Heparin: Effects upon the Glycocalyx and Endothelial Cells

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Abstract: Unfractionated heparin (UFH) is the most widely used injectable medication in the United States. UFH is a poly-dispersed, relatively impure combination of many polysaccharides known as a glycosaminoglycan. It is used as the primary anticoagulant for heart surgery as well as for active treatment of deep venous thrombosis, vascular thrombosis, stroke, and many other potentially catastrophic clotting syndromes. Many perfusionists and cardiac team members know little of the biology of UFH other than its use for cardiopulmonary bypass. UFH is very similar to heparin sulfate, found on the surface of endothelial cells. Heparan sulfate protects endothelial surfaces from inflammatory attack and serves as a mechno-transducer for vascular shear. UFH and all glycosaminoglycans have far reaching pleotropic actions. This review elaborates on some of fascinating unique biology of these polysaccharides. Perhaps a number of the complex complications attributed to CPB are either caused by, or set up to occur by the complicated biology of UFH?


We depend on heparin for usual anticoagulation in heart surgery. However, it is not a substance that normally occurs in circulation. Unfractionated heparin (UFH) is a mucopolysaccharide isolated from mast cells. It is found in high concentrations either in porcine mucosa (vast majority of commercial heparin in the United States today) or in bovine lung (available in some other parts of the world). The glycosaminoglycans (GAGs) are a family of polysaccharides based on repeating units of various saccharides (1,2). As such, they form long, straight chain molecules that have a wide range of cellular activities based on their charge, the specific sequence of their sugar moieties, and their attachment to various proteins. GAGs do not have the globular shape of proteins, and they do not share the dynamic ability of proteins to flex, fold, and unfold. Nevertheless, the long-chain polysaccharides do have the ability to rotate and move in solutions, presenting varying aspects of their side charge moieties to cells, receptors, and proteins. The polysaccharides (heparin and heparan sulfate) are built from the same repeating units of glucosamine and iduronic acid with varying amounts of sulphydryl groups.

These sequences bear N-sulfate, N-acetyl, and O-sulfate side chain substitutions in different conformations and in different densities of sulfation. Hyaluronic acid, heparin, and heparin sulfate all contain glucosamine. Chondroitin sulfate and dermatan sulfate are GAGs found in connective tissue, joints, and cartilage. These can have some anti-coagulant behaviors, especially when their sulphydration is altered pharmaceutically. However, it has been shown that such alteration makes them highly toxic—a different subject entirely. Keratan sulfate, another connective tissue building block, is made up of repeating units of N-acetyllactosamine and sulfated galactosamine. That means there is a huge biologic variability in these sugar moiety chains as building blocks and cell signalers. Small differences in saccharide sequence and level of sulfation and negative charge connoto highly variable differences in biologic activity (1–4).

Contained within the polydispersed long-chain polysaccharides are specific sequences of units that signal various cellular activities (1–4). We in cardiac surgery are focused on the ability of heparin to bind and activate antithrombin (AT), a circulating zymogen protein (3,5–8). The specific pentasaccharide sequence that triggers the conformational change in AT is unique in terms of its saccharides and side chain charges. That pentasaccharide sequence has been created as a drug by starch chemists, and it is highly active as an anticoagulant by itself (fondaparinux). Furthermore, the use of chemical methods to purify heparins have been used to create low molecular weight
Heparins with perhaps fewer side effects than the UFH—decreased but not absent heparin antibody formation, for example. This manuscript will not focus on indications for these various forms of heparin but rather will talk about how the drugs can affect endothelial cell functions. This pharmaco-biological interaction is a newly appreciated forefront of some research.

Heparin is released from mast cells and basophils when they are stimulated by an extracorporeal invasion of bacteria or particulates (6–8). These inflammatory first responder cells are concentrated in the lung and gut. The outside world meets the inside physiology in these two hollow organs. Therefore, they are constantly bombarded with low- to high-level invasions of microorganisms. The gut, particularly, contains friendly saprophytic organisms that create vitamins and help digest foods. However, if those organisms are not held in equilibrium and in the gut lumen, it can result in invasion, overgrowth, or dysfunctional disease. Heparins—GAGs, hyaluronic acid, chondroitin sulfate, and heparan sulfate—literally have multitudes of known physiologic functions (2–16).

When released from mast cells, heparin enters the interstitial space. Ideally, there is very little AT in the interstitial space. So it appears that the primary function of AT in the interstitial space is to enhance white blood cell chemosignaling or attractant for in UFH in the interstitial space. Ideally, there is very little AT in the interstitial space. So it appears that the primary function of AT in the interstitial space is to enhance white blood cell chemosignaling or attractant for in UFH in the interstitial space. Ideally, there is very little AT in the interstitial space.

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When released from mast cells, heparin enters the interstitial space. Ideally, there is very little AT in the interstitial space. So it appears that the primary function of UFH in the interstitial space is to enhance white blood cell movement and chemosignaling or attractant for inflammation in that space (2–16). It would make sense as well that if heparin was to function as an inflammation promoter, it should have anti-coagulant effects, especially if plasma were to leak into the interstitium. There are estimated to be 10,000 different functions of heparin, including: inflammatory chemo-attraction; white blood cell diapedesis and movement between cells; upregulation of overall inflammation as well as cellular repair; vessel growth; and even anticancer functions. Interestingly, fibroblast growth factors and platelet growth factors release is activated by heparin (2–16). Platelets and endothelial cells as well as white blood cells are also activated by low- to moderate-dose heparin (10–13). The contact of platelets with heparin leads them to express many or most of their protected or invaginated cell membrane glycoprotein binding sites. This includes the expression of platelet factor 4 (PF-4) as well as glycoprotein Ib and glycoprotein IIb/IIIa. We know that after cardiopulmonary bypass (CPB), many of these glycoprotein binding sites are dysfunctional or competitively bound with fibrin degradation or serine protein products. PF-4 provides natural defense by circulating platelets and white blood cells for heparan sulfate released from injured endothelial cells. The protein binds heparan sulfate and heparin, clearing the circulation of these anti-coagulant, AT-activating polysaccharides. PF-4/heparin complexes are highly antigenic, and given enough time and exposure, more than 50% of humans will express antibodies against the complex (14). This leads to the heparin-induced thrombotic thrombocytopenia (HITT)—the most highly pro-thrombotic disease known to medicine. HITT is caused by our intravenous use of heparin. Unfortunately, we do not yet have widespread, readily reversible, highly or easily titrated intravenous drugs that can replace heparin. Direct thrombin inhibitors offer advantages but to date are not rapidly reversed. Heparin remains the most widely used intravenous drug in the world. For all of its problems, the antibody formation via PF-4 seems to be one of the worst. PF-4 has an interesting three-dimensional structure quite similar to some cytokines—particularly interleukin-8 (IL-8), also known as leukocyte chemoattractant factor (17). Both heparin and heparan sulfate bind to and facilitate IL-8 activity, stimulating white blood cell trafficking (diapedesis) through endothelial cell tight junctions. The effects of UFH in terms of white blood cell tight junctions during CPB are yet unknown.

HEPARAN SULFATE

Heparan sulfate is not the same as heparin (1–4). Heparan sulfate is created constantly by normal, healthy endothelial cells. It is made up of a group of long-chain mucopolysaccharides with the unique pentasaccharide sequence somewhere (perhaps created by chance alone) within its chain length. Heparan sulfate has different saccharide building blocks with substantially more variable proportions than heparin. Heparin has much more sulfhydryl substitution on the side moieties than heparin sulfate, and heparan sulfate seems to have a number of saccharide sequences that function simply as spacers (1). What is highly confusing is the fact that these substances, both naturally occurring and both polysaccharides, are named so similarly. Interestingly, the cell surface slime of Escherichia coli is essentially relatively unsulfated heparin (1). Heparan sulfate is tethered to the surface of endothelial cells by its attachment to a protein backbone. That protein backbone connects through a number of proteins, but ones very often investigated are the syndecan proteins. Various lengths of heparin sulfate polysaccharides stream from the tree trunk-like syndecans, and all together they form something similar in appearance to a kelp bed, with feathery projections waving in the plasma stream of the circulation.

This endothelial glycocalyx (EG) coating—a mixture of polysaccharides and proteins—creates a “non-wettable” surface on endothelial cells. The polysaccharides create a strong negative (anionic) charge similar to many of the circulating serine proteases so that these proteins are actually repelled by charge density alone. Embedded within the EG are a large number of captured enzymes, proteins,

and a particularly high concentration of albumin. Albumin, it turns out, is a great antioxidant protein. Today we are learning that albumin may have several functions in addition to maintenance of oncotic/osmotic pressures within the vasculature. In recent studies in cardiac surgery, it seems that patients given albumin during their cardiac surgery have superior outcomes compared to those given only crystalloids (18). One of the enzymes embedded in the EG is superoxide dismutase. This enzyme quickly detoxifies superoxide radicals released from white blood cells as a natural killing mechanism for bacteria.

The heparan sulfate not only helps to create the charge density and “non-wettable” surface of the glycocalyx/endothelial cell surface, but it plays a role in the mechanotransduction for the endothelial cells. Endothelial cells are sensitive to and dependent on shear forces. Too little shear and they are dysfunctional; too much shear and they lose EG as well as undergo stress, making them susceptible to inflammation (19,20). The heparan–syndecan combination tethers transmembrane to a calcium channel and other internal biochemical mechanisms, making the endothelial cell highly responsive to shear forces. This is its normal environment. Different endothelial cells have adapted to perform several different functions. For example, the endothelial cells of the renal tubules are highly sensitive to solutes pressure and shear forces, as are those in the pulmonary circulation. Each has an EG layer and each senses its environment, but because of embedded enzymes each has different functions responses to pressure and oxygen concentrations. Hypertension, high cardiac outputs, and high shear all affect endothelial cells signaled by the glycocalyx. Diabetes through oxidative stress on the EG damages endothelial cells, as does stress from hypertension, vascular stretch, and turbulence. The complexity of diabetic oxidative stress with vascular biology is a great part of the investigations regarding long-term endothelial stress leading to atherosclerosis generation (21).

**OXIDATIVE STRESS**

Endothelial cells are negatively affected by oxidative stress and by anoxic stress (21). It may well be that anoxia is not as damaging as is reperfusion injury on top of the loss of functional, normal, antioxidant, and homeostatic mechanisms. The width of the EG is normally 2.1–2.5 μm. After ischemia and reperfusion injury, the thickness can decrease to 50–90% (22). Of course, in CPB there are localized vascular beds of ischemia and reperfusion injury, such as the pulmonary and coronary vasculature. Hyperkalemia is particularly damaging to the EG and, of course, our standard of care in CPB-hyperkalemic cardioplegia to stop the heart (19). Various organs spontaneously release heparan sulfate during and for transplantation when they are made ischemic (23). This spontaneous heparinization can lead to clinical bleeding in liver transplantation. In the process of extracorporeal membrane oxygenation, the spontaneous release of heparan-like substances is noted in 56% of patients (24). The heparan sulfate was seen in patients who received just bivalirudin, a direct thrombin inhibitor. It is therefore believed that either the underlying vascular, whole-body inflammatory events necessitating the extracorporeal membrane oxygenation or the extracorporeal circuit was stimulating an inflammatory reaction to cause endothelial cells to release heparan sulfate (24).

Since the 1970s, we have known that endothelial cells exposed to UFH solutions lose their heparan coating (1–4). We also know that during CPB, the syndecan-1 protein skeletons can be found in ever-increasing concentrations in the plasma. Heparan sulfate is found there, as well. It is very tempting to therefore say that heparin somehow makes endothelial cells more susceptible to ischemia and inflammatory attack during CPB. That may, however, be too simplistic. It is quite likely that UFH competitively binds to the syndecan-1 proteoglycan binding sites and that it releases heparan sulfate, but we do not yet know just how much that exposes endothelial cells to inflammatory attack. If heparin has replaced the syndecans, we do not know how dysfunctional that substitution may become. Other effects of ischemia, shock, endotoxin, etc. are important when trying to understand these complex interactions.

**SHOCK**

Heparan sulfate is released in both endotoxin/septic shock and hemorrhagic shock. In a dog model of septic shock, as exposure to E. coli endotoxin increased, the levels of syndecan-1 and heparan sulfate correlated with the levels of cytokines—IL-6 and tissue necrosis factor-α (6). Of interest, the shedding of the native heparan sulfate and syndecan-1 was reduced when UFH was administered. The mechanism by which the levels of endothelial shedding were reduced may have to do with the pleotropic effects of these polysaccharides. They both bind to and react to cytokines (1–4,6). If there is more circulating polysaccharide, it may well bind to the free cytokines and therefore reduce the effects of those cytokines on endothelial cells. We are all aware that variable and potentially high levels of inflammatory cytokines are seen during CPB. Much more work needs to be done to understand the complexity of these interactions.

In trauma, the systemic attack on vascular endothelial cell function is real, extensive, and highly dependent on both the severity of injury (e.g., severe crush injury or...
bone/fat embolism injury) as well as the severity of hemorrhagic shock (e.g., ischemia/reperfusion injury). Trauma-induced coagulopathy is poorly understood, dynamic, and highly variable. Certainly, trauma creates an initial insult of high levels of circulating tissue factor, which rapidly stimulates thrombin generation. Tissue factor along with thrombin are highly pro-thrombotic and highly inflammatory proteins that amplify many other cellular processes, including platelet activation and white blood cell chemotaxis. In a study of 77 major trauma patients from Denmark, about 5% showed a high degree of auto-heparinization (24). Those who auto-anticoagulated had the highest levels of syndecan-1, high prothrombin times (international normalized ratio), and higher thrombomodulin levels (endothelial response to thrombin exposure). These patients also had received more allogenic blood transfusions—a high inflammatory load by themselves—and a high level of endothelial insult through dysfunctional red blood cell-to-endothelial interactions. The take-home point from such studies on trauma is that endothelial shedding of heparan may well contribute to trauma-induced coagulopathy that is difficult to characterize/treat. A study on the use of protamine infusions in such patients might be of interest, but it has not yet been done.

WATER/SOLUTE FLUX

The health of the EG has effects on vascular leakage of water and solutes (1–4). The levels of heparan/heparin affect the vascular extravasation of many molecules. In mice treated with heparin, hyaluronidase, saline, and dextran, the clearance of dextrans from the circulation increased by the use of hyaluronidase and UFH (25). The dilator response of arterioles was reduced after exposure to UFH. It was thought that the lack of dilation was due to reduced nitric oxide bioavailability. Nitric oxide production is highly oxygen-dependent and is affected by endothelial cell nitric oxide synthase as well as multiple upstream biochemical reactions related to cellular viability. The transfer of shear messages to the endothelial cells was thought to therefore be affected by the external administration of UFH. Shear stress induces coronary vasodilation as a reflex response (26). An intact EG is important for this shear stress response, and in at least one study looking at albumin vs. other colloidal intravenous solutions, it was only the albumin that participated in the shear stress phenomenon (24). When heparinase was infused and the EG was partially or totally obliterated, the vascular hyperactivity and vascular spasm increased 10–15 times (26,27).

Sodium plays multiple roles in cell ion fluxes. Aldosterone levels and small increases in plasma sodium concentration can stiffen vascular endothelium, probably via water fluxes. Endothelial cell sodium channels are ATPase dependent and are embedded not only in the cell membranes but across the glycocalyx (28,29). If the EG is denuded by the enzyme heparinase, then sodium permeation into the vascular cells increases. This is in the context that ischemia and reperfusion injury can reduce EG thickness and therefore could lead to endothelial cell damage, increasing stiffness due to loss of heparan sulfate alone (28,29). Essentially, the EG functions as a firewall for sodium movement into cells. How that is affected by heparin and its replacement of or release of heparan sulfate is as of yet unstudied.

Reactive oxygen species are produced by inflammation as well as ischemia and reperfusion. Heparan sulfate, the glycocalyx, and embedded antioxidants are all very important in protecting endothelial cell functions (29). The EG is rapidly and dynamically modified by ischemia and reperfusion. Xanthine oxidoreductase is hypothesized to be displaced by heparin in the glycocalyx. If heparin displaces some of the protective enzymes that down-regulate oxidative stress, then the endothelial cells are prone to attack by a wide range of toxic events. Loss of albumin alone means the loss of antioxidant effects.

There is growing literature about the importance of AT in endothelial health and resistance to injury. In CPB, the levels of AT drop by variable amounts in response to UFH, length of surgery, overall inflammatory load, and the preoperative use of UFH. The variability of AT levels is quite large, with some patients hitting levels of below 50% activity and some even falling as low as 20% activity. The use of AT and its biologic activity for normal endothelial cell function is a major area of investigation.

MICROBUBBLES

CPB universally creates microbubbles. Those microbubbles have been studied with respect to endothelial cell function (31,32). It makes great sense that the temporary plugging of the microcirculation by microbubbles is one major mechanism responsible for diffuse vascular ischemia and reperfusion injury. Permanent neuropsychiatric deficits have been reported in 20–40% of CPB patients, and greater than 50% of patients have at least transient neuropsychiatric deficits. The interaction of bubble surfaces with endothelial cells is quite complex and stunning. If cell-cultured endothelial cells are exposed to air bubbles, they will have early cell death (31–33). If the heparan sulfate matrix of the glycocalyx is denuded, the potential for bubbles to lodge in vascular structures increases and further enhances the risk of endothelial cells for early death. It appears that the bubbles trigger cellular calcium release as digestion of heparan sulfate leads to fast influx of calcium into endothelial cells, potentially triggering some elements
of apoptosis (32). Again, we do not know if the competitive shedding of heparan sulfate by UFH leads to an increased reactivity to bubble loads in small vascular structures. Surfactants and perfluorocarbon emulsions have been shown to be vascular endothelial protectants against air bubbles. Studies of how these agents work with preservation of the glycocalyx have not yet been performed.

PROTAMINE

Heparan and heparin bind positively charged polyanions. Histone proteins are notorious for their interaction in binding with negatively charged heparins/heparans. Obviously, we use this pharmaceutical interaction every day when intravenous heparin is reversed with intravenous protamine. Histone proteins are found in nature coating the outside of the double helix of DNA, maintaining its tightly folded configuration. Do protamine sulfate or other naturally occurring histone proteins bind to or alter the function of EG-tethered heparan? A Swedish study of glomerular filtration protamine sulfate demonstrated that infusion of the histones could dynamically decrease the molecular size selectivity of the rat glomerulus (34). Protamine sulfate binds into the glomerular EG and creates alterations of the negative surface charge by binding to native heparin. When rats infused with polydispersed tracer molecules (n-Ficoll) charged (anionic) or uncharged molecules were studied those that had received protamine sulfate had glomerular restricted molecular size discrimination (30). Of interest when heparinase was infused the same thing happened suggesting that any attack on the homeostasis of the glomerular EG can be detrimental. We still do not have an answer as to whether the use of large dosages of protamine lead to the production of tethered native heparan sulfates.

CONCLUSION

In the 1970s, it was shown that UFH competitively releases heparan sulfate from glycocalyx. Today that fact is still used in research labs as a way to harvest native heparan sulfate from endothelial cells. Ischemia, reperfusion, hyperkalemia, air emboli, and other factors all put the EG at risk for loss of syndecan and heparan sulfate. Loss of these protective and important cell-signaling, surface-coating molecules puts the endothelial cells at risk for inflammatory attack. UFH and heparan sulfate both bind to and react to cytokines, seemingly acting as messengers but potentially maintaining homeostasis by pulling the cytokines out of circulation. The implications of this interaction are not clear, but it is puzzling that these polysaccharides have such complex interactions with protein inflammatory cell signaling. We are now beginning to appreciate that heparin/heparan may have more than 10,000 different biological activities. In cardiac surgery, anesthesia, and perfusion, we focus on only the interaction of heparin with AT. Furthermore, we foment academic arguments about heparin protamine ratios, re-heparinization, and other topics long of interest to us, yet the large question of what UFH does intravascularly to potentiate widespread vascular damage needs to be investigated. The deeper one delves into the biology, the more puzzling the conundrums of daily clinical medicine vs. complex biochemistry and pathophysiology become. It remains this author’s steadfast belief that UFH has toxicity potential that we to date do not fully comprehend.

REFERENCES

HEPARIN: EFFECTS UPON THE GLYCOCALYX AND ENDOTHELIAL CELLS


