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Unfractionated and Low Molecular Weight Heparin Affect Fibrin Structure and Angiogenesis in Vitro

Annemie Collen, Susanne M. Smorenburg, Erna Peters, Florea Lupu, Pieter Koolwijk, Cornelis Van Noorden, and Victor W. M. van Hinsbergh

Gaubius Laboratory TNO-PG, 2333 CK Leiden, the Netherlands [A. C., E. P., P. K., V. W. M. v. H.]; Departments of Cell Biology and Histology [S. M. S., C. V. N.] and Vascular Medicine [S. M. S.], Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, the Netherlands; Vascular Biology, Thrombosis Research Institute, SW3 6LR London, United Kingdom [F. L.]; Department of Physiology, Institute for Cardiovascular Research, Vrije Universiteit, 1081 BT Amsterdam, the Netherlands [V. W. M. v. H.]

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). Because 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 bFGF-induced proliferation of human microvascular endothelial cells (hMVECs) to the same extent (36–60%). VEGF165-induced proliferation was inhibited to a lesser extent (19–33%). 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, whereas the presence of UFH resulted in a more opaque more porous network with thick fibrin fibers. We used a human in vitro angiogenesis model, which consisted of hMVECs seeded on top of a fibrin matrix, and stimulated the cells with basic fibroblast growth factor plus tumor necrosis factor α 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 compared with a control matrix for both 1.5 and 10 units/ml LMWH), whereas matrices polymerized in the presence of UFH facilitated tubular structure formation (72 and 36% stimulation compared with a control matrix for 1.5 and 10 units/ml UFH, respectively). Similar results were obtained for cells stimulated with vascular endothelial growth factor plus tumor necrosis factor α. These data demonstrate the inhibitory effect of heparins on proliferation of hMVECs 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 UFH3 has been the standard initial treatment, but recent randomized clinical trials have shown that s.c. fixed doses of 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 3-month survival compared with 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 Ref. 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 MVECs, 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 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). To date, 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 MVECs with the matrix that 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 neo-vascularization (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 alter its sensitivity to 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 what extent LMWH or UFH affects 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 MVECs (hMVECs). Both compounds reduce proliferation of hMVECs to a rather similar degree. However, the presence of LMWH during the polymerization of fibrin decreases the formation of tubular endothelial structures, whereas 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 Earle’s Balanced Salt Solution, l-glutamine, and HEPES (both obtained from BioWhittaker (Verviers, Belgium). Trichloroacetic acid and trypsin 1–300 (370 USP/mg) were obtained from ICN (Costa Mesa, CA), and heat-inactivated newborn calf serum was from Life Technol-
cies (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 was prepared from bovine brain (30). Thrombin and UFH were obtained from Leo Pharmaceutical Products (Weesp, the Netherlands), and tissue culture plastics were from Costar (Cambridge, MA). Human fibrinogen (batch X 0379-51) containing 3.2 μg of plasminogen and 5 μg of plasmin per g of fibrinogen was purchased from Chromogenix AB (Mölndal, Sweden), and the LMWH, Reviparin, was from Knoll (Ludwigshaven, Germany). VEGF165 was a kind gift from Dr. H. Weich, (GBF, Braunschweig, Germany), and TNFα, containing 2.45 × 10^7 units/mg of protein and <40 ng of lipo polysaccharide per mg of protein, was from Dr. J. Tavernier (Biogent, Gent, Belgium). Recombinant human bFGF was purchased from PeproTech (Rocky Hill, NJ), and [3 H]thymidine was from Amersham (Buckinghamshire, United Kingdom).

Cell Culture. HUVECs (31) and human foreskin hMVECs were isolated, cultured, and characterized as described previously (32, 33). Cells were cultured until confluence in a 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 μg/ml endothelial cell growth factor, 100 IU/ml penicillin, and 100 μg/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 passages 9–11 for hMVECs and at passage 2 for HUVECs.

Proliferation Assay. Incorporation of [3 H]thymidine in DNA was determined as described previously (18).

Preparation of Fibrin Matrices. Human fibrin matrices were prepared by the addition of 0.1 units/ml thrombin to 300 μl of 3 mg/ml fibrinogen dialyzed against PBS [140 mM NaCl, 13.4 mM Na₂HPO₄·2H₂O, 138 mM NaH₂PO₄·2H₂O (pH 7.4)] in a 1-cm² well of a 48-well plate. 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 h 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 h and once for 24 h with 0.5 ml of M199 containing 10% human serum and 10% newborn calf serum.

Fig. 1. Incorporation of [3 H]thymidine in DNA of hMVECs (A and B) or HUVECs (C and D). The inhibition of growth factor-induced proliferation was studied by the addition of 0.5 or 10 units/ml UFH or 0.5 or 10 units/ml LMWH. Data are the means of three independent experiments for bFGF (A and C) and for VEGF165 (B and D); bars, SE. * P < 0.01; ** P < 0.05.

Fig. 2. Effect of polymerization conditions on the structure of the formed fibrin matrix: A and B, prior to polymerization, the pH of the buffer was altered between pH 7.0 and 7.8 (A), or 1.5 or 10 units/ml UFH and 1.5 or 10 units/ml LMWH were added (B). After 4 h of polymerization, the structure of the fibrin was determined by measurement of the turbidity at 340 nm (A₅₄₀). Data are expressed as the means of three independent experiments; bars, SE. 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).
The influence of heparins on the structure of the fibrin matrix was studied by the addition of 1.5 or 10 units/ml UFH or 1.5 or 10 units/ml LMWH prior to the polymerization. After 4 h of polymerization, the matrices were washed with culture medium.

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-l-lysine, by the addition of 1 unit/ml thrombin in the presence or absence of 10 units/ml LMWH or UFH. After repeated washing with water, the specimen was dried and stained with 2% phosphotungstic acid for 1 min, and the fibrin network formed was analyzed in a Philips 201 electron microscope.

**RESULTS**

**Effect of Heparin on Proliferation of hMVECs and HUVECs.** Proliferation was measured as the incorporation of [3H]thymidine in hMVECs 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 units/ml of UFH or 0.5 units/ml of LMWH) to bFGF-stimulated cells resulted in a significant decrease of [3H]thymidine incorporation (58 ± 3% and 36 ± 3% inhibition, respectively, compared with control). The addition of higher concentrations of both heparin preparations resulted in similar or even further inhibition (60 ± 9% and 50 ± 10% inhibition, respectively; Fig. 1A). In addition, VEGF$_{165}$-induced proliferation was inhibited by the addition of UFH and LMWH, although to a lesser extent (33 ± 3% for 0.5 units/ml UFH and 0.5 units/ml LMWH, and to 28 ± 3% and 19 ± 3% for 10 units/ml UFH and LMWH, respectively; Fig. 1B).

Interestingly, this effect of heparin was specific for hMVECs because UFH or LMWH did not significantly affect the proliferation of HUVECs induced by bFGF (8 ± 2% and 5 ± 8% for 0.5 units/ml UFH and LMWH, respectively, and 15 ± 5% for 10 units/ml; Fig. 1C) or VEGF$_{165}$ (2 ± 4% and 9 ± 7% for 0.5 units/ml UFH and LMWH, respectively, and 3 ± 5% and 4 ± 10% for 10 units/ml; Fig. 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 and more porous network, whereas those polymerized in a more basic environment (pH 7.8) had a low absorbency, indicative of a transparent, dense, and rigid network (Fig. 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 (Fig. 2B).

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

**Formation of Capillary-like Tubular Structures in Various Fibrin Matrices.** hMVECs 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 Fig. 3, A and B). The overall length of tubular structures was considerably higher in more porous fibrin matrices produced at pH 7.0 than in fibrin matrices produced at pH 7.8 in both bFGF/TNFα- and VEGF$_{165}$/TNFα-stimulated cells (Fig. 4).

The presence of UFH and LMWH during polymerization altered the fibrin matrices and also affected the extent of tube formation by hMVECs (Fig. 3). The total lengths of the tubes formed after stimulation with bFGF/TNFα of the cells grown on fibrin matrices polymerized in the presence 1.5 units/ml and 10 units/ml UFH were increased by 72 ± 10% and 36 ± 8%, respectively, compared with
control matrices (Fig. 3 and Table 1). Similar results were obtained if cells were stimulated with VEGF<sub>165</sub>/TNF<sub>a</sub> (Table 1). However, the presence of similar amounts of LMWH during matrix polymerization caused a decrease in the length of tube formation compared with control for bFGF/TNF<sub>a</sub>-stimulated cells (Fig. 3 and Table 1) and for VEGF<sub>165</sub>/TNF<sub>a</sub>-stimulated cells (Table 1).

When UFH or LMWH was added after polymerization of the fibrin and seeding of the hMVECs, no significant effects on tube formation were observed for bFGF/TNF<sub>a</sub>-stimulated cells (81 ± 12% and 85 ± 10% of control for 1.5 units/ml UFH, respectively, and 100 ± 11% and 97 ± 8% for 1.5 units/ml and 10 units/ml LMWH, respectively; n = 7; control was 294 ± 44 mm/cm²). This indicates that the effects of UFH and LMWH were mainly the result of 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/TNF<sub>a</sub>-stimulated cells (Table 2).

**Table 2 Accumulation of u-PA and PAI-1 antigen in conditioned media of tube-forming hMVECs on different fibrin matrices**

<table>
<thead>
<tr>
<th>Addition</th>
<th>PAI-1 antigen (ng/10&lt;sup&gt;5&lt;/sup&gt; cells/6 days)</th>
<th>u-PA antigen (ng/10&lt;sup&gt;5&lt;/sup&gt; cells/6 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>678 ± 50</td>
<td>21 ± 0.4</td>
</tr>
<tr>
<td>1.5 U/ml UFH</td>
<td>694 ± 57</td>
<td>24 ± 0.1</td>
</tr>
<tr>
<td>10 U/ml UFH</td>
<td>685 ± 34</td>
<td>23 ± 0.3</td>
</tr>
<tr>
<td>1.5 U/ml LMWH</td>
<td>711 ± 20</td>
<td>23 ± 0.1</td>
</tr>
<tr>
<td>10 U/ml LMWH</td>
<td>682 ± 20</td>
<td>23 ± 0.4</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The present study reveals two mechanisms by which UFH and LMWH affect angiogenesis in vitro. UFH and LMWH inhibit the proliferation of hMVECs induced by the angiogenic factors bFGF and VEGF<sub>165</sub> 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, whereas UFH contributes to the formation of a more porous 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 hMVECs, whereas it was nonsignificant in HUVECs. 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, the 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, including fibrinogen, extravasate. The subsequently formed fibrinous exudate is a major component of the initial provisional scaffolding for invasive cells, thereby contributing to tumor growth and neovascularization. In vitro angiogenesis studies have 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, 41). In addition, the structure of fibrin itself plays an important role in endothelial cell invasion (25, 26), and as is shown here, heparins affect fibrin matrix formation. Differences in the fibrin network structure alter its sensitivity toward plasmin-dependent proteolysis (42, 29) and the array of epotopes involved in endothelial cell-matrix interaction during angiogenesis (43, 44). Heparins were present during the polymerization of the fibrin matrix, and because heparin is known to bind to fibrinogen (45, 46) as well as fibrin (47), it might still be present in the matrix. In our in vitro model,
however, the formation of capillary-like tubular structures was not affected by the addition of heparins after polymerization of the matrices. 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, 41). 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 different effects 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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