Heparins, cancer and thrombosis: clinical and experimental studies

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UNFRACTIONATED AND LOW MOLECULAR WEIGHT HEPARIN AFFECT FIBRIN STRUCTURE AND ANGIOGENESIS IN VITRO
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ABSTRACT
Cancer patients treated for venous thromboembolism with low molecular weight heparin (LMWH) have a better survival rate than patients treated with unfractionated heparin (UFH). Since fibrin-associated angiogenesis is an important determinant in the progression and metastasis of many solid tumors, the effects of heparins on *in vitro* angiogenesis were investigated. Both UFH and LMWH inhibited to the same extent bFGF-induced proliferation of human microvascular endothelial cells (hMVEC) as determined by \[^3H\]-thymidine incorporation (36-60 % inhibition). VEGF\(_{165}\)-induced proliferation was also inhibited, but to a to a lesser extent (19-33 % inhibition).

Turbidity measurements and electron microscopy showed that the presence of LMWH during polymerization of the fibrin matrix led to a more transparent rigid network with thin fibrin bundles, while the presence of UFH resulted in a more opaque malleable network with thick fibrin fibers. We used a human *in vitro* angiogenesis model, that consisted of hMVEC seeded on top of a fibrin matrix, and stimulated the cells with bFGF/TNF\(\alpha\) to induce capillary-like tubular structures. The formation of capillary-like tubular structures was retarded with matrices polymerized in the presence of LMWH (46% inhibition as compared to a control matrix for both 1.5 and 10 U/ml LMWH), while matrices polymerized in the presence of UFH facilitated tubular structure formation (72% and 36% stimulation as compared to a control matrix for 1.5 and 10 U/ml UFH respectively). Similar results were obtained for cells stimulated with VEGF/TNF\(\alpha\).

These data determine the inhibitory effect of heparins on proliferation of hMVEC and provide a novel mechanism by which LMWH may affect tumor progression, namely reduced ingrowth of microvascular structures in a fibrinous stroma matrix by rendering it less permissive for invasion.

INTRODUCTION
Patients with malignant diseases are at increased risk of venous thromboembolic complications (1). As a result, many cancer patients are treated with antithrombotic drugs, including heparins. Intravenous dose-adjusted unfractionated heparin (UFH) has been the standard initial treatment, but recent randomized clinical trials have shown that subcutaneous fixed doses of low molecular weight heparin (LMWH) are as safe and effective as UFH (2-4). Cancer patients who have been treated with LMWH for their venous thromboembolism were found to have a significantly improved three month survival as compared to UFH recipients, whereas incidences of hemorrhages and thromboembolic recurrences were comparable in both treatment groups (5).

Several experimental studies have reported the either stimulatory or inhibitory effects of heparins on tumor growth and metastasis (see (6) for review). These effects may not only reflect their anticoagulant function, but may involve other processes, such as angiogenesis (7). Animal studies have shown that LMWH and UFH differentially affect angiogenesis, but the mechanisms by which they act remain unclear (8,9).
Angiogenesis, the formation of new blood vessels, supports the expansion of many solid tumors and facilitates the escape of tumor cells and thus metastasis (10,11). Angiogenesis is driven by microvascular endothelial cells (MVEC), which upon activation degrade their basement membrane, migrate into the interstitial matrix, proliferate, and form new capillary-like tubular structures (12). Tumors release a number of angiogenic growth factors, such as vascular endothelial growth factors (VEGFs) (13), fibroblast growth factors (FGFs) (14) and scatter factor (15). Induction and maintenance of angiogenesis requires interaction of these growth factors with their respective receptors, which then activate endothelial cells (16,17), often in concert with other cytokines (18). Heparan sulfates and heparins modulate the binding of many angiogenic growth factors, and hence may affect endothelial cell responses (19,20). Thus far, the effects of heparins on angiogenesis have been attributed to their interaction with angiogenic growth factors. However, other steps in the process of angiogenesis may also be influenced, in particular the interaction of MVEC with the matrix which they invade.

In several tumor types, fibrin is a major component of the initial stroma (21,22). Fibrin provides scaffolding for both invasive cancer and endothelial cells, thereby contributing to tumor growth and neovascularization (23,24). The structural and mechanical properties of the fibrin matrix play a regulatory role in the formation of capillary-like tubular structures (25,26). Modifications of the structure of the fibrin network alters its sensitivity towards proteolytic degradation (27,28), which affects tube formation. Heparins also affect the structure of the fibrin clot, altering its sensitivity to plasmin degradation (29). However it is not known to which extent LMWH or UFH affect angiogenesis by altering the structure of this temporary matrix.

The present study evaluates the effects of UFH and LMWH on growth factor-induced proliferation and the formation of capillary-like tubular structures by human MVEC (hMVEC). Both compounds reduce proliferation of hMVEC to a rather similar degree. However, the presence of LMWH during the polymerization of the fibrin decreases the formation of tubular endothelial structures, while the presence of UFH enhances its formation. These data provide a novel mechanism by which LMWH may affect tumor progression, namely reduced ingrowth of microvascular structures in a fibrinous stroma matrix by rendering it less permissive for invasion.

**MATERIALS AND METHODS**

**Materials**

Penicillin/streptomycin, L-glutamine and medium M199 with or without phenol red, with EBBS, L-glutamine and HEPES were obtained from Biowithaker (Verviers, Belgium). Trichloroacetic acid (TCA), trypsin 1-300 370 USP/mg were obtained from ICN (Costa Mesa, CA) and heat-inactivated newborn calf serum from GIBCO BRL (Paisley, Scotland). Human serum was prepared from the pooled fresh blood of 10-20 healthy donors obtained from a local blood bank. Fibronectin was a gift from Dr J. van Mourik (Central Laboratory of the Blood Transfusion Service,
Amsterdam, The Netherlands). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine brain (30). Thrombin and unfractionated heparin (UFH) were obtained from Leo Pharmaceutical Products (Weesp, The Netherlands), tissue culture plastics from Costar (Cambridge, MA). Human fibrinogen, batch X 0379-51 containing 3.2 μg plasminogen and 5 μg plasmin per gram fibrinogen was purchased from Chromogenix AB (Möln达尔, Sweden) and the low molecular weight heparin (LMWH) Reviparin was from Knoll (Ludwigshaven, Germany). VEGF₁₆₅ was a kind gift from Dr. H. Weich, (GBF, Braunsweig, Germany), and TNFα containing $2.45 \times 10^7$ U/mg protein and less than 40 ng lipopolysaccharide per mg protein from Dr. J. Tavernier (Biogent, Gent, Belgium). Recombinant human basic fibroblast growth factor (bFGF) was purchased from PeproTech (Rocky Hill, NJ) and [³H]-thymidine from Amersham (Buckinghamshire, UK).

**CELL CULTURE**

Human umbilical vein endothelial cells (HUVEC) (31) and human foreskin microvascular endothelial cells (hMVEC) were isolated, cultured and characterized as previously described (32,33). Cells were cultured until confluence in 5% CO₂/95% air atmosphere on fibronectin-coated dishes in M199 supplemented with 2 mM L-glutamine, 20 mM HEPES (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 mg/ml ECGF, 100 IU/ml penicillin and 100 mg/ml streptomycin. The endothelial cells were then detached with trypsin/EDTA and transferred to new fibronectin-coated dishes at a split ratio of 1:3. Confluent endothelial cells were used at passage 9-11 for hMVEC and at passage 2 for HUVEC.

**PROLIFERATION ASSAY**

Incorporation of [³H]-thymidine in DNA was determined as previously described (18). Confluent cultures of HUVEC or hMVEC were detached by trypsin/EDTA solution and seeded at a density of 7500 cells per cm² on fibronectin-coated 24 well dishes in M199 medium containing 10% NBCS, penicillin/streptomycin, growth factor (2.5 ng/ml bFGF or 12.5 ng/ml VEGF₁₆₅). The inhibition of proliferation was studied by the addition of 0.5 or 10 U/ml UFH or 0.5 or 10 U/ml LMWH. After a preincubation period of 48 hours, a trace amount of [³H]-thymidine (0.5 μCi per 2 cm² well in 10 μl) was added and cells were incubated for another 8 hours. The cells then were washed with ice-cold PBS and [³H]-labeled cells were fixed with 0.5 ml 100% methanol, DNA was precipitated with 5% TCA, dissolved in 0.3 M NaOH and counted in a liquid scintillation counter.

**PREPARATION OF FIBRIN MATRICES**

Human fibrin matrices were prepared by the addition of 0.1 U/ml thrombin to 300 μl of 3 mg/ml fibrinogen dialyzed against phosphate-buffered saline (PBS, containing 140 mM NaCl, 13.4 mM Na₂HPO₄·2H₂O, 138 mM NaH₂PO₄·2H₂O, pH 7.4) in a 1 cm² well of 48-well plates. The structure of the fibrin clot was modified by varying the pH of the mixture before polymerization between pH
7.0 and 7.8 with NaOH or HCl. After 24 hours of polymerization, inactivation of thrombin and adjustment of the pH of the fibrin gels to pH 7.4 was carried out by equilibrating the gels twice for 12 hours and once for 24 hours with 0.5 ml M199 containing 10% human serum and 10% newborn calf serum.

The influence of heparins on the structure of the fibrin matrix was studied by the addition of 1.5 or 10 U/ml UFH or 1.5 or 10 U/ml of LMWH prior to the polymerization. After 4 hours of polymerization the matrices were washed with culture media.

In a parallel experiment the structure of fibrin fibers was monitored by turbidity measurement with a multichannel spectrophotometer at 340 nm (Titertek multiscan; Flows Labs, McLean, VA) and was plotted against the pH of the polymerization buffer or against the concentration of the added heparin.

For electron microscopy examination of the fibrin network structure, fibrinogen was clotted on formvar-coated 200 mesh nickel grids, which were dipped in poly-1-lysine, by the addition of 1 U/ml of thrombin in the presence or absence of 10 U/ml LMWH or UFH. After repeated washing with water the specimen was dried, stained with 2% phosphotungstic acid for 1 min and the fibrin network formed was analyzed in a Philips 201 electron microscope.

**IN VITRO ANGIogenesis Model**

After preparation of the fibrin matrices, confluent endothelial cells were detached from the fibronectin-coated dishes with trypsin/EDTA and seeded in a confluent density on the fibrin matrices. After 24 hours the medium was replaced with medium containing different mediators. Every 48 hours the medium was changed and collected, for a time period of six days. The formation of tubular structures of endothelial cells by invasion into the underlying matrix was analyzed by phase contrast microscopy. Quantification of the length of the structures formed was performed by a computer equipped with Optimas image analysis software connected to a monochrome CCD camera (MX5) (18).

**Antigen Measurement in Conditioned Media**

u-PA antigen was measured as previously described (18). Briefly, 96-well microtiter plates were coated with a mixture of two monoclonal antibodies, UK 2.1 and UK 26.15, recognizing different epitopes of the u-PA antigen. The next day, the plates were blocked with casein and serial dilutions of standard u-PA (Ukidan; Serono, Aubonne, Switzerland) or culture media were added. Finally, an incubation was performed with HRP-conjugated monoclonal anti-u-PA IgG (LMW 11.1), and then tetramethylbenzidine substrate was added to react. The reaction was stopped with H₂SO₄ after 15 min of incubation. The absorbance was measured with a multichannel spectrophotometer.
PAI-1 antigen was determined by ELISA of the conditioned media collected from cells grown on fibrin and stimulated with different factors in M199 supplemented with 10% human serum and 10% newborn calf serum, according to the instructions of the manufactures (Biopool, Umea, Sweden).

**Statistics**

Data were expressed as mean±SEM. Statistical significance of differences between groups were analyzed by one-way ANOVA followed by Bonferroni’s modified t-test. Differences were considered significant if P < 0.05.

**RESULTS**

**Effect of heparin on the proliferation of hMVEC and HUVEC**

Proliferation was measured as the incorporation of [3H]-thymidine in hMVEC stimulated with bFGF (2.5 ng/ml) or VEGF_{165} (12.5 ng/ml). Addition of a low concentration of UFH- or LMWH (0.5 U/ml of UFH or 0.5 U/ml of LMWH) to bFGF-stimulated cells resulted in a significant decrease of [3H]-thymidine incorporation (58±3 % and 36±3 % inhibition, respectively, as compared to control). The addition of higher concentrations of both heparin preparations resulted in similar or even

![Figure 1: Incorporation of [3H]-thymidine in DNA of hMVEC (A, B) or HUVEC (C, D): The inhibition of growth factor-induced proliferation was studied by the addition of 0.5 or 10 U/ml UFH or 0.5 or 10 U/ml LMWH. Data are the mean ± SE of three independent experiments for bFGF (A and C) and for VEGF_{165} (B and D). (* represents P<0.01 and ** represents P<0.05)
Further inhibition (60±9% and 50±10% inhibition, respectively) (Figure 1A). Also VEGF_{165}-induced proliferation was inhibited by the addition of UFH and LMWH, although to a lesser extent (33±3% for 0.5 U/ml UFH and 0.5 U/ml LMWH, and to 28±3% and 19±3% for 10 U/ml respectively) (Figure 1B).

Interestingly, this effect of heparin was specific for hMVEC, because UFH or LMWH did not significantly affect the proliferation of HUVEC induced by bFGF (8±2% and 5±8% for 0.5 U/ml UFH and 0.5 U/ml LMWH and to 15±5% for 10 U/ml) (Figure 1C) nor VEGF_{165} (2±4% and 9±7% for 0.5 U/ml respectively, and to 3±5% and 4±10% for 10 U/ml, respectively) (Figure 1D).

**Fibrin matrices polymerized in different conditions**

Previously it was shown that the structure of fibrin depends on the pH at which fibrin was polymerized (26,27,34). Fibrin matrices polymerized at pH 7.0 or lower had a high turbidity indicative of an opaque, malleable and porous network, while those polymerized in a more basic environment (pH 7.8) had a low absorbency indicative of a transparent, dense and rigid network (Figure 2A).

The presence of UFH and LMWH during fibrin polymerization also caused an alteration of the turbidity of the matrices formed. UFH induced an increase in turbidity and LMWH a decrease (Figure 2B).

Electron microscopic studies on fibrin matrices confirmed that the fibrin network formed in the presence of UFH was composed of thicker fibrin bundles in a more porous network, while bundles in the tighter network formed in the presence of LMWH were thinner and denser (Figure 2C-E).

**Formation of capillary-like tubular structures in the various fibrin matrices**

hMVEC grown on a fibrin matrix and stimulated with the combination of bFGF and TNFα (bFGF/TNFα) or VEGF_{165} and TNFα (VEGF_{165}/ TNFα) invaded the underlying fibrin matrix and formed capillary-like tubular structures (18, compare Figure 4A,B). The overall length of tubular structures was considerably higher in malleable fibrin matrices produced at pH 7.0 than in fibrin matrices produced at pH 7.8 both in bFGF/TNFα and VEGF_{165}/ TNFα-stimulated cells (Figure 3).

The presence of UFH and LMWH during the polymerization altered the fibrin matrices and also affected the extent of tube-formation by hMVEC (Figure 4). The total length of the tubes formed after stimulation with bFGF/TNFα of the cells grown on fibrin matrices polymerized in the presence 1.5 U/ml and 10 U/ml UFH was increased with 72±10% and 36±8% respectively, as compared to control matrices (Figure 4, Table 1). Similar results were obtained if cells were stimulated with VEGF_{165}/ TNFα (Table 1). However, the presence of similar amounts of LMWH during matrix polymerization caused a decrease in the length of tube as compared to control for bFGF/TNFα-stimulated cells (Figure 4, Table 1) and for VEGF_{165}/ TNFα-stimulated cells (Table 1).
When UFH or LMWH were added after polymerization of the fibrin and seeding of the hMVEC no significant effects on tube formation were observed for bFGF/TNFα-stimulated cells (81±12% and 85±10% of control for 1.5 U/ml and 10 U/ml UFH respectively and 100±11% and 97±8% for 1.5 U/ml and 10 U/ml LMWH respectively, n=7, control is 294±44 mm/cm²). This indicates that the effect of UFH and LMWH were mainly due to their effect on the structure of the fibrin matrix. The differences in tube formation could not be explained by an altered u-PA activity, because the secretion of u-PA and PAI-1 antigen in all conditions was similar, as shown for bFGF/TNFα-stimulated cells (Table 2).

Figure 2: The effect of the polymerization conditions on the structure of the formed fibrin matrix: A,B: Prior to polymerization the pH of the buffer was altered between pH 7.0 and 7.8 (A) or 1.5 or 10 U/ml UFH and 1.5 or 10 U/ml LMWH were added (B). After 4h of polymerization the structure of the fibrin was determined by measurement of the turbidity at 340 nm (A 340). Data are expressed as the mean ± SE of three independent experiments.
C-E: electron micrographs of a fibrin network formed from purified fibrinogen after polymerization without (C) or in the presence of UFH (D) or LMWH (E).
Figure 3: The effect of fibrin structure on capillary tube formation: Fibrin matrices were polymerized at pH 7.0, pH 7.4 and pH 7.8. hMVEC were seeded on top of these fibrin matrices and stimulated with 5 ng/mL bFGF and 1 ng/mL TNFα (bFGF/TNFα) or 40 ng/mL VEGF and 1 ng/mL TNFα (VEGF/TNFα) to form invasive capillary-like tubular structures. The total length of tubular structures was quantified and expressed as % of the values obtained after stimulation with bFGF/TNFα or VEGF/TNFα on a control fibrin matrix (pH 7.4). Data are expressed as mean ± SEM of five different experiments performed with duplicate wells for bFGF/TNFα and three experiments in duplicate for VEGF/TNFα. The total tube length in control matrices (100%) was 229±12 mm/cm² for bFGF/TNFα and 77±2 mm/cm² for VEGF/TNFα.

Figure 4: The effect of fibrin structure on the formation of capillary-like tubular structures: Stimulation of endothelial cells with 5 ng/ml bFGF and 1 ng/ml TNFα for six days induced the cells to invade the fibrin matrix and form capillary-like tubular structures. A, C–F: Phase-contrast photomicrographs of tubular structures (A, arrowhead) formed by hMVEC cultured on fibrin matrices formed in the presence of 1.5 or 10 U/ml UFH (C and D), 1.5 or 10 U/ml of LMWH (E and F) and control matrices (A). Bar represents 300 μM. B: Histological cross-section: a tubular structure, indicated by an arrowhead, is connected as became evident by serial sections. G: The total length of tubular structures formed after six days was quantified and expressed as % of the values obtained after stimulation with bFGF/TNFα on a control fibrin matrix (no heparin added). Data are expressed as mean ± SEM of six different experiments performed with duplicate wells (* represents P< 0.01). The total tube length in control matrices (100%) was 229±12 mm/cm².
Addition | bFGF/TNFα | VEGF/TNFα |
---|---|---|
None | 100 ± 1 | 100 ± 2 |
1.5 U/ml UFH | 172 ± 10* | 177 ± 5* |
10 U/ml UFH | 136 ± 8 | 138 ± 4* |
1.5 U/ml LMWH | 46 ± 6* | 45 ± 1* |
10 U/ml LMWH | 46 ± 4* | 43 ± 4* |

Table 1. The effect of the polymerization conditions on the structure of the formed fibrin matrix. Fibrin matrices were polymerized in the presence of UFH (1.5 U/ml and 10 U/ml) or LMWH (1.5 U/ml and 10 U/ml), or without addition (control matrix). hMVEC were seeded on top of these fibrin matrices and stimulated with 5 ng/ml bFGF and 1 ng/ml TNFα (bFGF/TNFα) or 40 ng/ml VEGF and 1 ng/mL TNFα (VEGF/TNFα) to form invasive capillary-like tubular structures. The total length of tubular structures was quantified and expressed as % of the values obtained after stimulation of hMVEC with bFGF/TNFα or VEGF/TNFα, respectively, on a control fibrin matrix (no heparin added). Data are expressed as mean ± SEM of five different experiments performed with duplicate wells (* represents P< 0.01). The total tube length in control matrices (100%) was 229±12 mm/cm² for bFGF/TNFα and 77±2 mm/cm² for VEGF/TNFα. 1 U/ml LMWH represents 1 aXa/ml LMWH.

Addition | PAI-1 antigen | u-PA antigen |
---|---|---|
none | 678 ± 50 | 21 ± 0.4 |
1.5 U/ml UFH | 694 ± 57 | 24 ± 0.1 |
10 U/ml UFH | 685 ± 34 | 23 ± 0.3 |
1.5 U/ml LMWH | 711 ± 20 | 23 ± 0.1 |
10 U/ml LMWH | 682 ± 20 | 23 ± 0.4 |

Table 2: Accumulation of u-PA and PAI-1 antigen in conditioned media of tube forming hMVEC on different fibrin matrices. Stimulation of hMVEC with 5 ng/ml bFGF and 1 ng/ml TNFα induced the cells to invade the fibrin matrix and form capillary-like tubular structures. Every two days the supernatant media were renewed. The total concentrations of u-PA and PAI-1 antigens secreted in the conditioned media were measured by specific ELISA and expressed as the total amount (ng) secreted over a period of six days. Data are the mean± SEM of three different experiments performed with duplicate wells.

**DISCUSSION**

The present study reveals two mechanisms by which UFH and LMWH affect angiogenesis in vitro. UFH and LMWH inhibit the proliferation of hMVEC induced by the angiogenic factors bFGF and VEGF to a similar degree, and differently affect fibrin matrix formation. LMWH causes the formation of more rigid fibrin matrices that inhibit capillary-like tubular structure formation, while the presence of UFH contributes to the formation of a more malleable fibrin matrix and thus facilitates angiogenesis.

Angiogenesis is required for the expansion of many solid tumors and facilitates the metastasis of tumor cells to other organs (10). Factors altering angiogenesis may therefore influence these processes and thereby the prognosis of cancer patients. Various studies have suggested that heparins affect the proliferation of endothelial cells by their effects on angiogenic growth factors, in particular FGFs and VEGFs (35-36). Both endothelial heparan sulfates and heparins can promote the interaction of these growth factors with their receptors. One may anticipate that LMWH might inhibit angiogenesis by competing with cellular heparan sulfates for the binding of these growth factors (37,38). However, no major differences between UFH and LMWH on endothelial cell proliferation were observed. Interestingly, the inhibitory effect of heparin on proliferation was
relatively strong in hMVEC, whereas it was non-significant in HUVEC. Because angiogenesis is driven by microvascular endothelial cells, this effect is probably relevant for tumor neovascularization.

The migration and invasion of cells depend on their detachment from and the new attachment of invading cells to their extracellular matrix. Tumor cells induce a state of hyperpermeability in the surrounding vasculature by the release of vascular permeability factors such as VEGF (39). Plasma proteins, in particular fibrinogen, extravasate. The then formed fibrinous exudate composes a major component of the initial tumor stroma (22,39). This temporary matrix provides an important provisional scaffolding for invasive cells, thereby contributing to tumor growth and neovascularization. *In vitro* angiogenesis studies revealed that the formation of capillary-like tubular structures by endothelial cells in a fibrin matrix depends on local and controlled matrix degradation mediated by cell-bound urokinase and plasmin (40-43). In addition, the structure of fibrin itself plays an important role in the invasion of the matrices by (25,26) and as is shown here, heparins affect fibrin matrix formation. Differences in the fibrin network structure alter its sensitivity towards plasmin-dependent proteolysis (44,29) and the display of epitopes involved in endothelial cell-matrix interaction during angiogenesis (45,46). Heparins were present during the polymerization but the amount of heparin remaining did not significantly affect endothelial cell behaviour. The formation of capillary-like tubular structures was not affected by the addition of heparins in our in vitro model. Furthermore, the amount of heparin remaining did not affect endothelial cell proliferation. This suggestion is strengthened by our previous observation that the formation of tubular structures does not critically depend on endothelial proliferation (18,43). Finally, the effects of UFH and LMWH on hMVEC proliferation were similar, whereas their effects on capillary-like tube formation paralleled their effects on the fibrin structure. Thus heparins may influence angiogenesis differentially, by their alternate effect on the fibrin structure in the fibrinous stroma of a tumor.

These data provide a novel mechanism by which LMWH may affect tumor progression, namely reduced ingrowth of microvascular structures in a fibrinous stroma matrix by rendering it less permissive for invasion.

In conclusion, our data indicate that heparins not only affect the proliferation of endothelial cells, but also affect angiogenesis by altering the structural and mechanical properties of the fibrin network. Whereas the structural alterations of the fibrin matrix by UFH enhanced the invasion of the matrix by capillary-forming endothelial cells, LMWH reduced it. These findings may contribute to the elucidation of the mechanisms by which heparins may affect cancer progression differentially.
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CHAPTER 6 EFFECTS OF HEPARINS ON ANGIOGENESIS


