth, metastasis and angiogenesis; GRO-alpha appears to be pivotal in this reaction (80–82). Thrombin also acts as a MC secretagogue, since MCs express a thrombin receptor. Secreting, i.e. degranulating MCs, release heparin (83) in addition to a large number of pharmacologically potent agents, several growth factors (including VEGF and bFGF) and enzymes (84). MC degranulation in connection with thrombosis formation causes the release of heparin that rapidly induces the release of HGF in the circulation of rats and it is claimed that HGF is a major factor in heparin-induced angiogenesis (83, 85). Thrombosis formation may thus act pro-angiogenically in a systemic fashion. MC-mediated angiogenesis, which was first conclusively demonstrated by our laboratory in the 1980s and 1990s using the rat mesentery assay, might, in fact, depend on a multitude of mechanisms, as reviewed in (84).

By inhibiting thrombosis formation in cancer patients using anti-coagulant heparins, at least a dual effect is achieved, i.e. (i) reducing thrombosis formation and harmful or life-threatening thromboembolism and (ii) thereby interfering with the systemic release of pro-angiogenic factors from MCs that are able to enhance tumor angiogenesis, growth and progression.

As noted, heparins also interact with platelet factor-4 (Table 1), a CXC chemokine anti-angiogenic factor (86) that is released in large amounts at sites of vascular injury. Platelet factor-4 is able to interact directly with angiogenic growth factors such as bFGF and VEGF-A (86).

TISSUE FACTOR AND HEPARANASE IN TUMOR GROWTH AND EVIDENCE OF PATHWAYS OF BLOOD COAGULATION AND ANGIOGENESIS BEING RECIPROCALLY LINKED

As mentioned above, TF and heparanase bond to heparin. They are individually able significantly to influence tumor angiogenesis, growth, invasion and metastasis formation.

Until recently, the principal established role of TF has been that of the primary cellular trig-
Heparins and Angiogenesis

The overexpression of the heparanase enzyme, which preferentially occurs in tumors, confers a highly invasive phenotype and metastasis formation (97-101). Heparanase also releases angiogenic factors from the tumor microenvironment and induces an angiogenic response (102-104). Notably, heparins inhibit heparanase.

A Brief Note on Methods for Assessing Angiogenesis

As discussed below, the reported effects of heparins on angiogenesis appear to a large extent to be assay dependent. Some of the main in vitro and in vivo assays that have been used to study the effect of UFH and heparin derivatives on angiogenesis are briefly described.

In vitro assays

In cultures of ECs, variables such as proliferation, migration, microtube formation (a variable related to EC maturation), receptor expression, angiogenic growth factor gene and mRNA expression and protein production are assessed, as these features are components of angiogenesis in vivo. Not only the species origin but also the anatomic origin of the ECs may influence the phenotype of the ECs. One very good EC model to study in vitro is probably the human microvascular type, since microvascular ECs are involved in clinically relevant angiogenesis capillary sprouting in vivo. Matrigel, a chemically undefined extract of the Engelbreth-Holm-Swarm tumor composed of basement membrane components, is often used in studies of EC capillary tube formation. Matrigel is reported frequently to contain traces of growth factors. Clearly, the cell culture methodology used is also important for the outcome. The aortic ring assay, which is primarily suitable for studies of the migration, sprouting and proliferation of rat, mice and chick aortic ECs, is an important ex vivo assay (105). Obviously, in certain respects, such as selected EC properties, in vitro assays constitute an essential complement to in vivo assays.

In vivo assays

There are a number of assays with distinct biological characteristics (105). Those that are referred to in this paper are: (i) the corneal microsurgical assay in rodents, in which use is made of the fact that the cornea is normally avascular, but which naturally poses the question of whether the regulation of angiogenesis in the cornea is atypical for normal adult vascularized tissue; it is a very successful assay that has been used in many crucial angiogenesis studies since its introduction in 1974 by Folkman and associates; (ii) the embryonic chick cho-
rioallantoic membrane (CAM) assay in the egg (can also be performed in Petri dishes in vitro), in which the stimulation or suppression of ongoing organogenic angiogenesis, which is governed by VEGF-A and various additional unidentified growth factors, is assessed; largely owing to its simplicity and low cost, the CAM is the most widely used in vivo angiogenesis model; it was introduced in 1974 by Folkman and co-workers; (iii) the Matrigel plug assay, which is considered one of the best for the rapid screening of potential pro- and anti-angiogenic compounds; it was introduced in 1992 by Pas-saniti et al. (see 105). Matrigel is liquid at 4°C and forms a gel when warmed to 37°C. As it contains traces of growth factors, growth factor-reduced Matrigel is often used. When known angiogenic factors are mixed with the Matrigel and injected s.c., ECs migrate and form vessels in the plug that has been produced. Since no tissue is involved, endogenous factors that can modify angiogenic reactions, including pro- and anti-angiogenic factors, are not present. Moreover, there is (iv) the adult rat mesentery assay that was introduced in 1986 by Norrby and associates (106, 107, 114). The test tissue is the multiple, very thin membranous, translucent parts of the mesentery in which the angiogenic response is recorded following the i.p. injection of a pro-angiogenic agent and systemic treatment with UFH or a heparin derivative.

As the mesentery assay is less widely used than the other models and not discussed in (105), it needs to be briefly described. The normal mesentery is vascularized and lacks significant physiologic angiogenesis in adult rats (as a result of balanced activity of local pro- and anti-angiogenic factors), as in almost all normal adult tissues; no surgery is used thereby avoiding wound-healing-induced angiogenesis; the test tissue is mechanically untouched; because the test tissue is extremely thin, it can be analyzed while microscopically intact and spread on objective slides. The entire microvascular tree in each specimen is visualized immunohistochemically (108), as illustrated in Fig. 3. This enables the detailed analysis of a number of objective variables related to the virtually two-dimensional network, including microvessel spatial extension, density, pattern formation, and capillary sprouting. The test tissue displays angiogenesis by sprouting (109), which is predominant in adult mammalian organisms and tumors. The angiogenic response to pro-angiogenic agents injected i.p. is agent- and dose-specific in terms of swiftness, potency and duration (110, 111).

In view of the different biological features and differences in the power and precision of recording pertinent angiogenesis variables between the various in vivo assays, they should probably not be expected to be completely interchangeable. Moreover, the microvascular ECs in different organs and tissues are heterogeneous, which does not necessarily permit data on anti-angiogenesis obtained in one tissue to be fully translatable to other tissues (112, 113).

ANGIOGENESIS-MODULATING EFFECTS OF LMWH TINZAPARIN FRACTIONS OF VARYING MEAN MOLECULAR WEIGHT

Since the effect of heparin fractions on angiogenesis appears to be largely dependent on the assay used and the type of angiogenesis reaction studied, it is deemed necessary to provide fairly detailed information on the experimental conditions in the various studies in the following sections. Data on the effect of anti-coagulant LMWHs on angiogenesis in vivo and related features in vitro are given in Table 5.

In the rat mesentery assay in vivo

Mast-cell (MC)-mediated angiogenesis was first described at our laboratory (84, 114). As the MC is the only cellular source of heparin, we initiated studies with the aim of elucidating the role of UFH and fractions thereof in angiogenesis. We therefore established contact with the Heparin Research Laboratory of Novo Nordisk A/S, Denmark, as this company had thorough knowledge of heparins, including its LMWH tinzaparin (Logiparin® previously sold by Novo Nordisk and Innohep® that is presently sold by Leo Pharma, Denmark, are identical products).

In initial studies in the early 1990s, the selective MC secretagogue compound 48/80 or saline containing minute concentrations of endotoxin was injected i.p. to induce angiogenesis in the mesenteric test tissue (84, 115, 116) (see Table
HEPARINS AND ANGIOGENESIS

Fig. 3. The rat mesentery angiogenesis assay. Because the membranous parts of the true mesentery are extremely thin (~5–10 μm thick when seen under the transmission electron microscope) the intact tissue can be spread on slides and, following staining with an antibody directed against rat endothelium, the entire microvessel network is visualized in each specimen. Such specimens create ideal conditions for the quantification of objective microvessel variables such as microvessel spatial extension, density, and so on, as described in (106, 107, 152). The adult test tissue is normally sparsely vascularized and lacks physiologic angiogenesis. The distance between two adjacent lines in part 4 is 10 μm.

5). UFH or a tinzaparin fraction with an MW of 3.3-kDa was administered s.c. daily for 15 days. Only UFH significantly modified the angiogenic response, as it augmented the response induced by the endotoxin-containing saline compared with vehicle control and the 3.3-kDa fraction. Following the same angiogenic i.p. treatment with endotoxin-containing saline, heparin fractions with a MW of 2.5-, 8.0-, 15.0- and 22-kDa, as well as UFH, were injected s.c. twice daily for 16 consecutive days. All fractions had a similar sulfate content of 10–12%. The 2.5- and the 8.0-kDa fractions were derived from tinzaparin, whereas the 15- and 22-kDa fractions were derived directly from UFH. A strong positive correlation (linear regression analysis, \( r = 0.97 \)) was found between the MW and the angiogenic response in terms of microvessel spatial extension (117) (see Table 5). In fact, the UFH was shown for the first time to contain chain-length-related fragments which, following heparinase digestion (MW 2.5-kDa), exerted a significant anti-angiogenic effect compared with that of the UFH when administered systemically (117). By contrast, the MW 22-kDa fraction stimulated angiogenesis compared with that of the UFH (117). The angiogenic response appeared to be unrelated to the molecular concentration, the degree of anticoagulant activity, and the sulfate content of the heparins.

Basic FGF is a prototypic heparin-binding angiogenic growth factor (Table 1). We therefore studied the effect of various heparin frac-
<table>
<thead>
<tr>
<th>Assay</th>
<th>Angiogenesis-inducing treatment</th>
<th>Heparin type and administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat mesentery <em>in vivo</em></td>
<td>Endotoxin in minute concentration</td>
<td>UFH and an 3.3 kDa MW tinzaparin fraction were given s.c. daily for 15 days</td>
</tr>
<tr>
<td>Rat mesentery <em>in vivo</em></td>
<td>Endotoxin in minute concentration</td>
<td>UFH, tinzaparin fractions with MW of 2.5, 8.0 kDa and UFH fractions with MW of 15 and 22 kDa given s.c. daily for 16 days</td>
</tr>
<tr>
<td>Chronic foot ulcer in diabetics. <em>Clinical data</em></td>
<td></td>
<td>Dalteparin administered s.c.</td>
</tr>
<tr>
<td>Stable coronary disease. <em>Clinical data</em></td>
<td></td>
<td>Dalteparin administered s.c.+exercise</td>
</tr>
<tr>
<td>Aortic arch derived bovine endothelial cells <em>in vitro</em></td>
<td>VEGF-A</td>
<td>UFH and heparin fractions with MWs of 3, 6, 10 and 15 kDa, which were chemically modified</td>
</tr>
<tr>
<td>Rat mesentery <em>in vivo</em></td>
<td>bFGF</td>
<td>One tinzaparin fraction with MW of 2.5 kDa and four with MW~22 kDa derived from UFH and showing different degree of sulfation. S.c. daily for 14 days</td>
</tr>
<tr>
<td>Rat mesentery <em>in vivo</em></td>
<td>VEGF-A</td>
<td>Tinzaparin fractions: with MWs of 2.5 and 5.0, and one fraction with MW of 16.4 kDa derived from UFH, all with similar degree of sulfation. S.c. daily for 14 days</td>
</tr>
<tr>
<td>Human microvessel endothelial cells on fibrin bed <em>in vitro</em></td>
<td>bFGF, VEGF and TBF-alpha</td>
<td>Reviparin and UFH</td>
</tr>
<tr>
<td>HUVECs <em>in vitro</em></td>
<td>bFGF, VEGF and Matrigel</td>
<td>Heparin fractions with MW between 3 and 18 kDa, dalteparin,exoxaparin, fondaparinux and tinzaparin</td>
</tr>
<tr>
<td>HUVECs <em>in vitro</em></td>
<td>bFGF and Matrigel</td>
<td>Dalteparin</td>
</tr>
<tr>
<td>Aortic ring assay, chick and rat <em>ex vivo</em></td>
<td></td>
<td>Dalteparin</td>
</tr>
<tr>
<td>CAM human prostate cancer xenograft <em>in vivo</em></td>
<td>bFGF</td>
<td>Dalteparin</td>
</tr>
<tr>
<td>HUVECs <em>in vitro</em></td>
<td>bFGF</td>
<td>Tinzaparin and rTFPI</td>
</tr>
<tr>
<td>CAM <em>in vivo</em></td>
<td>bFGF, VEGF and endotoxin</td>
<td>Tinzaparin</td>
</tr>
<tr>
<td>CAM tumor xenograft <em>in vivo</em></td>
<td>bFGF</td>
<td>Tinzaparin</td>
</tr>
<tr>
<td>HUVECs <em>in vitro</em></td>
<td>bFGF, TF/vIIa</td>
<td>Tinzaparin fractions in the range of 1.5 to 12.6 kDa MW</td>
</tr>
<tr>
<td></td>
<td></td>
<td>As above, and also rTFPI</td>
</tr>
<tr>
<td>HUVECs <em>in vitro</em></td>
<td></td>
<td>Tinzaparin fractions with varying degree of sulfation</td>
</tr>
<tr>
<td>Mouse Matrigel plug assay <em>in vivo</em></td>
<td>Matrigel only (growth factor-reduced)</td>
<td>UFH and dalteparin were included in the Matrigel</td>
</tr>
<tr>
<td></td>
<td>VEGF-A included in the Matrigel (growth factor-reduced)</td>
<td>UFH and dalteparin injected s.c. daily</td>
</tr>
<tr>
<td>Rat mesentery <em>in vivo</em></td>
<td>VEGF-A</td>
<td>Dalteparin alone, epirubicin alone and the combination of the two. S.c. continuous infusion for 14 days</td>
</tr>
</tbody>
</table>

VEGF-A is the 165 or 164 isoform.
### TABLE 5. Continued

<table>
<thead>
<tr>
<th>Result</th>
<th>Comment or conclusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFH stimulated angiogenesis compared with the 3.3 MW fraction or the vehicle control</td>
<td>The heparins had similar degree of sulfation</td>
<td>116</td>
</tr>
<tr>
<td>The 2.5 kDa fraction inhibited and the 22 kDa fraction stimulated angiogenesis compared with UFH. Strong correlation between MW and angiogenic response ( \rho = 0.97 )</td>
<td>All heparins had similar degree of sulfation</td>
<td>117</td>
</tr>
<tr>
<td>Stimulation of wound healing</td>
<td>The local capillaries appeared increased in number sic!</td>
<td>127</td>
</tr>
<tr>
<td>Reduced myocardial ischemia, possibly due to enhanced collateral function</td>
<td>Angiogenesis was believed to have occurred</td>
<td>128</td>
</tr>
<tr>
<td>The binding of VEGF-A to its receptors on the endothelial cells was inhibited by fragments of 4.8 to 5.4 kDa MWs, while fragments with MWs &gt; 6.0 kDa potentiated the binding</td>
<td>The binding of VEGF to its receptors may require an interaction with cell surface heparin binding molecules</td>
<td>121</td>
</tr>
<tr>
<td>The 2.5 kDa fraction suppressed angiogenesis compared with vehicle control and also compared with the 22 kDa fractions</td>
<td>The degree of sulfation had no effect. The 2.5 kDa fraction was identical to that used in (117)</td>
<td>118</td>
</tr>
<tr>
<td>The 5.0 kDa fraction inhibited angiogenesis compared with the vehicle control, the 2.5 kDa and 16.4 kDa fractions</td>
<td>The 2.5 MW fraction was identical to that used in (117 and 118)</td>
<td>119</td>
</tr>
<tr>
<td>Microtubule formation was inhibited by reviparin and stimulated by UFH</td>
<td></td>
<td>131</td>
</tr>
<tr>
<td>The release of TFPI increased with increasing MW and increasing degree of sulfation</td>
<td>No effect of fractions with MWs &lt; 2.4 kDa</td>
<td>22</td>
</tr>
<tr>
<td>No effect on HUVEC proliferation (serum-free medium). Maximum inhibition of microtube formation (serum-containing medium) by fractions with MWs = 6 kDa</td>
<td></td>
<td>136</td>
</tr>
<tr>
<td>Microtubule formation was stimulated, whereas HUVEC proliferation was unaffected</td>
<td></td>
<td>125</td>
</tr>
<tr>
<td>Angiogenesis was inhibited</td>
<td></td>
<td>126</td>
</tr>
<tr>
<td>Tumor growth/angiogenesis was inhibited</td>
<td></td>
<td>126</td>
</tr>
<tr>
<td>Microtubule formation was inhibited by tinzaparin and rTFPI, which was reversed by a monoclonal antibody against TFPI</td>
<td>Anti-tumor/anti-angiogenic effect</td>
<td>122</td>
</tr>
<tr>
<td>Angiogenesis was inhibited dose-dependently. The highest efficacy was displayed by MW fractions in the 6.0 to 8.0 kDa MW range</td>
<td>This effect was unrelated to tinzaparin's anti-Factor-Xa activity</td>
<td>122</td>
</tr>
<tr>
<td>Anti-tumor/anti-angiogenic effect</td>
<td>Release of TFPI was greatest in the MW range of 8.0 to 12.6 kDa</td>
<td>123</td>
</tr>
<tr>
<td>Microtubule formation was inhibited by rTFPI and tinzaparin fractions. The highest efficacy was seen in the MW range of 8.0 to 12.6 kDa</td>
<td>Microtubule formation and release of TFPI increased with increasing MW and increasing degree of sulfation</td>
<td>124</td>
</tr>
<tr>
<td>Both UFH and dalteparin induced angiogenesis</td>
<td>Both UFH and dalteparin inhibited vessel formation in the plug</td>
<td>130</td>
</tr>
<tr>
<td>Dalteparin alone stimulated angiogenesis, while epirubicin alone exerted no significant effect. However, the combination of the two significantly inhibited angiogenesis</td>
<td>The doses were in the range used therapeutically in the clinic</td>
<td>129</td>
</tr>
</tbody>
</table>
tions – one tinzaparin fraction with a MW of 2.5-kDa, identical to the fraction used in the previous study (117), and four fractions derived from UFH with MWs of approximately 22-kDa – on bFGF-mediated angiogenesis (118). The heparins were injected s.c. twice daily for 14 days. The fractions displayed varying degrees of polydispersity (a measurement of the degree of molecular weight variation), charge density (degree of sulfation) and anti-coagulant activity. The angiogenic reaction was significantly reduced by the 2.5-kDa fraction compared with vehicle controls and the 22-kDa fractions (118). The data suggested that only molecular mass was decisive for the angiogenesis-modulating effect.

VEGF-A is heparin binding (Table 1) and VEGF-A-mediated angiogenesis is a characteristic of tumor angiogenesis, as in hypoxic tissue. We therefore studied the systemic effect of heparin fractions of varying molecular mass on VEGF-A-mediated angiogenesis. Fractions with MWs of 2.5-kDa (identical to the 2.5-kDa fraction that suppressed saline/endotoxin- and bFGF-mediated angiogenesis in (117) and (118)) and 5.0-kDa, both derived from tinzaparin, as well as 16.4-kDa, derived directly from UFH, were injected s.c. twice daily for 14 consecutive days (119). Treatment with the 5.0-kDa fraction suppressed angiogenesis significantly compared not only with the saline vehicle control but also with both the 2.5- and the 16.4-kDa fraction. Notably, the anti-angiogenic 5.0-kDa fraction displayed a narrow distribution of fragment sizes (polydispersity 1.04). As a result, there was only a small overlap of fragments present in either the 2.5-kDa or the 16.4-kDa fraction. Furthermore, the 5.0-kDa MW fraction significantly shortened the capillary sprouts and reduced the number of microvessel segments and number of branching points per unit tissue volume in the mesenteric test tissue as compared with the vehicle control (120). It is noteworthy that heparin fragments with MWs of 4.8- to 5.4-kDa inhibit the binding of $^{125}$I-VEGF-A to the VEGF-A receptors on cultured bovine aortic arch ECs, while fragments with MWs of >6.9-kDa enhance the binding (121). Collectively, these findings (116–120) demonstrate that tinzaparin exerts a systemic fragment-mass-specific and angiogenesis-type-dependent anti-angiogenic effect in vivo using the rat mesentery assay.

In other angiogenesis assays

As mentioned earlier, TFPI is involved in the negative regulation of angiogenesis, tumor growth and tumor metastasis. Vascular TFPI is synthesized and constitutively secreted by ECs, while tinzaparin releases TFPI from cultured human umbilical vein ECs (HUVECs) (22).

In vitro

The release of TFPI from HUVECs was directly dependent on the molecular weight and the degree of sulfation of tinzaparin (22). HUVEC microtube formation induced by bFGF was inhibited by tinzaparin in a dose-related fashion and by recombinant TFPI, rTFPI (122). The inhibitory effect of tinzaparin or rTFPI on bFGF-induced HUVEC tube formation was totally reversed by a specific monoclonal TFPI antibody (122, 123). Moreover, the effect of different MW fractions of tinzaparin (ranging from 1.7- to 12.6-kDa) on TFPI release from HUVECs showed that fractions in the range of 8.0- to 12.6-kDa were most effective in stimulating the release. There was no release of TFPI by a pentasaccharide (MW 1.5-kDa), but, as the MW increased above 2- to 3-kDa, there was a significant increase in TFPI release (see Table 5).

When the effects of tinzaparin and rTFPI on inhibiting either bFGF- or TF/fVIIa-induced HUVEC capillary tube formation were compared in an extended study, it was found that tinzaparin and rTFPI blocked the tube formation in a concentration-dependent manner (123). Tinzaparin fractions with MWs ranging from 3.2- to 12.6-kDa were tested. Fragments in the MW range of 8.0- to 12.6-kDa were once more reported to produce the maximum TFPI release.

It was subsequently demonstrated that the higher the molecular weight and degree of sulfation of tinzaparin fractions, the greater the release of TFPI from HUVECs and the greater the efficacy in inhibiting HUVEC tube formation (124). Notably, the release of TFPI and the inhibitory effect on tube formation was not related to the anti-Factor Xa activity of tinzaparin.
In vivo

In the CAM assay, tinzaparin significantly inhibited angiogenesis augmented by bFGF, VEGF or endotoxin and also in a CAM tumor xenograft model (122). Sterile filter disks with the angiogenic test agent were placed on growing CAMs. The organogenic angiogenesis in the CAM is driven by VEGF-A, as in all embryonic tissues, in addition to a number of undefined growth factors. The tinzaparin fractions suppressed the various angiogenesis-enhancing responses in a concentration-dependent manner (122). The most effective fractions were in the 6- to 8-kDa MW range. Tinzaparin fractions in the range of hexasaccharide (MW 1.8-kDa) had minimal TFPI release and were associated with minimal anti-angiogenic efficacy (see Table 5).

There was obviously a dissociation between the maximum release of TFPI by tinzaparin fractions with MWs between 8.0- and 12.6-kDa in the cultured HUVECs (123) and the optimal anti-angiogenic effect of tinzaparin in the CAM assay, in which fractions ranging in MW from 6- to 8-kDa exerted the strongest anti-angiogenic effect (122). In the light of the substantial variation in fragment size within most LMWH fractions (Fig. 1), the result in the CAM assay is compatible with the previous finding of an MW 5.0-kDa tinzaparin fraction exerting a significant anti-angiogenic effect in VEGF-A-mediated angiogenesis in vivo in the rat mesentery assay (119).

EFFECT OF NON-TINZAPARIN COMMERCIAL LMWHS ON ANGIOGENESIS

Dalteparin

Using a Matrigel model, bFGF-mediated capillary tube formation and alpha-6 expression in cultured HUVECs were stimulated by dalteparin (Table 3), MW 6.0-kDa (125). It was concluded that alpha-6 overexpression triggered the differentiation of HUVECs but prevented proliferation. However, UFH and dalteparin displayed anti-angiogenic activity in the ex vivo chick and rat aortic ring assays, as well as in a human prostate cancer CAM model in vivo (126). In the clinic, dalteparin administered s.c. has been shown positively to influence the healing process of chronic foot ulcers in diabetic patients, perhaps by improving the capillary circulation in the ulcer region (127). In patients with stable coronary artery disease, exercise in combination with s.c. dalteparin treatment reduced myocardial ischemia, which was likely due to enhanced collateral function (128). Using the rat mesentery assay in vivo, dalteparin continuously infused s.c. as monotherapy significantly stimulated VEGF-A-mediated angiogenesis in an inversely dose-dependent fashion, whereas angiogenesis was significantly inhibited when the animals were continuously co-treated with dalteparin and the cytotoxic agent epirubicin (129). Notably, epirubicin (an intercalating topoisomerase-targeting drug of the anthracycline class) as monotherapy did not affect angiogenesis. Clearly, dalteparin and epirubicin interacted in a complex fashion.

This appears to be the first demonstration that any LMWH in its own right is able to augment angiogenesis in vivo. The pharmacologic profile of dalteparin in this respect is favorable, as its anti-angiogenic effect occurred only in combination with epirubicin. The pro-angiogenic effect of dalteparin when administered alone is desirable when it comes to the treatment of thromboembolism unrelated to tumors, since thromboses and emboli cause hypoxia, which potentially leads to tissue necrosis. Compensatory VEGF-A-mediated angiogenesis, which is powerfully driven by hypoxia in poorly perfused tissues, should be stimulated by dalteparin and the same thing probably applies to wound healing.

Recently, it was reported that UFH and dalteparin exerted similar effects on angiogenesis using the Matrigel plug assay in mice (130). A mixture of growth factor-reduced Matrigel with either PBS-saline, UFH or dalteparin was injected s.c., thereby forming a plug. Both heparins were able to induce angiogenesis in their own right when mixed with the Matrigel measured as the hemoglobin content in the plug. When the Matrigel was loaded with VEGF-A, both UFH and dalteparin, given s.c. daily at an anti-coagulant dose, inhibited vessel formation in terms of vessel area compared with the s.c. injection of PBS-saline.

Reviparin and other LMWHs

The stimulation of capillary-like tube formation by bFGF or VEGF-A in combination with
TNF-alpha in human microvascular ECs seeded on top of a fibrin matrix was inhibited by reviparin (MW 4.2-kDa) and stimulated by UFH (131). Reviparin (Table 3) and UFH in therapeutic doses affected neither the development of metastases nor blood vessel formation in a colon cancer metastasis model in the rat liver, however (132). Immunohistochemistry was used to visualize the ECs and thereby indirectly estimate tumor-associated angiogenesis in tissue microtome sections in terms of vessel density as a ratio of tumor-cell density (132). As has been clarified more recently in greater detail, vessel density assessment in microtome-sectioned tissue is not a measurement of angiogenesis and, furthermore, there are no means of quantitatively assessing the anti-angiogenic effect per se of drugs in tumors (133–135). Consequently, the anti-angiogenic effect per se of any agent must be assessed in a surrogate non-tumor tissue using angiogenesis assays such as those mentioned above.

Recently, microtube formation and the proliferation of primary cultures of HUVECs were studied in Matrigel exposed to bFGF or VEGF-A in combination with heparins differing in MW between 3- and 18-kDa (prepared from UFH by controlled deaminative cleavage and subsequent purification), UFH, or the commercial LMWHs dalteparin, enoxaparin (MW 4.5-kDa), fondaparinux (a synthetic 1.7-kDa pentasaccharide) and tinzaparin (136). For proliferation studies the HUVECs were plated in gelatin-coated wells and grown in a serum-free medium. No inhibition of proliferation was observed with heparin tetrasaccharide (MW 1.2-kDa), pentasaccharide (MW 1.7-kDa, i.e. fondaparinux), or octasaccharide (MW 2.4-kDa). For microtube formation, the culture medium contained serum. The maximum inhibition of HUVEC proliferation and tube formation occurred at a MW of ~6-kDa in both bFGF- and VEGF-A-mediated stimulation. There was no significant difference in effect between fractions with MWs of 3-kDa, 6-kDa, or between tinzaparin and dalteparin. It was concluded that the heparin inhibition of HUVEC proliferation and microtube organization requires a chain length of >8 saccharide units (MW 2.4-kDa) with maximum inhibition at approximately 20 saccharide units (MW 6-kDa). Moreover, the 6-kDa MW fraction inhibited vessel formation in a placental explant assay (136).

EFFECT OF HEPARIN-LIKE MOLECULES, LMWH DALTEPARIN, OR A VERY LOW-MOLECULAR-WEIGHT HEPARAN SULFATE IN COMBINATION WITH STEROIDS OR A TYROSINE KINASE INHIBITOR ON ANGIOGENESIS AND TUMOR GROWTH

Pioneering studies by Folkman and associates showed that UFH, specific heparin fractions or a synthetic cyclic oligosaccharide convert certain steroids to angiogenesis inhibitors in the rabbit corneal and the chick CAM assays (137–139). It was postulated that heparins concentrate steroid molecules that bind to sulfated polyanion receptors on the ECs. The steroid suppressed murine EC proliferation and tumor growth in vivo (140, 141). Angiogenesis following the chemical cauteration of the rat cornea close to the corneoscleral limbus was studied by measuring the angiogenic response in terms of the vascularized corneal area on different days post-treatment (142). The cautery induced bFGF immunoreactivity in the traumatized corneal epithelium and stroma. Angiogenesis was assessed after treatment with two drops of test solution/eye four times daily for 6 days. A very low-molecular-weight heparan sulfate (MW < 1.0-kDa) or UFH + hydrocortisone, but not UFH alone, inhibited angiogenesis (142).

In mice bearing the human U87 glioblastoma xenograft, tumor growth was moderately suppressed by SU5416 and unaffected by daily s.c. injections of dalteparin. However, co-treatment with SU5416 and dalteparin reduced the level of VEGF in tumors and increased the growth-inhibitory effect of SU5416 (143). SU5416 is a small molecule inhibitor of tyrosine kinase receptors, including the VEGF receptor 2 and the stem cell factor receptor c-kit.

CONTROL OF ANGIOGENESIS AND TUMOR METASTASIS BY NON-ANTI-COAGULANT HEPARIN DERIVATIVES LINKED TO ANTI-ANGIOGENIC MOLECULES OR INTERFERING WITH GROWTH-FACTOR RECEPTOR BINDING

Cancer patients are particularly prone to develop bleeding complications while receiving anti-coagulant treatment with warfarin (an oral
HEPARINS AND ANGIOGENESIS

anti-coagulant), but LMWHs are associated with a lower risk of adverse events.

As noted, recent clinical trials with LMWH and meta-analyses of earlier clinical trials with UFH indicate that these heparins have an anti-metastatic activity. Animal studies using non-anti-coagulant heparin suggest that it is possible to distinguish the anti-metastatic and anti-coagulant activities of heparin. Non-anti-coagulant heparins have clinical potential because they can be administered at a higher dose, thereby fully exploiting the anti-metastatic component of heparin activity, and because they can be used in cancer patients with bleeding complications, where the use of LMWH or UFH is currently precluded (144). The mechanism by which non-anti-coagulant heparin inhibits metastasis is not yet fully understood. Possible mechanisms include the inhibition of selectin-mediated cell-cell interactions, heparanase and angiogenesis, as well as the stimulation of TFPI release (144), because chemical modifications of UFH that diminish its anti-coagulant properties may preserve its anti-coagulant properties may preserve its heparanase-inhibitory, anti-tumor and anti-metastatic effects (145).

Various non-anti-coagulant low- or very-low-molecular-weight derivatives showing anti-angiogenic activity by interfering with the binding of heparin-binding pro-angiogenic factors to specific cell surface receptors have been developed as potential anti-cancer drugs, including undersulfated and glycol-split heparins (146). For instance, a heparin derivative (MW 9.8-kDa), obtained from UFH by nitrous degradation and modification of the fragments, was more effective than UFH in inhibiting the mitogenic activity induced by bFGF in cultured transformed fetal bovine aortic ECs (147). This 9.8-kDa fraction also inhibited angiogenesis in the CAM-sponge assay in vivo in which UFH was inactive. A recent study shows that heparin-like LMW-sulfated Escherichia coli capsule polysaccharide derivatives can act as bFGF antagonists and thereby exert anti-angiogenic effects in bovine endothelium GM7373 cultures and the CAM assay (148). These non-anti-coagulant molecules display MWs ranging from 4.2- to 6.0-kDa.

The structure/function of HSPGs on ECs and the angiostatic properties of synthetic heparin-like compounds, chemically modified heparin, and biotechnological heparins were recently reviewed in (149). The development of other heparinomimetic drugs is briefly discussed in (150).

CONCLUDING REMARKS

Venous thromboembolism is a significant cause of mortality in cancer patients and the main objective of thrombosis prophylaxis in these patients is to reduce the risk of fatal pulmonary embolism. The anti-coagulant therapy is currently the foremost clinical application of unfractionated heparin (UFH) and low-molecular-weight heparins (LMWHs). Several clinical trials demonstrate that treatments with various LMWHs (including certoparin, dalteparin, enoxaparin, reviparin and tinzaparin) for preventing or treating thromboembolism improve survival time compared with UFH or no heparin treatment in cancer patients receiving chemotherapy. The anti-neoplastic effects in these studies are thought to be largely unrelated to the inhibition of thrombosis formation. Through purported direct anti-neoplastic effects, anti-angiogenic effects and the modulation of the immune system, LMWHs thus appear to have significant anti-tumor activity in humans. As is well known, tumors are angiogenesis dependent.

Available preclinical data indicate that any effect on angiogenesis by anti-coagulant heparins is likely to depend primarily on a number of circumstances (Table 5): (i) the mean fragment size of the heparin derivative tested; (ii) the manufacturing process used for each type of LMWH; (iii) the angiogenesis assay used for assessing the effect (type of endothelial cell variable studied in vitro, in vitro vs in vivo, embryonic avian vs adult mammalian tissue); (iv) the type of angiogenesis reaction studied (such as VEGF-, bFGF-, or endotoxin-mediated angiogenesis). To date, only the LMWH tinzaparin (MW 6.5-kDa) or fractions thereof with MWs ranging between ~2.4- and 8-kDa are reported to inhibit angiogenesis in a tissue in vivo. A 5.0-kDa MW tinzaparin fraction systemically and specifically inhibits VEGF-A-mediated angiogenesis in the adult rat. Notably, heparin fractions with MWs of 4.8- to 5.4-kDa block the binding of VEGF-A to its specific receptor on endothelial cells in vitro. However, it was recently demonstrated that dalteparin (MW 6.0-
kDa) as monotherapy stimulates VEGF-A-mediated angiogenesis, which is opposite to the effect of tinzaparin in the same experimental system, suggesting that the manufacturing process for producing LMWHs might be decisive for the outcome. Several LMWHs are reported to suppress or, in exceptional cases, stimulate angiogenesis-related events in cultured endothelial cells. The angiogenesis-modulating efficacy of the heparins appears to be unrelated to the anti-coagulant effect of these molecules.

The molecular mechanisms involved in modulating angiogenesis by systemically administered heparins are probably complex, as heparins bind to, modify and release a multitude of circulating, cell-bound or ECM-bound pro-angiogenic factors, anti-angiogenic factors, other growth factors, enzymes, proteins and receptors that may influence the induction and progression of angiogenesis. VEGF-A, a pivotal pro-angiogenic factor in most tumors, bonds to heparin, as does bFGF, which is also a potent pro-angiogenic growth factor. Tissue factor (TF) and its natural inhibitor TFPI are also important heparin-binding proteins that perform well-recognized roles in the regulation of angiogenesis, tumor growth and metastasis.

Since directly comparative clinical trials have not been performed, it is not known which commercial LMWH exerts the greatest anti-tumor effect in cancer patients. Because of the extensive use of LMWHs in cancer patients, there is a call for systematic experimental studies of the angiogenesis-modulating effect of discrete LMWHs. The study of how the clinical practice of combined treatment with a LMWH and chemotherapy or surgery exerts anti-angiogenic and anti-tumor effects is still in its infancy. This significant point may be tricky to clarify in detail, however, as cytotoxics modulate angiogenesis in a drug-specific and dose-dependent way, as do LMWHs (at least tinzaparin and dalteparin) in one and the same in vivo mammalian model. Prospective clinical trials directly comparing the effects of two or more discrete LMWHs on the survival benefit, disease progression and well-being of cancer patients therefore appear to be warranted. Tinzaparin, or anti-coagulant fractions with certain MWs thereof, is the only LMWH that has been shown to suppress angiogenesis in any type of tissue in vivo in its own right. Hypothetically, tinzaparin could be the anti-coagulant heparin that is capable of exerting the most potent anti-tumor effects in cancer patients receiving chemotherapy, and possibly also when administered during primary surgery.

The Swedish Cancer Foundation (grant 020503) supported the study.

REFERENCES

HEPARINS AND ANGIOGENESIS


97
42. Affara NI, Robertson FM. Vascular endothelial growth factor as a survival factor in tumor-associated angiogenesis. In Vivo 2004;18:525–42.
65. Hettiarachchi RJK, Smorenburg SM, Ginsberg J, Levine M, Prins MH, Büller HR. Do heparins...


