Heparin Inhibition of Endothelial Cell Proliferation and Organization Is Dependent on Molecular Weight

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Objective—Studies have shown improved survival in cancer patients treated with low molecular weight heparins (LMWHs). Tumors depend on an expanding vasculature, and heparins may affect vessel growth and function. We investigated the effect of heparins differing in Mn on selected endothelial cell properties.

Methods and Results—Human umbilical vein endothelial cells were cultured with fibroblast growth factor-2 and heparins differing in Mn. Cell proliferation was assessed by [3H]thymidine incorporation, and vascular organization was assessed by in vitro assays. Maximum inhibition of 94±2% was observed with 6-kDa LMWH, greater than the inhibition seen with unfractionated heparin (58±8%) or 3-kDa LMWH (60±9%, P=0.02 for both). No inhibition of proliferation was observed with heparin tetrasaccharide, octasaccharide, or pentasaccharide (fondaparinux). Three- and 6-kDa fractions decreased endothelial tube formation in Matrigel to 58±15% and 67±9% (P<0.05), respectively, of that with fibroblast growth factor-2, whereas no inhibition was observed with unfractionated heparin, tetrasaccharide, pentasaccharide, or octasaccharide. LMWH (6 kDa) also inhibited vessel formation in a placental explant.

Conclusions—Heparin inhibition of endothelial cell proliferation and organization requires a chain length of >8 saccharide units, with maximal inhibition at Mn of 6 kDa. This Mn dependence differs from that required for anticoagulant activity. (Arterioscler Thromb Vasc Biol. 2003;23:2110-2115.)

Key Words: low molecular weight heparins ■ cancer ■ angiogenesis ■ endothelium ■ thrombosis

Unfractionated heparin is a heterogeneous mixture of polysaccharide molecules with a mean Mn between 12 and 15 kDa containing glycosaminoglycan chains from 200 to 300 saccharide units.1 Low molecular weight heparins (LMWHs) are fragments of unfractionated heparin produced by controlled enzymatic or chemical depolymerization with lower mean Mn between 3 and 6 kDa and chain lengths of 12 to 18 saccharide units.2 These structural differences result in important functional and pharmacokinetic differences, inasmuch as LMWHs have an increased anti-Xa/anti-IIa activity ratio, reduced plasma protein binding, decreased interaction with platelets and platelet factor 4, a prolonged half-life, and increased bioavailability after subcutaneous administration.2,3 These changes give LMWHs an improved therapeutic profile compared with unfractionated heparin and have led to their increased use in the treatment of venous and arterial thrombosis.

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Individual studies4,5 and meta-analyses6,7 have found significant improvements in 3- and 6-month mortality in patients with cancer who were treated with LMWHs compared with unfractionated heparin. This survival advantage was found in subgroups differing in age, sex, and primary site of malignancy and could not be attributed to differences in thrombosis or bleeding complications.8 The mechanism of this effect remains unknown but appears to be independent of the anticoagulant action of LMWH and may be related to effects on the vasculature. Prior studies have shown the effects of heparin on angiogenesis in rat mesentery9 and cornea10 models and have suggested a differential effect of fractions varying in Mn, without defining a relationship between Mn and specific vascular cell functions.

Tumor growth is dependent on an expanding vascular supply, and the development of metastasis involves the transvascular passage of malignant cells and new blood vessel development at the site of new tumor formation. These are complex processes requiring endothelial and progenitor cell recruitment, proliferation, migration, and organization into new vessels.11 Glycosaminoglycans play a role in endothelial cell function, inasmuch as heparin and heparan sulfate can both modulate the activities of angiogenic growth factors, including fibroblast growth factor (FGF)-2,12 by facilitating the interaction of FGF-2 with its receptor and promoting receptor activation.13,14 Of note, specific structural variations in heparin and heparan sulfate can result in differential effects on FGF-2 signaling and downstream effects.15,16

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We hypothesized that the effect of heparins on endothelial cells is dependent on $M_r$ and have evaluated this by using in vitro models of endothelial cell function, including proliferation and organization, with the use of unfractonated heparin, heparin fractions varying widely in $M_r$, and commercial LMWHs. The results demonstrate a difference between the $M_r$ dependence of the effects of heparin on endothelial cells and on coagulation. Maximum inhibition of endothelial cell proliferation and organization occurred at $\approx 6$ kDa $M_r$, with less inhibition observed at both higher and lower $M_r$ fractions.

Methods

Cell Culture

Primary endothelial cells were obtained as described previously, seeded on 0.2% (wt/vol) gelatin-coated 25-cm$^2$ tissue culture flasks, and cultured in McCoy's 5A medium (Flow Laboratories) containing 20% FBS and 50 $\mu$g/mL endothelial cell growth supplement (ECGS) (Collaborative Research, Inc) until they reached confluence, within 2 or 3 days. The cells were passaged up to 2 times before use and then placed in suspension by trypsinization of monolayers. Cells were suspended by rinsing in Hanks' balanced salt solution, followed by brief incubation with trypsin-EDTA (GIBCO Life Technologies, Inc). The cells were pelleted by centrifugation for 10 minutes at 2000 rpm and resuspended in McCoy's 5A medium in the absence of serum. This wash procedure was repeated twice before use in experimental protocols.

$[^3]$HThymidine Incorporation

Approximately $2 \times 10^6$ endothelial cells suspended in McCoy's 5A medium supplemented with 20% FBS and 50 $\mu$g/mL ECGS were plated in gelatin-coated 12-well, non–tissue culture-treated plates (Becton Dickinson & Co) and allowed to adhere for 6 hours. The medium was then removed, and the cells were washed twice with serum-free McCoy's 5A medium. Serum-free medium containing 1% Nutridoma (Boehringer-Mannheim Corp), 25 ng/mL human recombinant FGF-2 (R&D Systems, Inc), or 25 ng/mL vascular endothelial growth factor (VEGF165, R&D Systems, Inc) and 1 $\mu$g/mL $[^3]$Hthymidine (New England Nuclear) was then added. To this medium was added 10 $\mu$g/mL of various heparins. Tetrasaccharide at 1.2 kDa and octasaccharide at 2.4 kDa (Neoparin Inc) were prepared from unfractionated heparin by controlled deaminative cleavage and subsequent purification. LMWH at 3 kDa, LMWH at 6 kDa, and unfractionated heparin (Sigma) were manufactured from porcine intestinal mucosa. Tinzaparin (DuPont Pharma), enoxaparin (Aventis Pharmaceuticals), dalteparin (Pharmacia and Upjohn Co), and fondaparinux (Sanofi-Synthelabo) were obtained as pharmaceutical products. After incubation at 37°C for 24, 48, or 72 hours, nonadherent cells were removed by washing twice with ice-cold PBS. To each well, 500 $\mu$L of 10% ice-cold trichloroacetic acid was added, and precipitates were collected on a filter by using a filtration manifold. Filters were washed twice with ice-cold 5% trichloroacetic acid, followed by 95% ethanol. They were allowed to air-dry and were then suspended in scintillation fluid. Acid-precipitable counts were quantified by using a scintillation counter.

In Vitro Models of Vascular Development

Matrigel Model

Matrigel (BD Biosciences) was thawed on ice at 4°C, and 24-well plates were coated with 300 $\mu$L Matrigel per well for 15 minutes at 25°C. Confluent human umbilical vein endothelial cells (HUVECs) were detached with trypsin-EDTA as described above and suspended in McCoy's 5A medium containing 20% FBS, and 192 $\mu$L of the suspension was plated into each well. Heparins were added at a total volume of 8 $\mu$L, followed by incubation in 5% CO$_2$/95% air. After 16 hours, medium was aspirated from each well, and 200 $\mu$L Diff-Quik fixative (Dade Behring) was added and incubated for 30 seconds. The fixative was then aspirated, and the cells were stained for 2 minutes with Diff-Quik solution II (Dade Behring) diluted 1:1 with water. Tube structures were observed by using an inverted microscope, photographed, and quantified as (1) the average total length of tube structures per low-power field (LPF) and (2) the number of tube structures per LPF with the use of Image-Pro Plus 3.0 software (Media Cybernetics).

Placental Explant Model

A placental explant model was prepared as previously described. Briefly, superficial vessels were excised from the apical surface of a placental explant within 24 hours of elective cesarean births. The vessels were cut into 1- to 2-mm fragments with the use of fine dissecting forceps and placed into Hanks' balanced salt solution containing 2.5 $\mu$g/mL fungizone (GIBCO Life Technologies). Fifteen microliters of thrombin was added to each well of a 48-well culture plate, followed by 0.5 mL of 3 mg/mL fibrogen per well (Enzyme Research) in medium 199 (GIBCO Life Technologies). One vessel fragment was placed quickly in the center of the well before clot formation. After gel formation, 0.5 mL medium 199 per well supplemented with 20% FBS (Gemini Bioproducts), 1-glutamine, gentamicin (GIBCO Life Technologies), and fungizone was added. Vessels were cultured at 37°C in an incubator for 18 days, and the medium was changed every 2 days. New vessel growth was observed microscopically and was quantified by using Image-Pro Plus 3.0 software every second day.

Anticoagulant Activity

Anticoagulant activity of various heparins was determined by using a heparin anti-Xa chromogenic assay. The anticoagulant activity of heparins declined progressively with size from 176 anti-Xa units per milligram for unfractionated heparin, to 85 anti-Xa units per milligram for 6-kDa LMWH, 29 anti-Xa units per milligram for octasaccharide, and 2 anti-Xa units per milligram for tetrasaccharide.

Statistical Analysis

Results are expressed as mean±SE. Statistical significance of differences in means between groups was determined by using a 2-tailed Student $t$ test or ANOVA. A value of $P<0.05$ was considered significant for our analysis. All experiments were repeated at least twice, in duplicate or triplicate.

Results

The effect of heparins on endothelial cell proliferation was evaluated as $[^3]$Hthymidine incorporation at 48 hours, selected on the basis of previous experience indicating that this was an optimal time to observe inhibitory effects. Compared with medium alone, FGF-2 increased cell proliferation by 60±14% ($P=0.02$), as expected (Figure 1). The addition of unfractionated heparin, 3-kDa or 6-kDa fractions, inhibited FGF-2–dependent proliferation as observed microscopically, although the cells appeared morphologically normal otherwise. As quantified by $[^3]$Hthymidine incorporation, the 6-kDa fraction inhibited proliferation by 94±2%, significantly greater than that with unfractionated heparin (58±8%) or with the 3-kDa fraction (60±9%, $P=0.02$ for both). The addition of heparin fractions of 1.2 kDa (tetrasaccharide), 2.4 kDa (octasaccharide), and 1.7 kDa (pentasaccharide [fondaparinux]) appeared to have no inhibitory effects microscopically. This was confirmed by using $[^3]$Hthymidine uptake, which increased by 61±10% ($P=0.04$) with octasaccharide, 8±15% ($P=0.7$) with pentasaccharide, and 38±9% ($P=0.14$) with tetrasaccharide, compared with culture with FGF-2 alone. Similar results were observed when HUVECs were stimulated with the use of a different angiogenic growth
For 6-kDa LMWH, progressive inhibitory activity was seen at higher concentrations, up to 5 μg/mL EGS and allowed to adhere for 6 hours. The cells were then washed twice with McCoy’s medium and incubated for 48 hours in serum-free medium containing 1% Nutridoma, 1 μCi/mL of [3H]thymidine with 25 ng/mL FGF-2, and 10 μg/mL of the following heparins: unfractionated heparin (UFH), 6-kDa fraction, 3-kDa fraction, 2.4-kDa octasaccharide, 1.7-kDa pentasaccharide (fondaparinux), and 1.2-kDa tetrasaccharide. Isotope incorporated into DNA was precipitated with trichloroacetic acid, collected by vacuum filtration, and measured by scintillation counting. For each condition, data are shown normalized to cells incubated with FGF-2 with no heparin.

We further characterized the time dependence of inhibition with the 3-kDa and 6-kDa fractions, which showed the greatest activity (please see online Figure I, available at http://atvb.ahajournals.org). The addition of FGF-2 increased growth over medium alone at each time point from 935 cpm at 24 hours to 27,280 ± 5874 cpm at 72 hours. FGF-2–stimulated proliferation was inhibited by the 3-kDa and 6-kDa heparin fractions at each time point, with inhibition by the 6-kDa fraction of 84 ± 4%, 90 ± 3%, and 93 ± 3% at 24, 48, and 72 hours, respectively (P<0.05 for each). The cells remained viable at 72 hours, with a normal microscopic appearance in the presence of the heparin fractions. Inhibition with the 3-kDa fraction was less but significant, with reduction of [3H]thymidine incorporation by 75 ± 2%, 81 ± 1%, and 78 ± 3% compared with FGF-2 at 24, 48, and 72 hours, respectively (P<0.05 for each).

The concentration dependence of FGF-2–stimulated HUVEC proliferation was characterized at concentrations of various heparin fractions from 0.1 to 50 μg/mL (Figure 2). For 6-kDa LMWH, progressive inhibitory activity was seen at higher concentrations, up to 5 μg/mL (P<0.05 for each). Maximum inhibition of proliferation was achieved with 6-kDa LMWH concentrations at 5 μg/mL without an increase in inhibition at higher concentrations. Inhibition was also observed with 3-kDa LWMH and unfractionated heparin, although the degree of inhibition was less than that observed with 6-kDa LMWH. Heparin fractions of 1.2 kDa (tetrasaccharide), 2.4 kDa (octasaccharide), and 1.7 kDa (pentasaccharide) were not inhibitory at any of the concentrations tested (please see online Figure II, available at http://atvb.ahajournals.org).

The effect of heparins on endothelial cell function was also evaluated by culturing the cells in Matrigel, leading to the formation of capillary-like tube structures (Figure 3A through 3F). In the presence of medium alone, there was little cell organization, whereas a complex network developed in the presence of FGF-2. The inclusion of unfractionated heparin in the medium appeared to have no effect on tube length. In contrast, only minimal tube formation was observed visually in the presence of LMWH fractions, and the complex networks observed in the presence of FGF-2 or unfractionated heparin were not observed in any area (Figure 3A through 3F). When analyzed quantitatively, unfractionated heparin had no significant effect on tube formation (Figure 3G). However, compared with FGF-2 alone, both the 6-kDa and 3-kDa LMWH fractions had similar inhibitory activity, decreasing tube length by 67 ± 9% and 58 ± 15%, respectively (P<0.05 for each). Inhibition was also observed with all 3 commercial LMWHs: enoxaparin, 46 ± 3%; tinzaparin, 58 ± 22%; and dalteparin, 68 ± 13% (P<0.05 versus FGF-2 for each). Tetrasaccharides, pentasaccharides, and octasaccharides had no significant inhibitory effects, as observed with cell proliferation. Similar results were obtained when organization was quantified as the average number of endothelial tubes observed per LPF (please see online Figure III, available at http://atvb.ahajournals.org). In the presence of 6-kDa LMWH, 65 ± 11% inhibition was observed, and in the presence of 3-kDa LMWH, 64 ± 10% inhibition was observed (P<0.05 versus FGF-2 for each). Similar amounts of inhibition were seen with the 3 commercial LMWHs: enoxaparin, 52 ± 10%; dalteparin, 61 ± 8%; and tinzaparin, 50 ± 21%
In the presence of the tetrasaccharide, pentasaccharide, octasaccharide, and unfractionated heparin, no significant inhibitory effects were seen. Similar results were observed when the cells were stimulated by using a different angiogenic growth factor, VEGF165. LMWH at 6 kDa inhibited VEGF165-stimulated tube formation by 60% (P<0.05), whereas no inhibition was observed with unfractionated heparin.

The inhibitory effect of 6-kDa LMWH was confirmed by culturing vessel fragments obtained from human placentas in a fibrin clot over 18 days (Figure 4). In the absence of 6-kDa LMWH, initiation of new vessel growth was observed as early as day 2. New vessel formation continued to occur thereafter, reaching a peak on day 8 and persisting up to day 18. In wells containing 6-kDa LMWH, new vessel growth initiation was observed at day 6. Although this initial growth was not significantly different from that observed with medium alone, growth subsequent to day 6 was considerably inhibited. After day 14, nearly 100% inhibition was seen in the presence of 6-kDa LMWH, whereas new vessels continued to be observed in wells without 6-kDa LMWH.

**Discussion**

The vascular adaptations required for tumor growth and metastasis are complex. We have chosen to evaluate the specific endothelial cell properties of proliferation and organization into tubular structures. The findings presented demonstrate that heparins have significant effects on these activities that are dependent on M_r. Unfractionated heparin inhibited endothelial cell proliferation but had no effect on endothelial tube formation, whereas heparin fractions with a mean M_r of 3 and 6 kDa had greater inhibitory effects on proliferation and also reduced tube formation, with maximum inhibition observed in the presence of the 6-kDa fraction. No inhibitory effects on either proliferation or tube formation were observed with fractions <3 kDa. The anticoagulant
activity of heparin was also related to size, but the $M_c$ dependence of the endothelial and anticoagulant activities differed (Figure 5).

Heparin and LMWHs are the most widely used parenteral anticoagulants, and they accelerate the rate of $\text{Xa}$ and thrombin inhibition through binding to antithrombin.$^2,^{19}$ The anticoagulant properties of heparins are related to size and chain length, as reflected by the increased anti-Xa/anti-IIa activity of LMWHs, resulting from loss during depolymerization of the larger molecules, which are needed for anti-IIa activity.$^2$

Other functional properties that also depend on chain length include half-life, bioavailability after subcutaneous administration, protein binding, release of tissue factor pathway inhibitor, and interactions with platelet factor 4.$^4,^{20,21}$ The mean $M_c$ value of LMWHs in clinical use ranges from 4 to 5.5 kDa, close to that of the 6-kDa fraction, which we found to exert maximum endothelial inhibitory effects.

Previous reports have evaluated the effects of heparins in models of angiogenesis but have not defined specific antiproliferative or antiangiogenic effects on $M_c$ or chain length. Folkman et al$^{22}$ demonstrated angiogenesis inhibition and tumor regression with unfractionated heparin or a hexasaccharide heparin fragment, but they observed this only in the presence of cortisone. Norrby and Ostergaard$^8$ showed that FGF-2–mediated angiogenesis in a rat mesentery model was more effectively suppressed by a 2.6-kDa fraction compared with 4 higher $M_c$ fractions varying in charge density and anticoagulant activity.$^9$ More recently, Collen et al$^{23}$ evaluated the effect of unfractionated heparin and LMWHs on vessel development in vitro in a fibrin matrix. They demonstrated similar inhibition of endothelial cell proliferation by both unfractionated heparin and LMWH and observed that the formation of tubular structures was inhibited by LMWH but stimulated by unfractionated heparin. They related these effects to alterations in the fibrin matrix structure caused by heparins. However, such effects on fibrin cannot explain the differential effects observed in our experiments in the absence of fibrin.

FGF-2 interacts with both specific tyrosine kinase receptors and with heparan sulfate proteoglycans (HSPGs) as small as disaccharides and trisaccharides,$^{24}$ which function as coreceptors, promoting receptor binding and activation.$^{13}$ This is consistent with our findings and explains the potentiation of FGF-2–mediated proliferation and endothelial cell organization observed with heparin tetrasaccharides and octasaccharides. Interestingly, Ornitz et al$^{24}$ observed that small and large heparin fractions could both induce FGF receptor dimerization; however, at higher concentrations, the amount of dimerization remained elevated with the disaccharide or trisaccharide but approached basal levels with hexadecasaccharide. The authors suggested that the larger hexadecasaccharide sterically inhibits receptor dimerization at high ratios of heparins to FGF. This is consistent with the differential effects of tetrasaccharide and octasaccharide compared with the 3- and 6-kDa fractions observed in the present study.

In addition to chain length, specific structural modifications also play an important role in HSPG function. The formation of a ternary complex of FGF-2, heparin, and FGF receptor 1 can be prevented by certain structural changes, such as selective 6-O-desulfation.$^{16,25}$ Similarly, specific saccharide sequences within HSPGs play critical roles, influencing which FGF receptor isomorph is activated and also acting as positive or negative regulators of FGF signaling.$^{15,26}$ Liu et al$^{27}$ have demonstrated that tumor cell surface heparan sulfate contains both cryptic promoters and inhibitors of growth and metastasis that can be differentially released by specific heparanases, and these influence the activity of FGF-2. Similarly, Zhang et al$^{28}$ have recently shown that membrane-associated HSPGs were effective promoters of FGF-2–initiated FGF receptor 1 phosphorylation, whereas equal amounts of soluble trypsinated HSPG were not. However, the soluble HSPG could be converted into a potent activator of FGF receptor signaling on further treatment with heparitinase.$^{28}$ The relation of specific structural modifications in the processing of heparin fractions to inhibitory activity against endothelial cell properties is not known.

The antiproliferative effect on endothelial cells may be relevant in the biology of malignancy and also important therapeutically. Two recent meta-analyses evaluating randomized controlled trials of LMWHs versus unfractionated heparin have found a significant risk reduction in mortality rates in the LMWH arm.$^6,^7$ Because the cytotoxic effects of LMWHs have not been reported, the effects on endothelial cells and vascularity may explain slower tumor growth. Prospective randomized trials are currently under way to evaluate effects on survival. Our results demonstrate critical and variable effects of the heparin chain length on coagulation and endothelial cell proliferation that must be considered in interpreting the results, inasmuch as a variety of antithrombotic agents are now available for use.$^{29,30}$

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References

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