Glycosaminoglycans: Anticoagulant and Nonanticoagulant Actions: A Short History of Symposia Held at Villa Vigoni

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Abstract

Heparin, a sulfated polysaccharide belonging to the family of glycosaminoglycans, was discovered in the beginning of the 20th century and was initially identified as a procoagulant isolated from liver tissue. After the first application in patients approximately 30 years later, further purification identified the major as well as minor, but important, component units of the complex chain mixtures constituting heparin and the multiplex actions became a scientific challenge recently. A series of “Glycosaminoglycan symposium—anticoagulant and nonanticoagulant actions” developed over the past 20 years and focused on this topic has published research data in three issues of Seminars in Thrombosis & Hemostasis and in several other international scientific journals.

Keywords
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► nonanticoagulant effects

Heparin was first described by Howell and McLean when they analyzed a thromboplastin substance from the brain and later from liver. Jay McLean was aware of several research results from Germany reporting on peptones from organs acting as thromboplastin.1 In 1915 and 1916, he purified in the laboratories of W. H. Howell something from the heparphospatide which contaminated the cephalins obtained also from other brain and heart tissues without telling Howell about the results.2 When Howell became aware of the data his student had published, he went on to analyze the contaminated cephalin and identified them as anticoagulants naming them antithrombin and heparin.3 Two batches of the purified compound were injected intravenously into dogs to demonstrate the anticoagulant heparin inhibition of blood coagulation.4

The founding Editor in Chief of Seminars in Thrombosis & Hemostasis, Eberhard Mammen, contributed to the pathophysiology of blood coagulation and the biological actions of glycosaminoglycans from the mid-20th century.5 In 1962, he described the benefit of heparin in many indications such as hemodialysis patients.6 During the period of discovery of low-molecular-weight heparin (LMWH), he investigated their pharmacodynamic effects and speculated about their reduced risk of heparin-induced thrombocytopenia.7 In a preface to a Glycosaminoglycan Symposium held at Villa Vigoni, he wrote:

The introduction of LMW heparins has opened new avenues for the management of patients with...
In 2002, Eberhard Mammen (Fig. 1) participated in one of the series of the Glycosaminoglycan Symposia held in the Villa Vigoni at Menaggio situated at Lake Como in north Italy. The participants of the symposia published articles within Seminars in Thrombosis & Hemostasis subsequent to the 3rd, 10th, and 14th symposium, in volumes each appearing 1 year later (in 1994,9 2002,10 and 200711). In the preface of the volume of 2002, Mammen wrote that

...most widely known GAGs are heparins and LMWHs and they are best known for their anticoagulant properties. Although the clinical usefulness of heparins cannot be disputed, there are serious side effects associated with their use, more with UFH than with LMWH. These problems have spurred continuous efforts to develop modifications of UFH in order to make compounds safer without compromising the desired anticoagulant activities.8

The subsequent symposia continued with the presentation of research on the various problems of anticoagulation and are also presented during the 22nd edition of the symposium.

Structural Analysis of Glycosaminoglycans

Antithrombin is required to potentiate the anticoagulant action of heparin LMWH,12 fondaparinux,13 and other heparin-derived oligosaccharides on coagulation proteases.14 Structural aspects of heparins and derivatives are analyzed today using high performance size exclusion chromatography,15 capillary electrophoresis,16 antithrombin affinity or other matrices in connection with electrophoreses methods,17 nuclear magnetic resonance techniques,18 matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry19 and fast atom bombardment, electrospray ionization, and tandem mass spectrometry.20 These methods were designed to differentiate isomeric heparin disaccharides, to determine sulfation positions and uronic acid epimerization in oligosaccharides of chondroitin sulfate (another member of the GAG family), and to study the effect of the positions of sulfate groups on heparin binding.20

Anticoagulant Actions of Glycosaminoglycans

The effect of glycosaminoglycans on the blood coagulation system depends on its affinity to bind to antithrombin and results in inhibition of the activity of many serine proteases involved in this process. The initial coagulation cascade described by Ratnoff and Menzie21 included an intrinsic and an extrinsic pathway activation of the factor–tenase complex. The steps followed by conversion of prothrombin to thrombin to initiate clot formation by splitting fibrinopeptides A and B from the α and β chains of fibrinogen to build fibrin. Substantial modifications of this initial concept adapted the system of blood coagulation to include activation of both pathways through tissue factor (TF), activation of the fibrinolytic system by coagulation proteases factors XII and thrombin and inactivation of fibrinolysis by activating thrombin-activated fibrinolysis inhibitor, multiple functions of thrombin by feedback activation of factor VIII, inhibition of factors V and VIII through binding to endothelially located thrombomodulin via the proteins C and S pathway, activation of factor XIII to activate glutaminase for stabilization of fibrin strains, binding to and activating protein-activating receptors on cell surfaces of platelets and leukocytes.22
Methods to Determine Anticoagulant Effects of Glycosaminoglycans

Overwhelming literature is available on the determination of the anticoagulant effects of various glycosaminoglycans, including heparins, LMWH, fondaparinux and heparin-derived oligosaccharides as well as other polysulfated polysaccharides. Many methods for the determination of heparins were presented at the Glycosaminoglycan symposia and some of them are commercially available today. The prothrombinase-induced clotting time revealed a higher sensitivity over prothrombin time and activated partial thromboplastin time assays for determination of heparins, LMWH, and fondaparinux. This assay was also independent of anticoagulant effects of a concomitant therapy with vitamin K antagonists. A specific assay was described for fondaparinux because heparanase does not degrade the pentasaccharide sequence constituting the antithrombin-binding region in contrast to other sequences in heparin and LMWH. The concentration of heparin can be detected by a polycationic ruthenium compound which quantifies heparin by monitoring 630 nm fluorescence. The compound is an example of a nonpolymeric low-molecular-weight agent which neutralizes the anticoagulant activity of heparin and LMWH in plasma samples. The fluorescent perylenediimide probes modified with 2, 4, 6, or 8 ammonium groups were synthesized and their binding to the antithrombotic drug heparin was studied by fluorescence spectroscopy in solution. The polyanionic polysaccharide strands of heparin bind more probe molecules per sugar unit when the charge of the latter is low, and stability of the probe-heparin complex increases with increasing probe charge. The red-fluorescent perylenediimide probe allowed sensitive quantification of heparin and LMWH in plasma and serum samples with minimal interference from matrix components. These are some of the new methods to determine heparins and were presented at the symposia at Villa Vigoni.

Treatment of Venous Thromboembolism with Glycosaminoglycans: Special Indications

Venous thromboembolism (VTE) is one of the relevant social burdens occurring as a complication of various underlying diseases leading to the postthrombotic syndrome if occurring in the extremities or to death in case of pulmonary embolism. The treatment of thromboembolic diseases is one of the main therapeutic areas in medicine, because up to 50% of patients with acute deep vein thrombosis will die without anticoagulant treatment within a few days. Reports on the prevention of postoperative VTE date back to the end of the 1930s. Crafoord reported the first prophylactic uses of repeated intravenous injections of heparin in postoperative medicine and in perinatal gynecology. Bauer, as reported by McLean, described the efficacy of heparin injection by reducing the incidence of mortality from 1 to 4% in 16,495 patients between 1939 and 1945 compared with 18% without heparin observed between 1929 and 1938 at the Mariestad hospital in Sweden. In these series, a reduction of fatal pulmonary embolism was found from 47 of approximately 25,000 cases to 3 of approximately 16,000 cases.

According to more recent results, heparin reduced the mortality rate to less than 5%. Unfractionated heparin (UFH) became the drug of choice in the mid-20th century for this indication. Because of the necessity of laboratory monitoring by the activated partial thromboplastin time, which had to be prolonged two to three times of the normal value LMWH have been developed.

Anticoagulation with heparin, LMWH, or fondaparinux is required to prevent VTE or to treat the acute event. The first report of the efficacy of heparin for postoperative prevention of VTE and for reduction of mortality were reported in the 1930s. LMWH showed an improved efficacy and reduced bleeding complications for prevention of VTE in postoperative care compared with UFH, in the prevention of myocardial infarction during an acute coronary syndrome, and for the treatment of acute VTE compared with UFH. In the initial treatment of VTE using LMWH once or twice daily subcutaneously, there did not appear any significant differences on the outcomes of recurrent VTE, major bleeding, or mortality. VTE is one of the major complications in patients suffering from cancer. A meta-analysis showed a reduction of VTE by 45% in cancer patients under treatment with LMWH and as a downside increased rates of bleeding complications (~30%) compared with placebo. However, mortality rates were not reduced by LMWH compared with no anticoagulant treatment. Recently, effective anticoagulant treatments of thrombotic occlusion at unusual sites of retinal and jugular veins, right heart thrombosis including thrombosis of coronary sinus and thrombosis of the azygos system, umbilical, renal, ovarian, spermatic, and iliac veins were reported.

Modifications and Synthesis of Glycosaminoglycans

Glycosaminoglycans have been modified because of the lack of oral absorption, adverse effects, and for investigations of structure function relationships with protein-binding. Some of the modifications took advantage of the end standing anhydromannose group of LMWH heparins by synthesizing lipophilic residues with intact anticoagulant activity and prolonged elimination half-life after administration into rats. Despite the demonstration of the feasibility of this approach for improvement of the oral or transdermal administration, this concept of lipophilic modification of heparins did not reach a clinical stage of development. Fluorescence isothiocyanate labeled LMWH showed unaltered anticoagulant actions in vitro and after intravenous injection into rats. Binding to leukocytes may influence the pharmacology of LMWH also. This was demonstrated by the binding of this fluorescent labeled LMWH to granulocytes, monocytes, and lymphocytes in vitro and ex vivo after administration to rats and by a similar elimination half-life from these cells as compared with plasma.

Seeberger and coworkers reported on the synthesis of different glycan structures, which were printed on
Nonanticoagulant Actions of Glycosaminoglycans

The nonanticoagulant action of glycosaminoglycans plays a major role in their biological activity. A series of symposia at Villa Vigoni began in 1991 and covering many of these topics. The participants of the 21st symposium published several articles within the last year on the analysis, synthesis, and action of glycosaminoglycans.

Reduction of heparin in the presence of periodate oxidation/borohydride led to glycol-split heparins lacking anticoagulant and anti-inflammatory effects to be used for anticancer and anti-inflammatory therapies.\(^\text{44}\) The nonanticoagulant action of glycol-split heparin inhibited hepcidin expression in hepatic HepG2 cells and primary hepatocytes and in mice suppressing liver hepcidin expression, reducing spleen iron, and reducing serum hepcidin levels.\(^\text{45}\) This was reproduced in an inflammation model with pretreatment with lipopolysaccharides followed by 1-week treatments with glycol-split heparin. In a model of inflammatory anemia, glycol-split heparin increased iron mobilization and reduced anemia. These data indicate that high levels of heparin in anemia and autoimmune intestinal bowel diseases may be treated with these nonanticoagulant heparins.\(^\text{46}\)

A modified sulfated nonanticoagulant heparin devoid of antithrombin (AT) binding and devoid of inhibition of systemic AT-dependent coagulation factors, and the LMWH tinzaparin both potently reduced adhesion and invasion of pancreatic cancer cells to the endothelial layer of umbilical cord vein in a dose-dependent manner. The sulfated heparin inhibited P-selectin mediated tumor cell adhesion, and inhibited cell adhesion and invasion similar to tinzaparin, indicating that systemic anticoagulation is not a necessary component for heparin attenuation of cancer cell adhesion, invasion, and metastasis.\(^\text{46}\) The integrin very late antigen (VLA-4) reduced metastasis production in a selectin-dependent manner. Nonanticoagulant heparin derivatives, enoxaparin, and tinzaparin effectively blocked VLA-4 cell binding, dominantly via the integrin's α-chain. Desulfation at 2-O-position, N-acetylation, or a size smaller than tetradeacascaride reduced VLA-4 inhibition. A derivative with 50% 6-O-desulfation was more effective than tinzaparin. This can distinguish anticoagulant and antiadhesive functions of heparin for antimetastatic therapies without the risk of bleeding complications.\(^\text{47}\)

In another tumor model, a murine mastocytoma cell line, which produce a highly sulfated heparin-like polysaccharide that lacks anticoagulant activity, transfection with a retroviral vector containing heparan sulfate 3-O-sulfotransferase-1 restored anticoagulant activity. These cell lines expressed N-acetylgalactosamine N-deacetylase/N-sulfotransferase-1, urosulfatase, and glucosaminyl 6-O-sulfotransferase-1, which was sufficient to make the highly sulfated heparin. Overexpression of this enzyme in mastocytoma cell lines resulted in a change in the composition of heparan sulfate/heparin and chondroitin sulfate/dermatan sulfate glycosaminoglycans. This model should provide a better understanding and a better control of the biosynthesis of heparin with different structures and activities.\(^\text{48}\) A series of polysulfated penta- and tetrascarharide glycosides were synthesized as heparan sulfate mimetics and evaluated for their ability to inhibit angiogenesis. The compounds bound tightly to fibroblast growth factors (FGF-1, FGF-2, and vascular endothelial growth factor [VEGF]) and strongly inhibited heparanase activity. The compounds also showed good anti-tumor activity in vivo in a mouse melanoma (solid tumor) model.\(^\text{49}\) Extracellular vesicle-mediated intercellular transfer of signaling proteins and nucleic acids are involved in cancer development. Heparan sulfate proteoglycans function as internalizing receptors of exosomes, which are colocalized with cell-surface localized syndecan and glypic type. Exosome uptake was specifically inhibited by free heparan sulfate chains, by using several cell mutants, providing genetic evidence of a receptor function of exosome uptake, which was dependent on intact heparan sulfate, specifically on the 2-O and N-sulfation groups. Intact and not genetically modified heparan sulfate proteoglycans are key receptors for functional activity of exosomes in tumor growth.\(^\text{50}\)

Heparanase is an endo-β-D-glucuronidase that cleaves heparan sulfate side chains of heparan sulfate proteoglycans on cell surfaces and the extracellular matrices in tumor metastasis and angiogenesis. Heparanase exerts also enzymatic-independent functions by upregulating VEGF-A, VEGF-C, and activation of intracellular signaling of cell survival and proliferation. Heparanase may also affect the hemostatic system in a nonenzymatic manner by expression of the blood coagulation initiator-TF and interaction with the TF pathway inhibitor (TFPI) on the cell surface membrane of endothelial and tumor cells. This leads to dissociation of TFPI and resulting in increased cell surface coagulation activity. It was shown that heparanase-enhanced TF activity resulted in factor Xa production. Inhibition of heparanase may be a good target for cancer therapy.\(^\text{51}\)

The established role of heparanase in cancer and its newly discovered roles in diabetes, inflammation, and vascular diseases have elevated the importance of developing clinically effective antiheparanase therapies. Accomplishing this goal will require not only a deeper understanding of the heparanase mechanism of action in disease, but also resolution of heparanase structure and substrate specificity.\(^\text{52}\)

Heparan sulfate was identified as a natural regulator of the cleavage of the amyloid precursor protein by β-secretase. During cleavage of the amyloid precursor protein, amyloid β-peptide is formed which is responsible for the formation of amyloid plaques present in the brains of Alzheimer patients. The deposition of insoluble accumulations of the amyloid β-peptide in the brain is critical in the development of Alzheimer disease. Heparan sulfate and nonanticoagulant heparins effectively inhibited β-secretase and progression of the disease. New insights into structure–activity
relationships for optimal β-secretase inhibition were provided using a library of 12- to 16-mer oligosaccharides of heparan sulfate to improve treatment of Alzheimer disease.53

**Perspectives**

The analyses of the interactions of glycosaminoglycans with coagulation proteases as well as with nonanticoagulant proteins are currently improved by very specialized analytical methods. The synthesis of oligosaccharides of original and modified heparin-like products improves the understanding of specific interactions with proteins. The ultimate goal of these investigations is the development of defined glycosaminoglycans for treatment of nonthrombotic diseases.

**Conflict of Interest**

The authors do not have any conflicts of interest to declare.

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