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

Alok A. Khorana, Abha Sahni, Owen D. Altland, Charles W. Francis

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 $M_r$ on selected endothelial cell properties.

Methods and Results—Human umbilical vein endothelial cells were cultured with fibroblast growth factor-2 and heparins differing in $M_r$. Cell proliferation was assessed by $[^{3}H]$thymidine incorporation, and vascular organization was assessed by in vitro assays. Maximum inhibition of $94 \pm 2\%$ was observed with 6-kDa LMWH, greater than the inhibition seen with unfractionated heparin ($58 \pm 8\%$) or 3-kDa LMWH ($60 \pm 9\%, P=0.02$ 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 $58 \pm 15\%$ and $67 \pm 9\%$ ($P<0.05$, 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 $M_r$ of 6 kDa. This $M_r$ 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 $M_r$ between 12 and 15 kDa containing glycosaminoglycan chains from 200 to 300 saccharide units. Low molecular weight heparins (LMWHs) are fragments of unfractionated heparin produced by controlled enzymatic or chemical depolymerization with lower mean $M_r$ between 3 and 6 kDa and chain lengths of 12 to 18 saccharide units. 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. 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 studies$^{4,5}$ and meta-analyses$^{6,7}$ 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 mesentery$^9$ and cornea$^{10}$ models and have suggested a differential effect of fractions varying in $M_r$ without defining a relationship between $M_r$ 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.$^{15,16}$

Received July 16, 2003; revision accepted July 31, 2003.
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© 2003 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol is available at http://www.atvbaha.org DOI: 10.1161/23
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We hypothesized that the effect of heparins on endothelial cells is dependent on $M$ and have evaluated this by using in vitro models of endothelial cell function, including proliferation and organization, with the use of unfractionated heparin, heparin fractions varying widely in $M$, and commercial LMWHs. The results demonstrate a difference between the $M$ dependence of the effects of heparin on endothelial cells and on coagulation. Maximum inhibition of endothelial cell proliferation and organization occurred at $\approx 6\text{-kDa } M$, with less inhibition observed at both higher and lower $M$ 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 μg/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.

$[^3H]$Thymidine Incorporation
Approximately 2×10$^5$ endothelial cells suspended in McCoy’s 5A medium supplemented with 20% FBS and 50 μg/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.

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 $[^3H]$thymidine 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 $[^3H]$thymidine 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) appeared to have no inhibitory effects microscopically. This was confirmed by using $[^3H]$thymidine uptake, which increased by 61±10% ($P=0.04$) with pentasaccharide, 8±15% ($P=0.7$) with octasaccharide, 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

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for 6-kDa LMWH, progressive inhibitory activity was seen at higher concentrations, up to 5 μg/mL. For 6-kDa LMWH, inhibition was also observed with 6-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 (tetrosaccharide), 2.4 kDa (pentasaccharide), and 1.7 kDa (octasaccharide) 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). Tetrosaccharides, 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±9% (P=0.04), 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.

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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 Mr. Unfractionated heparin inhibited endothelial cell proliferation but had no effect on endothelial tube formation, whereas heparin fractions with a mean Mr 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 Xa and thrombin inhibition through binding to antithrombin.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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 Östergaard\textsuperscript{9} showed that FGF-2-mediated angiogenesis in a rat mesentery model was more effectively suppressed by a 2.6-kDa fraction compared with higher $M_c$ fractions varying in charge density and anticoagulant activity.\textsuperscript{9} More recently, Collen et al\textsuperscript{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,\textsuperscript{24} which function as coreceptors, promoting receptor binding and activation.\textsuperscript{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\textsuperscript{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.\textsuperscript{16,25} Similarly, specific saccharide sequences within HSPGs play critical roles, influencing which FGF receptor isof orm is activated and also acting as positive or negative regulators of FGF signaling.\textsuperscript{15,26} Liu et al\textsuperscript{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\textsuperscript{28} have recently shown that membrane-associated HSPGs were effective promoters of FGF-2–initiated FGF receptor 1 phosphorylation, whereas equal amounts of soluble trypsinized HSPG were not. However, the soluble HSPG could be converted into a potent activator of FGF receptor signaling on further treatment with heparitinase.\textsuperscript{23} 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.\textsuperscript{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.\textsuperscript{29,30}

Acknowledgments

This work was supported by a grant (HL-30616) from the National Heart, Lung, and Blood Institute, Bethesda, Md. Dr Khorana was supported by the James P. Wilmot Cancer Research Fellowship.

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Arterioscler Thromb Vasc Biol. 2003;23:2110-2115; originally published online August 14, 2003;
doi: 10.1161/01.ATV.0000090671.56682.D7

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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