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Unfractionated and Low Molecular Weight Heparin Affect Fibrin Structure and Angiogenesis *in Vitro*[1]

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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). 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 the extent (36–60%). $VEGF_{165}$-induced proliferation was inhibited to a 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 UFH[3] 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 thromboembolisms 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 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 alter its sensitivity toward 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 were 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-
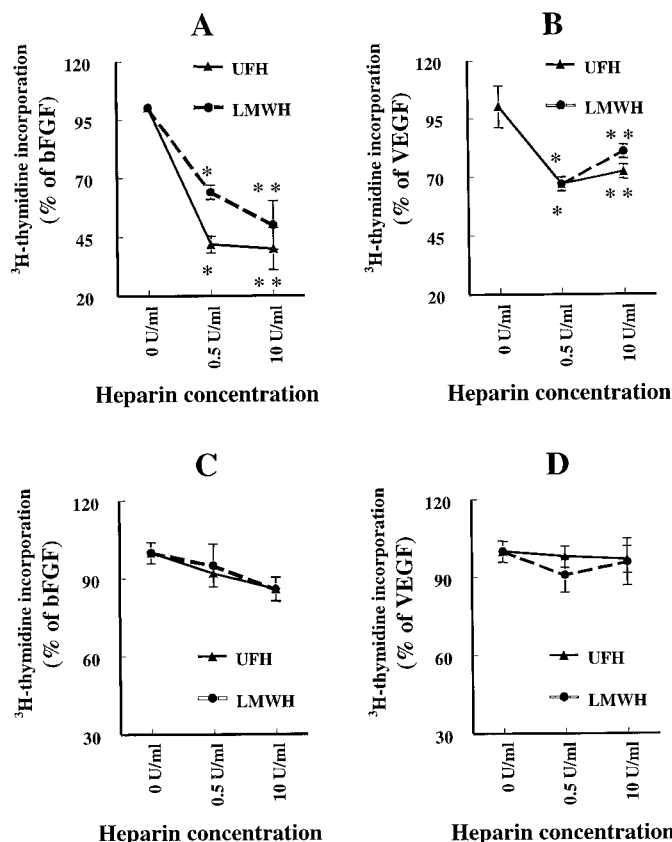
Fig. 1. Incorporation of [³H]thymidine in DNA of hMVECs (*A* and *B*) or HUVECs (*C* and *D*). The inhibition of growth factor-induced proliferation was studied by the addition of 0.5 or 10 units/ml UFH or 0.5 or 10 units/ml LMWH. Data are the means of three independent experiments for bFGF (*A* and *C*) and for VEGF$_{165}$ (*B* and *D*); *bars*, SE. *, $P < 0.01$; **, $P < 0.05$.

ogies (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). VEGF$_{165}$ was a kind gift from Dr. H. Weich, (GBF, Braunsweig, Germany), and TNFα, containing 2.45×10^7 units/mg of protein and <40 ng of lipopolysaccharide per mg of protein, was from Dr. J. Tavernier (Biogent, Gent, Belgium). Recombinant human bFGF was purchased from PeproTech (Rocky Hill, NJ), and [³H]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% CO$_2$-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 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 passages 9–11 for hMVECs and at passage 2 for HUVECs.

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

Preparation of Fibrin Matrices. Human fibrin matrices were prepared by the addition of 0.1 unit/ml thrombin to 300 µl of 3 mg/ml fibrinogen dialyzed against PBS [140 mM NaCl, 13.4 mM Na$_2$HPO$_4$·2H$_2$O, 138 mM NaH$_2$PO$_4$·2H$_2$O (pH 7.4)] in a 1-cm^2 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. 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 (A_{340}). 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*).
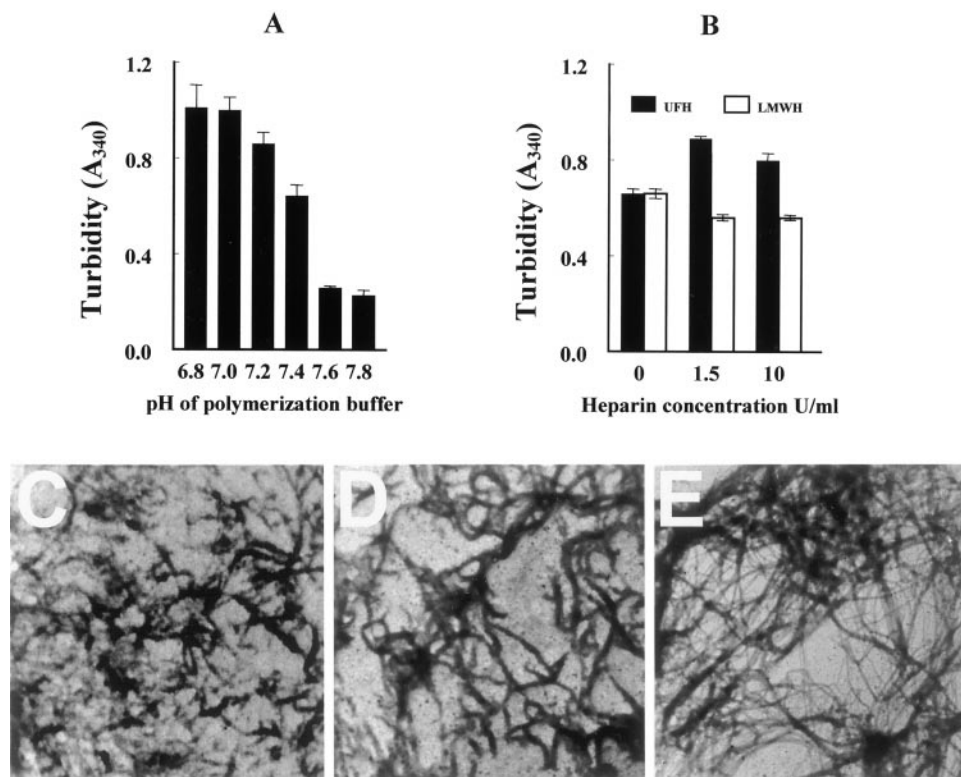
Fig. 3. Effect of fibrin structure on the formation of capillary-like tubular structures. Stimulation of endothelial cells with 5 ng/ml bFGF and 1 ng/ml TNFα for 6 days induced the cells to invade the fibrin matrix and form capillary-like tubular structures. *A* and *C–F*, phase-contrast photomicrographs of tubular structures (*A*, *arrowheads*) formed by hMVECs cultured on fibrin matrices formed in the presence of 1.5 or 10 units/ml UFH (*C* and *D*, respectively), 1.5 or 10 units/ml of LMWH (*E* and *F*, respectively) 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 6 days was quantified and expressed as percentage of the values obtained after stimulation with bFGF/TNFα on a control fibrin matrix (no heparin added). Data are expressed as mean of six different experiments performed with duplicate wells; *bars*, SE. ∗, $P < 0.01$. The total tube length in control matrices (100%) was 229 ± 12 mm/cm^2.
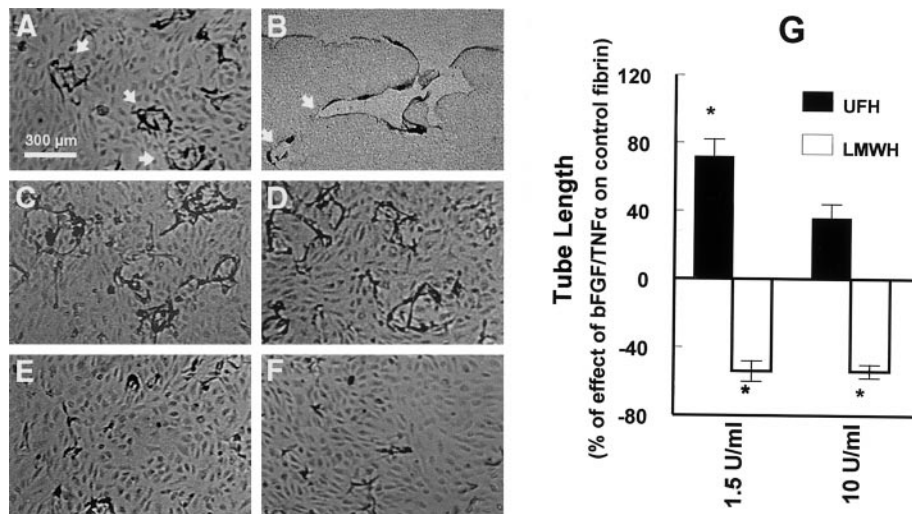
The influence of heparins on the structure of the fibrin matrix was studied by the addition of 1.5 or 10 units/ml UFH or 1.5 or 10 units/ml LMWH prior to the polymerization. After 4 h of polymerization, the matrices were washed with culture medium.

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.

***In Vitro* Angiogenesis Model.** Confluent endothelial cells were detached with trypsin-EDTA, suspended in medium, and seeded in a confluent density on the fibrin matrices. After 24 h, the medium was replaced with medium containing different mediators. Every 48 h, the medium was changed and collected, for a time period of 6 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; Ref. 18).

Antigen Measurement in Conditioned Medium. u-PA antigen was measured as described previously (18). As tagging antibodies, a mixture of two monoclonal antibodies, UK 2.1 and UK 26.15, which recognize different epitopes of the u-PA antigen, was used. Horseradish peroxidase-conjugated monoclonal anti-u-PA IgG (LMW 11.1) was used as a capping antibody, and u-PA (Ukidan; Serono, Aubonne, Switzerland) as standard.

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 manufacture (Biopool, Umea, Sweden).

Statistics. Data were expressed as mean ± SE. Statistical significance of differences between groups was analyzed by one-way ANOVA followed by Bonferroni's modified *t* test. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of Heparin on Proliferation of hMVECs and HUVECs. Proliferation was measured as the incorporation of [^{3}H]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 [^{3}H]thymidine incorporation ($58 \pm 3\%$ and $36 \pm 3\%$ inhibition, respectively, compared with control). The addi-

tion of higher concentrations of both heparin preparations resulted in similar or even further inhibition ($60 \pm 9\%$ and $50 \pm 10\%$ inhibition, respectively; Fig. 1*A*). In addition, VEGF$_{165}$-induced proliferation was inhibited by the addition of UFH and LMWH, although to a lesser extent ($33 \pm 3\%$ for 0.5 units/ml UFH and 0.5 units/ml LMWH, and to $28 \pm 3\%$ and $19 \pm 3\%$ for 10 units/ml UFH and LMWH, respectively; Fig. 1*B*).

Interestingly, this effect of heparin was specific for hMVECs because UFH or LMWH did not significantly affect the proliferation of HUVECs induced by bFGF ($8 \pm 2\%$ and $5 \pm 8\%$ for 0.5 units/ml UFH and LMWH, respectively, and $15 \pm 5\%$ for 10 units/ml; Fig. 1*C*) or VEGF$_{165}$ ($2 \pm 4\%$ and $9 \pm 7\%$ for 0.5 units/ml UFH and LMWH, respectively, and $3 \pm 5\%$ and $4 \pm 10\%$ for 10 units/ml, respectively; Fig. 1*D*).

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. 2*A*).

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. 2*B*).

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 $72 \pm 10\%$ and $36 \pm 8\%$, respectively, compared with

control matrices (Fig. 3 and 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 formation compared with control for bFGF/TNFα-stimulated cells (Fig. 3 and Table 1) and for $VEGF_{165}$/TNFα-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/TNFα-stimulated cells ($81 \pm 12\%$ and $85 \pm 10\%$ of control for 1.5 units/ml and 10 units/ml UFH, respectively, and $100 \pm 11\%$ and $97 \pm 8\%$ 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

Table 2 *Accumulation of u-PA and PAI-1 antigen in conditioned media of tube-forming hMVECs 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 2 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 6 days. Data are the means $\pm$ SE of three different experiments performed with duplicate wells.

Addition	PAI-1 antigen (ng/10⁵ cells/6 days)	u-PA antigen (ng/10⁵ cells/6 days)
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

in all conditions was similar, as shown for bFGF/TNFα-stimulated cells (Table 2).

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 $VEGF_{165}$ 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, 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, including fibrinogen, extravasate. The subsequently formed fibrinous exudate is 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 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 epitopes 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,
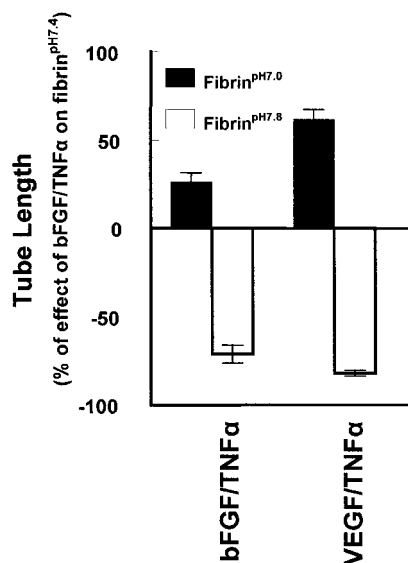

Fig. 4. Effect of fibrin structure on capillary tube formation. Fibrin matrices were polymerized at pH 7.0, pH 7.4, and pH 7.8. hMVECs on top of these fibrin matrices were stimulated with 5 ng/ml bFGF and 1 ng/ml TNFα (*bFGF/TNFα*) or 40 ng/ml VEGF and 1 ng/ml TNFα (*VEGF/TNFα*) to form invasive capillary-like tubular structures. The total lengths of tubular structures were quantified and expressed as percentages of the values obtained after stimulation with bFGF/TNFα or VEGF/TNFα on a control fibrin matrix (pH 7.4). Data are expressed as means of five different experiments performed with duplicate wells for bFGF/TNFα and three experiments in duplicate for VEGF/TNFα; *bars*, SE. The total tube length in control matrices (100%) was 229 ± 12 mm/cm² for bFGF/TNFα and 77 ± 2 mm/cm² for VEGF/TNFα.

Table 1 *Effect of UFH and LMWH on fibrin gel structure as measured by the length of endothelial tubular structures formed in the presence of a growth factor (bFGF or VEGF) and TNFα*

Fibrin matrices were polymerized in the presence of UFH (1.5 and 10 units/ml) or LMWH (1.5 and 10 units/ml), or without addition (control matrices). hMVECs on top of these fibrin matrices were 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 percentage 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 means $\pm$ SE of five different experiments performed with duplicate wells. 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 units/ml LMWH represents 1 aXa/ml LMWH.

Addition	bFGF/TNFα	VEGF/TNFα
None	100 ± 1	100 ± 2
1.5 U/ml UFH	172 ± 10[a]	177 ± 5[a]
10 U/ml UFH	136 ± 8	138 ± 4[a]
1.5 U/ml LMWH	46 ± 6	45 ± 1[a]
10 U/ml LMWH	46 ± 4	43 ± 4[a]

[a] $P < 0.01$.

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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.

ACKNOWLEDGMENTS

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