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Review Article

On Low-Molecular-Weight Heparin and Angiogenesis

Klas Norrby*

Department of Pathology, University of Gothenburg, Sweden

Abstract

Unfractionated heparin (UFH), made from very high-molecular mast cell proteoglycans, has the highest negative charge density of any known biological molecule. It binds to proteins, including many growth factors, facilitating the interaction between growth factors and their receptors. The degradation of native heparin includes continuous heparinase depolymerization. Low-molecular-weight heparins (LMWHs) used as anticoagulants are manufactured from UFH by diverse methods. Tinzaparin is the only LMWH that is produced enzymatically, by heparinase-depolymerization of UFH.

Data show that Tinzaparin (6.5 kDa) injected s.c. significantly suppresses angiogenesis mediated by VEGF, a key regulator of physiological and pathological angiogenesis. Notably, a 5.0 kDa fraction of Tinzaparin exercises an even more potent angiogenesis-suppressive effect, while UFH (c. 15 kDa) tends to stimulate and 22 kDa heparins significantly stimulate VEGF-mediated angiogenesis, demonstrating fragment-mass-specific effects. The anti-angiogenic effect of the 5 kDa Tinzaparin fraction is shown also in angiogenesis mediated by basic fibroblast growth factor or endotoxin. There is a strong correlation between heparin fragment-mass and angiogenesis-modulating effect ($r = 0.97$ in the examined range of 2.6, 8, 15 and 22 kDa). Contrarily to Tinzaparin, the LMWH Dalteparin (6 kDa), produced by chemical depolymerization of UFH, stimulates VEGF-mediated angiogenesis demonstrating a non-equivalent angiogenesis-modulating effect among LMWHs.

A novel hypothesis suggests that there is an *in vivo* '*intrinsic heparin-depolymerization angiogenesis-modulating process*' due to continual heparinase depolymerization of angiogenic high-molecular weight heparins, including those in UFH, into anti-angiogenic low-molecular-weight heparins. Further depolymerization would produce inactive heparin species, as judged by the data.

***Corresponding author**

Klas Norrby, Department of Pathology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, Email: klas.norrby@pathology.gu.se

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ABBREVIATIONS

VEGF: Vascular Endothelial Growth Factor; bFGF: basic Fibroblast Growth Factor; *mmw*: Mean Molecular Weight; kDa: kilo Dalton

INTRODUCTION

Unfractionated heparin (UFH) is a heterogeneous mixture of sulfated glycosaminoglycans [1] that binds to antithrombin, leading to inactivation of clotting factors. Heparin, displaying the highest negative charge density among known biological molecules, binds readily via electrostatic interactions to a wide range of molecules and cells including the large group of heparin-binding proteins, causing rather unpredictable pharmacokinetic and pharmacodynamic properties [2]. Some of the most potent angiogenic and anti-angiogenic proteins are heparin-binding. Low-molecular-weight heparin (LMWH) medications have been shown to be safe and effective anticoagulants, particularly in the prevention of venous thrombosis in high-risk patients for the treatment of venous thrombosis. The advantages of LMWHs compared to UFH as anticoagulants include lower risk of bleeding, osteoporosis and thrombocytopenia and they can be used subcutaneously (s.c.) rather than intravenously (i.v.).

Unfractionated Heparin (UFH)

Mast cells are the main repository of heparin in the body. Mast cell granules contain heparin proteoglycans and heparin is the medicinal counterpart of heparin proteoglycans. Rat mast cells release soluble heparin proteoglycans of very high molecular weight (~750 000 to ~ 950 000 kDa), which *in vivo* are degraded by heparinases and heparanases present in many cells and tissues [3]. Native heparin, largely made up of disaccharides, is cleaved not into short oligosaccharides. In this process, native heparin chains (60-100 kDa) are degraded to fragments the approximate size range of UFH [4].

Currently, UFH in the USA and Europe is prepared from porcine mucosa, and in other parts of the world bovine mucosal heparin is in use. There are strong similarities in heparin structures across the entire animal kingdom. Native heparin is a polymer with a molecular weight ranging from c. 3 kDa to 40 kDa, and the mean molecular weight (*mmw*) of most commercial UFH preparations is in the range of 12 kDa to 15 kDa. UFH, like native heparin, is a mixture of disaccharide chains consisting of a variably sulfated repeating disaccharide unit; not more than 20% material by weight is over 24 kDa, and about 10% is less than 8 kDa.

Differences in manufacturing processes of UFH have only a minor influence over molecular weight, so for the most part; UFHs retain the molecular weight it had on extraction from the porcine mucosa.

Low-Molecular-Weight Heparin (LMWH)

Most of the commercial LMWHs are a diverse group of depolymerized heparin preparations with *mmw* ranging from about 3 kDa to 9 kDa, depending on the manufacturing process. The Tinzaparin drug is unique among LMWHs as it is produced enzymatically by heparinase digestion of UFH. All other LMWHs are isolated from UFH by chemical means followed by gel filtration chromatography, differential precipitation with ethanol, and partial depolymerization.

The s.c. administration of LMWHs appears to be universally accepted as the anticoagulant treatment of choice for the post-surgical prophylaxis of deep vein thrombosis, pulmonary embolism, and in the treatment of myocardial infarction. However, the FDA indicates that the approved LMWH drugs should not be regarded as interchangeable.

Heparins as Anticoagulants in Patients with Malignant Tumors

UFH and LMWH are widely used for the treatment of patients with thrombo-embolic issues, which are particularly common in patients with cancer. When co-administered with chemotherapeutics, various LMWHs, as compared to co-treatment of chemotherapeutics with UFH or chemotherapeutics without heparin co-treatment, significantly increased patient survival in a number of studies, beyond the reduction of fatal thrombo-embolic events. The mechanisms behind this anti-tumor effect of LMWHs remain to be fully elucidated although anti-angiogenic effects may play a part [5].

Effects of Heparin beyond Anticoagulant Effects

As heparins enter the circulation, they bind to proteins, especially arginine amino acids, endothelial cells and other cells including blood cells and tumor cells. Therapeutic levels of heparin in the circulatory system trigger complex processes, causing the systemic release of heparin-binding proteins including angiogenic growth factors such as VEGF, which is a key regulator of physiological and pathological angiogenesis; VEGF-mediated angiogenesis is viewed as a proxy for tumor angiogenesis. Moreover, the angiogenic basic fibroblast growth factor, bFGF, and many other growth factors lodged on endothelial cells and in the extracellular matrix are released. Heparins also induce anti-oxidant activity that can significantly influence angiogenesis.

A Biologically Highly Relevant Angiogenesis Assay is Essential for Reliable Results

The rat mesentery angiogenesis assay, which was introduced by our group [6-9], exploits intact normal adult animals, is non-surgical and biologically distinctly relevant. An angiogenic agent is injected intraperitoneally (i.p.) and the animals are sacrificed and specimens are examined at appropriate times thereafter. The extremely thin small-intestine mesenteric test tissue is vascularized, albeit sparsely, and lacks significant physiological angiogenesis in adulthood. The assay allows quantification in detail of microvessels in spreads of the *intact* tissue.

Unintentional inflammation-induced angiogenesis is virtually absent (as judged by comparing untreated and vehicle-treated animals), which is a rare feature of *in vivo* angiogenesis models [8]. This guarantees a high degree of sensitivity since inflammation activates mast cells and induces angiogenesis. Toxic effects after treatment of any agent are readily controlled. The assay has proved well suited to drug testing and studies of molecular structure vs. activity, dose-response, and angiogenesis kinetics, which is exceptional for a mammalian system.

Using computer-aided morphometric microscopy it is possible to record any immunohisto-chemically identified blood vessels. The analysis yields objective, quantitative variables in terms of microvessel spatial extension, density and pattern formation, and regarding microvessel sprouting in terms of number, pattern formation and length of individual sprouts [10-12].

Clearly, the assay replicates the clinical situation, as angiogenesis-modulating test substances, such as heparins, are administered systemically (s.c., orally and i.v.), but not intraperitoneally to avoid any direct effect on target cells in the test tissue. The observed responses reflect the net effect of all molecular, cellular and metabolic alterations induced by the treatment.

The merits and limitations of the assay have been discussed and compared with all the other major *in vivo* angiogenesis models, in invited review papers [8,13]; the methodology is demonstrated in a DVD movie [9]. Arguably, the assay is capable of reflecting the clinical situation better than any other pre-clinical angiogenesis model described to date. Using this assay, mast-cell-mediated angiogenesis was originally shown [6,7]. Activated mast cells release a great number of bioactive molecules (including VEGF) that are able to initiate powerful angiogenesis [7], the majority probably via pathways activating VEGF, while one to date essentially unnoticed role of heparin would be to modulate angiogenesis by enhancing and decreasing influences, as discussed here.

Heparin systemically affects angiogenesis specifically, depending on its molecular mass and production method

Protamine, a highly cationic peptide rich in arginine, firmly binds to heparin via a stable ion pair, with capability of neutralizing the free heparin. Protamine sulfate suppresses mast-cell-mediated angiogenesis significantly when injected s.c. suggesting an angiogenic role for native heparin proteoglycans in rat mast cells [14].

VEGF-mediated angiogenesis

In collaboration with Novo Nordisk A/S, the then-manufacturer of the Tinzaparin drug (6.5 kDa *mmw*), which is derived by beta-eliminative cleavage by a naturally occurring heparinase of UFH, Norrby and co-workers studied the effect of s.c. injected Tinzaparin fractions with *mmw* of 2.5, 5.0 and 16.4 kDa on angiogenesis induced by VEGF. Treatment with the 5.0 kDa fraction suppresses angiogenesis significantly compared to

the effect of the 2.5 and 16.4 kDa fractions and the vehicle control [15-17].

As the overlaps between the molecular mass distributions of the three fractions tested were relatively small (polydispersity around 1.10), they essentially represent three different populations of heparin molecules. The peak molecular weight corresponds to the predominant number of sugar residues as follows: 2.5 kDa = 8 residues, 5.0 kDa = 16-18 residues and 16.4 kDa = c. 52 sugar residues. The doses of the heparins given were equal in terms of weight, but different in terms of the number of molecules and biologic activity. No significant effects were related to the degree of charge density and anticoagulant activity of the heparins. Moreover, in a subsequent study, the Tinzaparin drug significantly but to a slightly lesser degree than the 5.0 kDa fraction of Tinzaparin suppresses VEGF-mediated angiogenesis in statistical terms. Both the 2.5 kDa and 5.0 kDa fractions suppressed microvessel sprouting [21] but the impact of the 2.5 kDa fraction was not enough to suppress the over-all angiogenic response (recorded as vascularized area x microvessel density within this area, the mean of 3 randomly selected measuring field per specimen, the average of 4 mesenteric specimens per animal, and mean of c. 10 animals per treatment group at each observation time) in statistical terms [9].

Subsequently, the anti-angiogenic efficacy of Tinzaparin in VEGF-mediated angiogenesis has been substantiated *in vitro* in an endothelial cell tube formation assay and *in vivo* in the chick chorioallantoic membrane model [18].

The Dalteparin drug (6.0 kDa *mmw*), manufactured non-ezymatically, by nitrous acid degradation of UFH, injected s.c. significantly enhances VEGF-mediated angiogenesis, which is the opposite of the effect seen for the Tinzaparin drug with a comparable *mmw* and sulfate content [19]. Interestingly, Dalteparin is reported to promote wound healing in patients with peripheral arterial occlusive disease [20].

The s.c. injection of UFH (~15 kDa *mmw*) tends to enhance VEGF-mediated angiogenesis, while larger UFH-derived fragments (~22 kDa *mmw*) significantly stimulate VEGF-mediated angiogenesis in statistical terms [16].

Basic Fibroblast Growth Factor (bFGF)-mediated angiogenesis

The 2.5 kDa Tinzaparin fraction suppresses the angiogenesis significantly compared with the treatment with four c. 20 kDa fractions derived from UFH with varying degrees of polydispersity, charge density and anticoagulant activity [16].

Endotoxin-mediated angiogenesis

Many 'physiological' salt solutions used as vehicles contain traces of endotoxin, which, among other things, activates mast cells to secrete growth factors. The use of endotoxin-free saline vehicles enables uncompromised angiogenesis experiments to be performed, since endotoxin is angiogenic even at very low concentrations *in vivo,* as first reported by our group [22]. The response pattern of Tinzaparin-derived fractions on very-low-dose endotoxin-mediated angiogenesis is similar to that in VEGF-mediated angiogenesis, as the effects are closely related to the *mmw* of the various tinzaparin- and UFH-derived fractions that were injected s.c.; linear regression $r = 0.97$ in the examined wide range of 2.6, 8, 15 and 22 kDa *mmw* [23].

The heparins were in all experiments given s.c. at dosages which are approximately within the range used clinically.

Heparin Interacts with Many Other Drugs

This is a well-known fact. We have, for instance, found that low-dose metronomic Epirubicin cytotoxin monotherapy exercises no effect on VEGF-mediated angiogenesis while co-treatment with Epirubicin with Dalteparin, which is an anticoagulant and antioxidant with angiogenic properties, significantly inhibits angiogenesis, suggesting complex drug actions [19].

Hypothesis: Innate Heparin-Depolymerization, an Angiogenesis-Modulating Process In Vivo

The data on the systemic effect of Tinzaparin and fractions thereof on VEGF-mediated angiogenesis are significant as (i) VEGF is a key regulator of physiological and pathological angiogenesis, and (ii) Tinzaparin is produced by heparinase depolymerization of UFH, which is comparable to native heparin, and heparinases are present in many cells and tissues.

Following the release of angiogenic high-molecular-weight heparin proteoglycans by activated mast cells [14], which regularly occurs in numerous conditions, particularly in inflammation, wounding, wound healing, and tumors [7], one can surmise that continuous innate heparinase degradation of these heparins into anti-angiogenic Tinzaparin-like low-molecular-weight heparins takes place, which would suggest a novel '*intrinsic heparin-depolymerization angiogenesis-modulating process*'. Moreover, angiogenesis-suppressing low-molecular-weight heparins can obviously be depolymerized further into inactive species, as the data indicate. This proposed process may apply also to treatment with UFH since this medication contains disaccharide chains that enhance angiogenesis, supposedly those over 10-12 kDa in weight, as well as disaccharide chains that decrease angiogenesis, supposedly those less than 6-8 kDa in weight.

DISCUSSION & CONCLUSION

Low-molecular-weight heparins, as a rather poorly defined group of diverse LMWH drugs used in different clinical studies, prolong survival time in patients with malignant tumors when being co-treated with chemotherapeutics, compared to patients receiving chemotherapeutic in combination with UFH or chemotherapeutics without heparin co-treatment. This effect is beyond the reduction of fatal thrombo-embolic events. The mechanism behind this anti-tumor outcome is not clarified, but an angiogenesis-suppressing effect of LMWHs has been proposed, as tumors are angiogenesis-dependent. However, only Tinzaparin (6.5 kDa), which uniquely is enzymatically manufactured by heparinase depolymerization of UFH, has been show to significantly inhibit angiogenesis responses to diverse agents, such as VEGF, bFGF, and endotoxin, in an adult mammalian naively vascularized tissue lacking physiological angiogenesis. The very sensitive angiogenesis assay used in these studies is essentially non-inflammatory, which is an exceptional feature among in *in vivo* angiogenesis assays.

By examining fractions with different *mmw* of Tinzaparin and UFH, it is shown that there is a strong fragment-mass-specific effect on angiogenesis: $r = 0.97$ in the examined range of 2.6, 8, 15 and 22 kDa heparins. In other studies, the 5.0 kDa fraction of Tinzaparin significantly suppresses angiogenesis more than the 6.5 kDa Tinzaparin drug, which also significantly suppresses angiogenesis in statistic terms, while UFH stimulates and the 22 kDa fractions derived from UFH significantly stimulate VEGF-mediated angiogenesis following s.c. administration.

Interestingly, the LMWH drug Dalteparin (6.5 kDa), which is produced by chemical depolymerization of UFH, exerts the opposite effect of Tinzaparin as it significantly stimulates VEGF-mediated angiogenesis. In fact, Tinzaparin and Dalteparin, with similar *mmw* and sulfate content, differ with respect to their molecular, structural, physio-chemical, biophysical, and biological properties and are not clinically equivalent (FDA, EMA, WHO). Clearly, the angiogenesis-modulating effect among LMWHs is not equivalent.

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