Bioengineered heparin

Is there a future for this form of the successful therapeutic?

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Heparin is a widely used drug for the control of blood coagulation. The majority of heparin that is produced commercially is derived from animal sources, is poly-disperse in nature and therefore ill-defined in structure. This makes regulation of heparin challenging with respect to identifying its absolute structural identity, purity, and efficacy. This raises the question as to whether there might be alternative methods of producing commercial grade heparin. The commentary highlights ways that we might manufacture heparin using bioengineering approaches to yield a successful therapeutic replacement for animal-derived heparin in the future.

Introduction

Heparin is one of the oldest drugs that is still used in the clinic and that continues to save many lives each and every day by preventing unwanted thrombotic events that may have led to heart attack or stroke. It is one of the few carbohydrate based pharmaceuticals that is a naturally derived product and that is poly-disperse in nature and therefore ill-defined in structure.

Heparin was first isolated from canine liver and derived its name from the Greek terminology of "hepar," standing for liver, and its ability to inhibit the clotting of cat's blood was discovered serendipitously. The earliest measure of heparin activity was defined as the amount of heparin that caused 1 mL of cat blood to "half-clot" when left in the refrigerator for one night.[1]

From the earliest days of commercial scale isolation of heparin from animal tissues, the process has suffered from batch to batch variability, in potency of activity as well as issues of side-effects including headaches, nausea, and fevers. Today, heparin is predominantly sourced from porcine intestinal mucosa and bovine lungs as a by-product of the meat and livestock industry and is processed using very similar processes to those that have been in place for the last 20–30 years.

Recently, heparin has been reported to cause hypotension and approximately 100 deaths which when investigated was found to be caused by an uncontrolled anaphylactoid response.[2] This prompted a global recall of heparin sodium for injection by Baxter Healthcare in 2008 and the Food and Drug Administration identified that the contaminant was a synthetic form of over-sulfated chondroitin sulfate (OSCS) and that this contaminant was present at various levels in batches of heparin manufactured in China.[3] Some of these heparin batches were found to contain up to 30% w/w OSCS,[2] which has also shown to be a potent anti-coagulant mediated by an anti-thrombin III (ATIII)-independent mechanism.[2] As the majority of the heparin that is produced commercially is derived from animal sources, it makes the regulation of this type of drug very challenging with respect to issues surrounding its absolute structural identity, purity and efficacy, which raises the question as to whether there might be alternative methods of producing

commercial grade heparin for the anticoagulant market that does not suffer from these significant challenges.

What is the Biochemical Structure of Heparin?

Heparin derived from animal tissues is a polydisperse mixture of polysaccharide chains that have different molecular weights in the range of 5–40 kDa and different sulfation patterns along the chain with an average of 2.5 sulfate groups per disaccharide. The polysaccharide chains are composed of alternating and repeating co-polymers of a hexose-amine sugar, N-acetyl glucosamine (GlcNAc), and hexuronic acid with iduronic acid (IdoA) being the most predominant form. The basic biochemical structure of heparin is very similar to heparan sulfate (HS). In contrast to HS, heparin has a higher proportion of IdoA compared with glucuronic acid (GlcA) and greater amounts of sulfate groups modifying the polysaccharide chains with 75–95% of heparin composed of trisulfated disaccharides. Both heparin and HS together with chondroitin and/or dermatan sulfate CS/DS, keratan sulfate (KS), and hyaluronic acid (HA) belong to the glycosaminoglycan family of molecules and, with the exception of HA, are all found covalently attached to serine residues of the polypeptide chain of protein cores of proteoglycans. Heparin (and HS) are not synthesized as free glycosaminoglycan chains and as such do not have significant biological roles in the absence of the proteoglycans that they decorate.

Low molecular weight heparins (LMWH) were developed from an understanding of the biochemical mechanism of action of heparin in the coagulation cascade and the need to develop an anti-coagulant without the major hemorrhagic side effects associated with heparin. Fractionation of heparin based on size or chemical and/or enzymatic depolymerization has been used to make LMWH, which has a size less than 8 kDa. However the structure of the resultant LMWH is dependent on the structures present in the unfractionated heparin used as a starting material.

How is it Synthesized In Vivo?

With the exception of HA, glycosaminoglycans are covalently attached to proteoglycan protein cores and modified via the action of a large group of enzymes that reside in the Golgi and endoplasmic reticula of cells. Glycosaminoglycan chains are linked to the serine residue through a linkage tetrasaccharide structure consisting of GlcAβ1-3Galβ1-3Galβ1-4Xyl-ol-serine.[4] Heparin and HS chains are elongated via condensation polymerization reactions catalyzed by the EXT1/EXT2 complex of enzymes. Sulfate groups are added to the polysaccharide chain in a specific order with the sulfate groups added to GlcNAc via N-deacetylase/N-sulfotransferase (NDST) or to various positions on either the GlcNAc or hexuronic acids via specific sulfotransferases. The epimerization of GlcA residues to IdoA is performed via C5 epimerases, which are thought to provide flexibility to the relatively stiff polysaccharide chain. Further sulfate modification of the heparin/HS is performed either by GlcA or IdoA sulfotransferase enzymes. Arguably, the most important modification of heparin is performed by the 3-O-sulfotransferase family of enzymes, which are responsible for the addition of the sulfate group to the 3 position of the disulfated monosaccharide GlcNS(6S), which is important for the binding of ATIII and the overall anti-coagulant activity of heparin.[5] The ATIII binding site has been characterized and shown to have the following structure with the tri-sulfated glucosamine residue located centrally: GlcNAc(6S)β1-4GlcAβ1-4GlcNS(3,6S)β 1-4IdoA(2S)β1-4GlcNS(6S). With the sequencing of the human genome came the information that the number of sulfotransferases in humans compared with *C. elegans* and mouse has increased significantly throughout evolution suggesting the microstructure of heparin and other glycosaminoglycans have been very important in determining the human phenotype we see today, which is as a result of millions of years of evolution.[6] It is also tempting to speculate that if one of the major evolutionary advances that humans have over other species is a larger and more complex brain, then the increase in the complexity of heparin and HS microstructure by way of more intricate sulfation patterns has been instrumental in the increase of size and complexity of brain function in man.

Heparin polysaccharide chains have been shown to decorate the proteoglycan serglycin, which was named because of the relatively high proportion of serine and glycine amino acids arranged in serine-glycine repeats in its protein core. Serglycin can be decorated with as many as 8 glycosaminoglycan chains that can be CS/DS either in addition to, or instead of, heparin. It is not well understood what controls the switching between heparin and CS/DS chains on serglycin. Mast cells in culture decorate the protein core of serglycin with heparin and/or CS/DS chains with the extent of heparin decoration being variable. In the circulation, and when freshly isolated from the circulation, mast cells mostly synthesize CS/DS, whereas when the cells are co-cultured with fibroblasts[7] or seeded in collagen gels, they produce relatively more heparin suggesting that interactions with integrins on the cell surface and extracellular matrix may be important in this switch.

What is the Role of Heparin In Vivo?

Heparin is used therapeutically as an anticoagulant due to its ability to bind and promote the activity of the serine protease inhibitor, ATIII, which inhibits thrombin that normally cleaves fibrinogen to produce fibrin. ATIII also inhibits the activation of factor X, which once activated (Xa) has the ability to cleave pro-thrombin (factor II) to produce active thrombin (IIa). Therefore, heparin has a role and inhibits both the production of active thrombin and its ability to cleave of fibrinogen. Heparin has been shown to bind to other factors in the blood

including platelet factor 4, which causes a conformational change to the protein and in some cases resulting in antibodies being produced in the patient who was administered the heparin. This can have devastating effects in the patient due to the activation of platelets, which can cause uncontrolled thrombosis and lead to a drop in the number of platelets in the circulation; it is for this reason that these complex disorders are known as heparin-induced thrombocytopenia and thrombosis (HITT) and have over the years been successfully treated with LMWH. The more highly charged unfractionated forms of heparin have a greater potential of causing HITT whereas LMWH has a significantly lower potential, probably due to an increased relative affinity for the binding of ATIII to promote the inhibition of factor X compared with thrombin. As mentioned earlier, the ATIII binding site in unfractionated heparin contains a critical 3-O-S group in the middle of a pentasaccharide structure, which is the minimum size required for efficient binding and activation of ATIII. Oligosaccharides containing this pentasaccharide sequence have been isolated from unfractionated heparin via gel filtration and ATIII affinity chromatography. The specific ATIII pentasaccharide has been synthesized in vitro using solid-phase chemistry and has been marketed as a generic form of the drug Arixtra™.[8]

In order to investigate the importance of the ATIII binding sequence in vivo, the gene for the major isoform of the enzyme responsible for the addition of the 3 sulfate group to the glucosamine in the backbone of the HS chain has been knocked-out. The 3-O sulfotransferase enzyme has at least 7 isoforms in mammals, which is the largest number of sulfotransferase isoforms performing a single modification and which seem to be cell, tissue and context specific.[9] Mice lacking significant expression of the 3-O sulfotransferase 1 enzyme did not exhibit a hypercoagulative state indicating that the other sulfotransferases may be compensating for the lack of activity to generate ATIII binding sites. Interestingly, these animals exhibited vascular tone problems and may require

the 3 sulfated HS structures to control the movement of cytokines across the endothelial basement membrane.[10] Apart from this potential role, it has been shown that transient pulses of 3-O sulfotransferase activity occur in the murine developing salivary gland that seem to be timed with the expression and signaling of FGF receptors to control epithelial and mesenchymal cell differentiation.[11]

It is thought that the heparin structures expressed in mast cells are attached to the serglycin localized to the α-granules where it binds and controls the activities of the various proteases, which can be released in response to inflammatory signals[12] although mice that have reduced N-sulfation of heparin have also shown to be important for this activity.[13] Serglycin has also been shown to contain significant quantities of CS, which also seem to be active in binding and controlling the activity of proteases and it is not clear what controls the switch in these cells to cause them to increase the amount of heparin they produce to decorate the serglycin protein core.[14]

Can We Bioengineer Heparin?

While the majority of heparin on the market today is derived from animal tissues, there is currently one synthetic heparin, fondiparinux, which is a pentasaccharide that contains the minimum ATIII binding site in heparin and does not interact with platelets or platelet factor 4, thus avoiding the potential for HITT. Fondiparinux is chemically synthesized from $_D$-glucose and cellobiose in a process involving approximately 36 processing steps with an overall yield of 0.017%.[15]

Heparin mimetics have been explored as alternatives to heparin and are synthesized by sulfate modification of large molecular weight polysaccharides including starch, cellulose, chitin, chitosan, HA, and dextran. These sulfated polysaccharides exhibit anticoagulant properties, but the large molecular weight preparations were found to be toxic and produced bleeding in animal trials and also precipitated proteins from plasma affecting coagulation. Examples of

these types of molecules include sucrose octasulfate and a sulfated, phosphorylated carbohydrate compound that has been trialed in cancer patients, PI-88. These compounds may exhibit longer half-lives in vivo due to the absence of naturally occurring depolymerizing enzymes and it is still not known exactly how or when they are totally cleared from the body.

Bioengineered heparins may be a viable alternative source of heparin to the naturally sourced forms in the future. Unlike proteins, glycosaminoglycan synthesis does not rely on a DNA/RNA template and instead requires multiple Golgi and ER localized enzymes to be expressed by mammalian cells including those involved in glycosaminoglycan chain elongation, epimerisation of the GlcA moiety and modification by sulfate at multiple sites along the chain. Most cell types, and particularly those used for recombinant protein expression, do not express enzymes involved in heparin biosynthesis at a level that gives complete heparin chains, particularly the 3-O sulfotransferase enzyme. Linhardt and colleagues have metabolically engineered Chinese hamster ovary (CHO) cells to expressed the enzymes NDST2 and 3-O sulfotransferase isoform 1 (HS3st1) and while HS biosynthesis was dramatically increased, the level of anti-coagulant activity of the glycosaminoglycans produced was not as great as that of heparin isolated from natural sources, which may have also been due to incorrect targeting of the transfected enzymes.[16] More recently, when HS3st1 was localized in the Golgi on these same cells they produced the AT-binding motif, however not at the same density as observed in heparin.[17]

Another method that has been investigated to bioengineer heparin utilizes bacterial fermentation to synthesize heparosan, a bacterial capsular polysaccharide composed of repeating units of GlcA(1,4)GlcNAc, which can be subsequently sulfated by a multistep chemical and enzymatic approach using trimethylamine-sulfur trioxide complex and immobilized recombinant forms of the enzymes involved in HS/heparin biosynthesis.[18] This approach has produced milligram scale quantities

of anti-coagulant heparin containing trisulfated IdoA2S(1,4)GlcNS(6S). In a similar approach Lindahl and colleagues produced "neoheparin", from heparosan by utilizing C5 epimerase to convert GlcA to IdoUA followed by chemical per-O-sulfation and selective O-desulfation. This approach, although producing high yields on the gram scale of anti-coagulant and anti-thrombin-binding heparin, produces saccharides that do not occur naturally, including 3-O-sulfated GlcA.[19] Each of these bacterial approaches can be improved by reducing endotoxin levels.

These approaches to producing bioengineered heparin have gone some way to replicating the level of expression of enzymes involved in HS/heparin biosynthesis both in mammalian cells and in reaction vessels containing recombinantly expressed enzymes. As this significant research effort over the past 10 years has not been able to fully replicate HS/heparin biosynthesis by tissue-resident mast cells suggests that we still have much to learn about the location, timing and level of expression of enzymes involved in HS/heparin biosynthesis. Additionally, basic research focused on the mechanism of regulation of HS/heparin biosynthetic enzymes is needed to utilize the controlled chemical and enzymatic bioengineering approaches.

Given that the natural source of heparin is the proteoglycan, serglycin, produced by tissue-resident mast cells, an alternative strategy to bioengineer heparin in the future might be to harvest heparin from cultures of these cells under the correct environmental conditions. In a similar approach murine mastocytoma cells produce a highly sulfated form of HS that lacks anti-coagulant activity. These cells have been transfected with HS3st-1 and have recently been shown to produce an anti-coagulant form of HS/heparin.[20] Our laboratory has expressed serglycin in mammalian cells decorated with CS/DS and HS. This approach utilizes the natural protein template to control the type of glycosaminoglycans that decorate the protein core in mammalian cells replete with all the necessary HS/heparin biosynthetic enzymes. The challenge of this approach is to understand the mechanism of regulation of HS/heparin biosynthesis enzymes to ensure that the recombinant forms are decorated with heparin and not with CS/DS. Additionally, the challenge of this approach is to increase the yields beyond the milligram scale.

With the advent of both synthetic and systems biology approaches to produce and analyze the multitude of structures of bioactives produced by complex systems, we should be able to use these approaches to fine tune the production of bioengineered heparin. For example, the number of human proteins that could potentially interact with commercial batches of HS/heparin has been suggested to be well over 400 proteins[21] when at the same time we need to meet the challenge of producing a less heterogeneous population to increase yields of the required HS/heparin structures. This will be particularly useful if we can link this with the metabolic engineering approaches that will enable the production of more highly defined bioengineered heparin on a commercial scale by orchestrating the synthesis of these structures through the control of the expression and activity of the appropriate biosynthetic enzymes.

In summary, there are several approaches for the production of bioengineered heparin in the research phase. The promise of bioengineered heparin in a form of the successful therapeutic that can be produced on the commercial scale with controlled synthesis, structure and purity is worth pursing, however it will need a significant amount of basic and applied research activity around the globe before it will one day become a reality.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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