Heparin and anticoagulation

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TABLE OF CONTENTS

1. ABSTRACT

Heparin, a sulfated polysaccharide, has been used as a clinical anticoagulant for over 90 years. Newer anticoagulants, introduced for certain specialized applications, have not significantly displaced heparin and newer heparin-based anticoagulants in most medical procedures. This chapter, while reviewing anticoagulation and these newer anticoagulants, focuses on heparin-based anticoagulants, including unfractionated heparin, low molecular weight heparins and ultra-low molecular weight heparins. Heparin's structures and its biological and therapeutic roles are discussed. Particular emphasis is placed on heparin's therapeutic application and its adverse effects. The future prospects are excellent for new heparins and new heparin-based therapeutics with improved properties.

2. INTRODUCTION

Heparin is an essential drug and is the most widely used clinical anticoagulant worldwide (1). However, in the past decade there is a rising concern about the safety and supply security of heparin. An international heparin contamination crisis, which occurred in 2007 to 2008, was associated with more than 80 fatalities in the U.S. alone (2). Moreover, most of the world's supply of heparin comes from a single animal species, hogs, and is collected in a single country, China. This review presents the current fundamental knowledge of anticoagulants, including an overview of coagulation pathway, major diseases and conditions requiring anticoagulants, and the various classes of anticoagulant drugs. The focus of this review is on heparin and heparin-based anticoagulants, including their biochemistry, chemical structure and their wide range of biological activities. The therapeutic roles of heparin-based drugs, including unfractionated heparin (UFH), low molecular weight heparin (LMWH) (3), and synthetic heparins (4) are discussed. These heparin-based drugs are essential in the medical treatment of thrombosis, embolisms and in the prevention of clotting in surgery. As with any class of drugs, heparin-based therapeutics result in side-effects and complications. The redesign, re-formulation and appropriate application of these agents can reduce the risks associated with heparin-based therapy. However, contamination/impurities and bovine/porcine risks that have received much attention since the 2007-2008 contamination crisis are currently being addressed by considering the re-introduction of bovine heparin (5), through the introduction of bioengineered heparin (6), and in the improvement of synthetic heparin oligosaccharides (7). This review focuses on advances that have been made in the field over the past decade.

3. DETAILIED DESCRIPTION OF COAGULATION PATHWAY

Blood in a healthy individual freely circulates through arteries and veins. The normal vascular endothelium acts as an antithrombotic surface. However, when the hemostatic system is triggered it becomes active instantly due to the cascade reactions. When the blood vessel wall becomes damaged, platelets and fibrin aggregate to prevent hemorrhage. Although a rapid hemostasis is required to prevent the loss of blood, an excessive amount clotting can lead serious thrombotic complications.

The primary source of hemostasis is through platelet aggregation and adhesion to a damaged vessel. Secondary hemostasis is mediated by plasma-based coagulation factors, which undergo a biochemical cascade resulting in platelet-fibrin clots (8, 9). The heparan sulfate proteoglycan (HSPG) on the surface of the endothelium lining the lumen of the vessel also plays a role in controlling coagulation.

3.1. Primary hemostasis – platelets

Following vascular injury, platelets adhere to exposed endothelial collagen forming a 'platelet plug' resulting in primary hemostasis (Figure 1A). Von Willebrand factor (vWF) bridges between endothelial collagen and platelet surface receptors, mainly glycoprotein (GP) receptor Ib, promoting platelet adhesion and aggregation. An activated platelet degranulates to release various factors including serotonin, adenosine diphosphate (ADP), and thromboxane A2 (TXA2). Serotonin and TXA2 have vasoconstrictive effect. ADP and TXA2 stimulate further platelet aggregation. In addition, activated platelet exposes fibrinogen binding site, thus, fibrinogen bridges platelets to form a platelet plug. The generated platelet plug helps activated coagulation factors assemble on its surface and the secondary hemostasis, involving the plasma-based coagulation cascade follows.

3.2. Secondary hemostasis – biochemical cascade reactions

Secondary hemostasis involves a cascade of biochemical reactions (Figure 1C). This cascade is comprised of inactive zymogens (or pro-enzyme) called blood coagulation factors being activated to serine proteases (i.e. factor X to factor Xa) that can then go on to activate subsequent coagulation factors (i.e., factor Xa activates factor II to form factor IIa) that ultimately convert the soluble plasma protein fibrinogen, to the insoluble plasma protein, fibrin (comprising a clot). There are two traditional major secondary coagulation cascade pathways, the intrinsic pathway and the extrinsic pathway. The intrinsic pathway, which is also called contact pathway, is triggered by factors XII and XI. When factor XII contacts the negatively charged surface
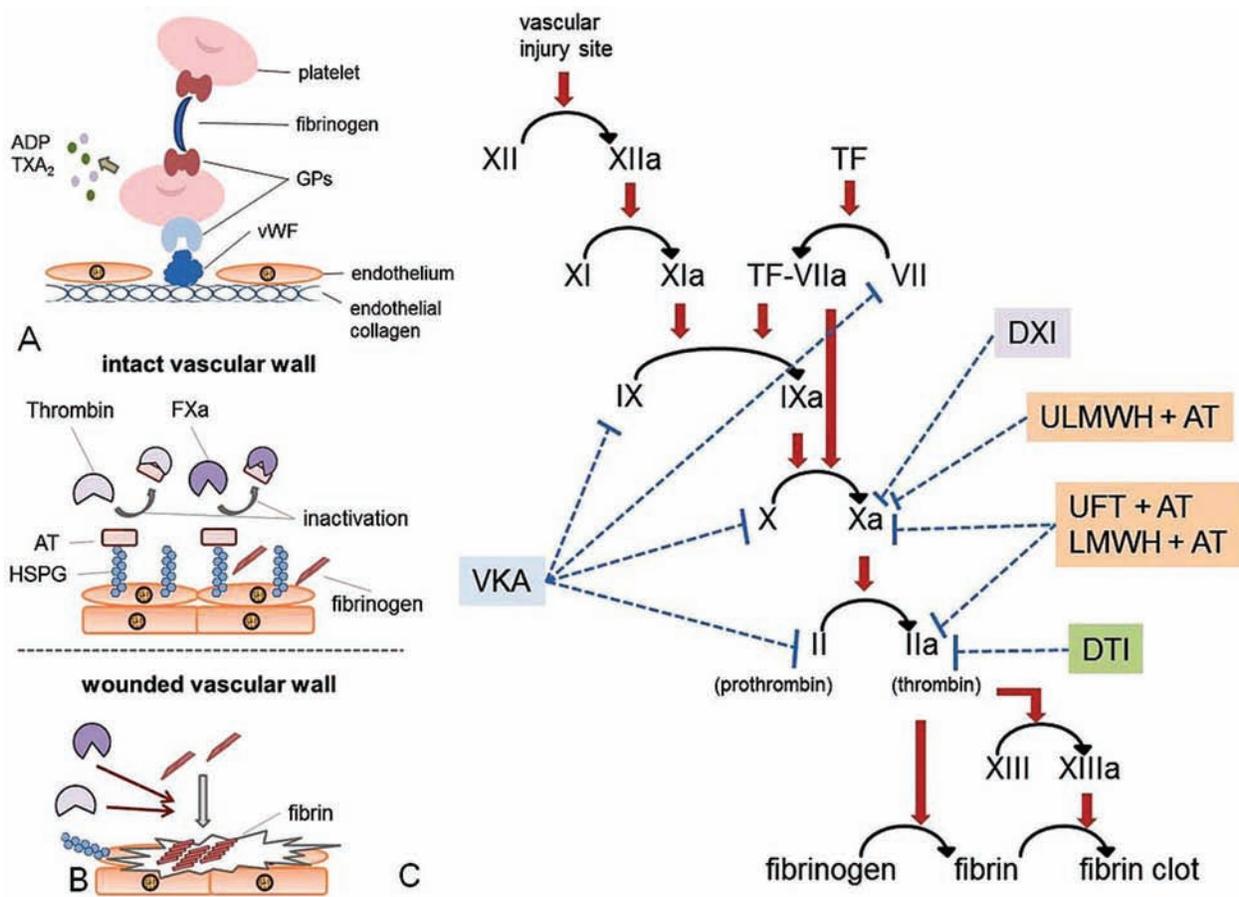
Figure 1. Coagulation pathway. A) Molecular mechanism of a platelet adhesion and aggregation involving von Willibrand factor (vWF) and glycoproteins (GPs), releasing adenosine diphosphate (ADP) and thromboxane A2 (TXA2). B) Endothelial heparan sulfate proteoglycan (HSPG) interaction with antithrombin (AT) resulting in the inactivation of thrombin and factor Xa (FXa) to control coagulation. C) Main reactions of the coagulation cascade after activation through vascular injury or by tissue factor (TF) and major clinical anticoagulants including direct factor Xa inhibitor (DXI), direct thrombin inhibitor (DTI) and vitamin K antagonists (VKAs).

(phospholipids exposed at a vascular injury site), it causes a local increase in its concentration, which then autoactivates to factor XIIa. Factor XIIa then catalyzes the conversion of prekallekerin to kininogen and factor XI to XIa (9, 10). Consequently, these activations lead to the formation of factor IXa.

The extrinsic pathway, which is also called tissue factor pathway, is the initial step in plasma-mediated hemostasis. The contact of the membrane-bound protein tissue factor (TF) with plasma containing factor VII triggers the extrinsic pathway, forming TF-VIIa complex. Alternatively the extrinsic pathway can also be initiated if monocytes and smooth muscle cells are exposed to cytokines or other inflammatory mediators. This also causes the release of tissue factor (9, 10). Once the TF-VIIa complex forms, it converts factor IX and factor X to factor IXa and factor Xa, respectively.

Once factor IXa is formed either by the intrinsic or extrinsic pathway, the 'tenase' complex, consisting

of factor IXa, factor VIIIa, calcium and phospholipids, generates. This tenase complex activates factor X. After the formation of the tenase complex, the prothrombinase complex, which consists of factor Xa, factor Va, calcium and phospholipids generates. Although factor Xa alone can catalyze prothrombin (factor II) into thrombin (factor IIa), this activation is greatly accelerated by factor Va and the complex. Thrombin, the final serine protease formed in the coagulation cascade has various roles in clotting (9). Thrombin activates various components of coagulation pathway, such as platelets, factors V, VIII and IX, protein C and thrombin-activatable fibrinolysis inhibitor to amplify the coagulation cascade. Most importantly, thrombin converts fibrinogen to fibrin, ultimately forming a clot.

The conversion from soluble fibrinogen to insoluble fibrin is the final step of the coagulation process. Factor XIIIa leads fibrin-monomer cross-linking to form a stabilized fibrin clot. In parallel, fibrinolytic system is activated to control the size of fibrin clots. Fibrinolysis

cleaves insoluble fibrin into fibrin degradation products, and rapidly clears it out to maintain hemostatic balance. Plasmin, which circulates as an inactive zymogen plasminogen, is the enzyme responsible for fibrinolysis.

Antithrombin (AT), previously known as antithrombin III, is a serine protease inhibitor that inactivates various activated coagulation serine proteases, including factors IXa, Xa, TF-VIIa complex and thrombin. AT covalently binds to serine residue of serine proteases causing their inactivation. However, in the presence of heparin or heparan sulfate (HS), the ability of AT to inhibit serine proteases is markedly enhanced and in the case of thrombin forming heparin-AT-thrombin ternary complex.

The intrinsic pathway and the extrinsic pathway were previously considered as independent pathways of factor X activation. However, it is now thought that the extrinsic pathway initiates thrombin generation (initiation phase) and the intrinsic pathway augments thrombin generation (propagation phase) (9, 10).

3.3. Endothelium
Thrombin promotes coagulation by catalyzing the conversion of fibrinogen to fibrin. In the healthy intact vessel, AT bound to the endothelial HSPG can inactivate thrombin (Figure 1B). AT-HSPG complex can also inactivate factor Xa through formation of AT-Xa complex. Since the healthy endothelium of an undamaged vessel wall is non-thrombogenic, clotting on healthy vessels is prevented. In contrast, endothelial damage in a wounded vessel, results in the loss of AT-HSPG, allowing thrombin to promote clot formation and coagulation (11). The endothelium is also responsible for the active transport and regulation of extravasation of fluid, solutes, hormones, macromolecules platelets and blood cells. This is accomplished through the use of smooth muscle cells, interendothelial junctions, vasodilation, and vasoconstriction. Dysfunction in the endothelium, i.e., failure to maintain blood fluidity, the appropriate concentration of factors, or inflammation, can result in catastrophic changes in coagulation (12).

Blood coagulation should occur rapidly but only if necessary. Otherwise, bleeding or thrombosis will occur. To achieve expediency the successive activation of pro-enzymes to enzymes, known as cascade reaction, triggers procoagulant activity explosively by producing an exponential increase in the number of these enzyme molecules. In contrast, under normal physiological conditions, inhibitors of coagulation (such as AT) limit the clot formation to avoid the thrombus formation.

4. DISEASES AND ABNORMALITIES

4.1. Venous thromboembolism
Venous thromboembolism is a coagulation disease that involves deep vein thrombosis and pulmonary embolism (13). Deep vein thrombosis results from clot formation in a major vein, normally one in the lower extremities. Unlike clots in superficial veins that stay in place, these clots can dislodge and, thus, are very dangerous. Clots that dislodge from the vein can travel into the lungs or other organs causing permanent damage by way of tissue death or loss of oxygen. There are several types of embolism that include a venous and arterial embolism and these can be anterograde and retrograde, depending on whether the embolus travels with or against the blood flow. There are several environmental and hereditary causes of deep vein thrombosis including factor deficiencies, hormone treatments, stress, diabetes, chronic inflammatory disorder, vein damage during surgery, broken bones, physical trauma, slow blood flow from lack of movement, pregnancy, venous catheters, old age, obesity and smoking (13-15). These clots can block blood flow leading to swelling, pain and death. Venous thromboembolism affects up to 5% of the population during their lifetime and approximately 20% of patients with pulmonary embolism die before diagnosis or within a day after diagnosis. Patients who survive past that period face an 11% mortality rate in the first 3 months, even with adequate therapy (14).

4.2. Venous thromboembolism treatments
The initial treatment venous thromboembolism relies on is heparin and LMWH anticoagulants. These acute treatments lower risk and costs associated and reduce risk of recurrence. Anticoagulant treatment is effective in thinning the blood preventing clot growth. While heparin does not remove the clot, it is slowly dissolved over the course of about 3 months. LMWH can only be at reduced doses with careful observation on patients with renal failure since LMWH is cleared through the kidney as described in Chapter 6.3. Vitamin K antagonists (VKAs), such as warfarin, used alone are not recommended for the initial treatment since a randomized trial demonstrated more recurrent symptomatic and asymptomatic events in patients treated with VKAs alone. However, after the initial use of heparin or LMWH, VKAs can be used to continue treatment. This improves its prospects as a long term treatment since VKAs are inexpensive. Conversely, rivaroxaban a direct oral factor Xa inhibitor (DXI) is easily administered but costly. Rivaroxaban treatment, like LMWH treatment, cannot be used in patients with renal failure. Thrombolytics, such as tissue plasminogen activator (t-PA), are used to dissolve the clot directly, however, due to the high bleeding risks, such agents are generally only used in emergency situations. Vena cava filters are another form of treatment primarily used to prevent the dislodging clots from migrating to vital organs by physically removing them. This treatment is preferred if the patient cannot take anticoagulants or in addition to thrombolytic treatment. Vena cava filters have the drawback of potentially causing thrombi formation at the insertion site but if the patient can take anticoagulants then this drawback can be remedied (13, 14).

4.3. Extracorporeal circuit

Clotting abnormalities can also be caused by non-biological processes. Many medical procedures require the separation, purification, or oxygenation of blood and the instruments used in this process need to be used in tandem with anticoagulants to prevent clot formation. An extracorporeal circuit or extracorporeal membrane oxygenator takes the patient's blood outside his or her body using plastic tubing, a hemodialysis machine, a dialyzer, or an oxygenator (16). There are two different types of extracorporeal membrane oxygenators (ECMOs), Veno-arterial and veno-venous oxygenerator. Veno-arterial ECMO is used for heart failure providing both gas exchange and hemodynamic support for a failing cardiopulmonary system. This means deoxygenated venous blood is drained into an oxygenator, oxygenated, and is returned into circulation. Veno-venous ECMO involves only gas exchange without the use of hemodynamic support and is used for refractory hypoxic respiratory failure with preserved cardiac function. Deoxygenated venous blood is drained into an oxygenator and hyperoxygenated arterial blood is returned to venous circulation. The blood interacts with the nonendothelial surfaces of the ECMO causing widespread inflammatory and prothrombotic response. Within minutes of initiation there is a widespread activation of the clotting cascade and also a dilution of coagulation factors in the blood. Platelets adhere to surface fibrinogen, causing platelet activation and platelet aggregation, resulting in platelet loss, thrombocytopenia. These complications require the use of anticoagulants. Heparin is used in the coating of these circuits to free tissue factor pathway inhibitor, and augment AT-dependent inhibition, freeing factors Xa, XIa, and VIIa/TF. AT has been shown to decrease with the initiation of the extracorporeal circuit leading to a procoagulative statedecreased heparin responsiveness. This means that AT should also be monitored and kept above 60% to prevent venous thrombosis. Understanding of the extracorporeal circuit and what coagulation factors need to be monitored when blood is pumped outside the body is important when performing cardiopulmonary bypass, hemofiltration, dialysis, and surgery.

4.4. Disseminated intravascular coagulation

Disseminated intravascular coagulation (DIC) is an acquired syndrome characterized by an unregulated systemic activation of coagulation, leading to excessive formation of microthrombi, microcirculation obstruction, resulting in organ dysfunction and death (17, 18). In addition, the extravagant consumption of platelets and coagulation factors cause serious hemorrhagic complications, which is called consumption coagulopathy. Therefore, a DIC patient can present thrombotic and bleeding symptoms concomitantly. DIC is commonly caused by sepsis, malignancy, pregnancy complications and massive inflammation. Regarding DIC diagnosis, a combination of laboratory tests such as prothrombin time, activated partial thromboplastin time, platelet count, fibrin

related marker, are conducted. DIC treatment should be based on the proper remedy of the underlying disorder. But sometimes, supportive treatment by anticoagulants is also required. UFH and LMWH are used for the treatment and prophylaxis of thrombosis and embolic complications associated with DIC. Concentrated platelets or fresh frozen plasma are transfused to replenish consumed platelets or clotting factors.

Recently, a recombinant thrombomodulin was approved for DIC. The International Society on Thrombosis and Haemostasis harmonized the guideline for diagnosis and treatment of DIC in 2013 (19). An observational study conducted in 2014 showed the gradual improved in-hospital mortality of DIC patients associated with infectious diseases and this study assumed that recombinant thrombomodulin and the new practice clinical guideline contributed this improvement (18).

5. ANTICOAGULANTS

5.1. Classes of anticoagulants

Anticoagulant drugs are categorized into four broad types: heparins, direct inhibitors, VKAs, and others. Heparins include UFH, LMWH and ultra-low-molecular weight heparin (ULMWH). UFH acts with AT and inactivates both FXa and thrombin. LMWH acts with AT to inactivate FXa and to a lesser degree thrombin (FIIa) and ULMWH acts with AT to exclusively inactivate thrombin. In contrast, direct inhibitors work independently of AT and inhibit either FXa or thrombin (Figure 2). Warfarin is the most commonly used of the VKAs. Other anticoagulants include several peptide and small molecules with a variety of mechanisms of actions.

5.2. Heparins

Heparin is a highly sulfated glycosaminoglycan. UFH is extracted and purified from animal tissues including porcine intestine and bovine lung and intestine. LMWH is produced through the controlled depolymerization of UFH. ULMWH is a synthetic specific pentasaccharide, which is similar to a pentasaccharide sequence found within UFH and LWMH. Heparin was discovered in 1916 by medical student Jay McLean (20). The clinical use of UFH started in 1930s. LWMHs have been clinically used since 1980s. ULMWH is the most recent subtype of heparin, which was approved by US Food and Drug Administration (FDA) in 2000s. Heparins work by primarily inhibiting thrombin (FIIa) and/or FXa. The ratios of anti-Xa activity to anti-IIa activity of different heparins differ. Shorter heparin chains having a low averaged-molecular weight display higher anti-Xa/anti-IIa ratios.

5.3. Vitamin K antagonists

Coumarins work as VKAs acting as anticoagulants by inhibiting the biosynthesis of several vitamin K-dependent clotting factors, including Factor
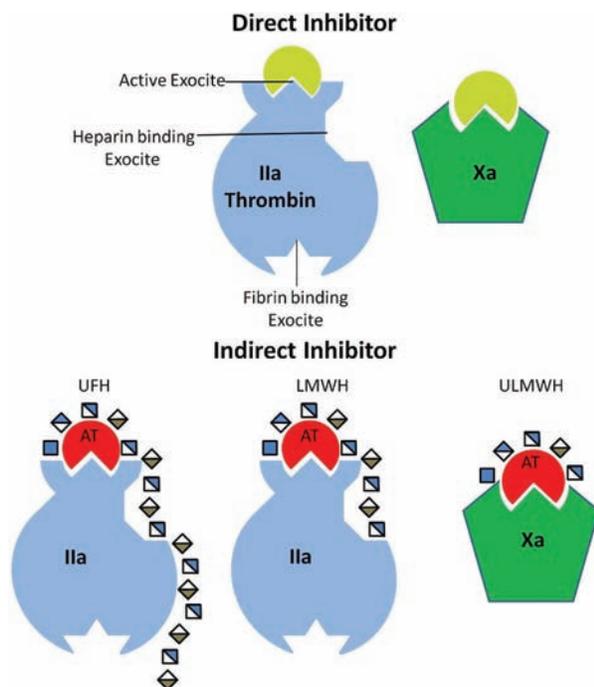
Figure 2. Schematic representation of direct inhibitors, including direct thrombin (FIIa) inhibitors (DTIs), Direct factor Xa inhibitors (DXIs) and indirect inhibitors, antithrombin (AT) with unfractionated heparin (UFH), low molecular weight heparin (LMWH) and ultra low molecular weight heparin (ULMWH), of factor IIa and factor Xa. The heparin sequence displayed in this figure corresponds to the pentasaccharide binding site (containing *N*-acetylglucosamine, glucuronic acid, glucosamine, idouronic acid, and glucosamine residues, respectively) and repeating idouronic acid, and glucosamine residues. Sulfo groups are not shown for simplicity. The symbols used are defined in Figure 5.

VIIa, IXa, Xa and thrombin. Warfarin, a specific member of the coumarin family, has been widely used as a clinical anticoagulant for more than half a century. However, its major shortcomings are the drug-food interactions and need for regular monitoring of the blood drug concentration. In addition, a drug interaction is another issue because warfarin is mainly metabolized by CYP2C9. Until recently, warfarin had been the only clinically used oral anticoagulant.

5.4. Direct inhibitors

Direct inhibitors are the newest class of oral anticoagulants. FDA-approved DXIs include rivaroxaban, apixaban and edoxaban. FDA-approved direct thrombin inhibitors (DTIs) include dabigatran (21). These new direct inhibitors provide wider therapeutic options than before. At the same time, their high cost, lack of antidotes and limited information on their long-term use represent serious drawbacks.

5.5. Others

Other anticoagulants include AT, synthetic serine protease inhibitor, and thrombomodulin. AT is a glycoprotein and the major inhibitor of clotting factors

and the cofactor through which heparin exerts its activity. Thus, concentrated AT can be used as an anticoagulant. Nafamostat and gabexate are the synthetic serine protease inhibitors. These drugs are competitive inhibitors and do not require AT for their mechanism of action. Thrombomodulin is an about 75 kDa integral membrane protein. Thrombomodulin has functions of both in the inhibition of thrombin and in the conversion of protein C into activated protein C, which exerts anticoagulant activity.

In addition to anticoagulants, many hemostatics and antithrombolytics are also available. Major types of hemostatics are capillary stabilizers (carbazochrome, vitamin C, etc.), coagulation accelerator (vitamin K, etc.), and antiplasmimn (tranexamic acid, etc.). Antithrombolytics include antiplatelet drugs (ticlopidine, aspirin, beraprost, etc.).

5.6. Comparison of anticoagulants

When selecting anticoagulant drugs, the cost, availability of antidotes, route of administration, safety and efficacy are important factors. Two other critical factors are their therapeutic indications and contraindications.

Heparins and warfarin are much less expensive than direct inhibitors. Protamine neutralizes heparins, and vitamin K is an antidote for VKAs, but there is currently no antidote for direct inhibitors. While warfarin and the direct inhibitors are oral drugs, heparins are used intravenously (UFH) or subcutaneously (LMWH and ULMWH). Direct inhibitors do not have yet sufficient safety and efficacy data for long-term use, patients during pregnancy, patients with mechanical heart valves, etc. Thus, heparins and warfarin remain the agents of choice. The period of administration is another important factor to consider when selecting an anticoagulant. In the treatment of venous thromboembolism there are three phases of treatment: acute, long-term and extended. Heparins are generally used in acute phase treatment while VKAs are used for both the long-term and the extended phase. A number of phase III studies are ongoing to evaluate direct inhibitors for the treatment of venous thromboembolism (22).

5.7. Recent topics

As of 2013, the annual sales worldwide of LMWH, VKAs, and direct inhibitors were, US$ 6.5 billion, 0.6 billion, 4.7 billion, respectively (Figure 3). It is estimated that the sales of LMWH and VKAs in 2018 will decrease by 27% and 18% from 2013, respectively. The sales of direct inhibitors are expected to increase by 181% during this period (23).

Recently, direct inhibitors have attracted much attention. Several new direct inhibitors and those antidotes are in development (22). For example, phase III clinical trials of a candidate antidote are ongoing (25, 26).
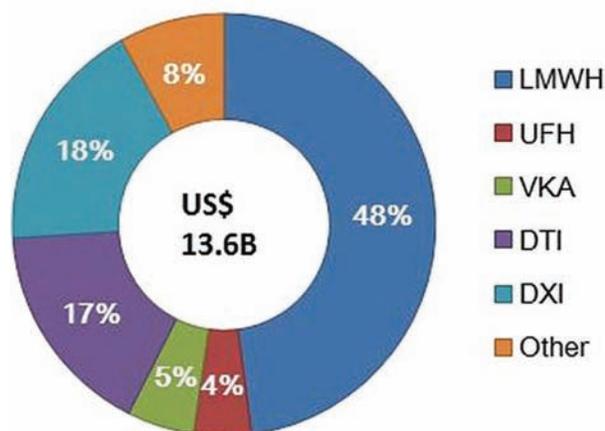
Anticoagulant Market 2013 (estimate)

Figure 3. Anticoagulant drug market sales in 2013 are estimated based on (23, 24). The market share of low molecular weight heparins (LMWHs), direct factor Xa inhibitors (DXIs), direct thrombin inhibitors (DTIs), vitamin K antagonists (VKAs), and unfractionated heparin (UFH) are shown.

Comparison of direct inhibitors and existing anticoagulants is also under intensive study. A meta-analysis of direct inhibitors suggested that both apixaban and rivaroxaban are associated with lower acute coronary events than dabigatran (27).

6. CLASSES OF HEPARIN

6.1. Heparin

Heparin is a highly sulfated polysaccharide that is used as a major clinical anticoagulant (1, 28, 29). Heparin is also a linear glycosaminoglycan (GAG) with an average molecular weight of between 15 and19 kDa (1, 30). It is made up of trisulfated disaccharide glucosamine and uronic acid repeating units, together with less sulfated and variably sulfated domains (Figure 4A). Heparin is biosynthesized in the endoplasmic reticulum and the Golgi of mast cells that are present in larger numbers in the liver, intestines, and lungs (Figure 5). It is extracted from food animal sources including cows and pigs, with porcine intestinal mucosa being the standard species and tissue source (1, 30). Heparin's anticoagulant activity is due to its ability to inhibit multiple factors in the coagulation cascade. Heparin binds to AT, as serine protease inhibitor, and targets coagulation proteins including factor Xa, and factor IIa (thrombin). AT binds a variably sulfated pentasaccharide sequence having a central 3-*O*-sulfoglucosamine residue. Only about 30% of the heparin chains contain this sequence. AT bound to heparin undergoes a conformational change, exposing a reactive loop that is acted upon and by factor Xa and thrombin catalyzing their inactivation. A heparin polysacaride of at least 18 saccharide residues is required to bind thrombin and AT in a ternary complex (31, 32). Heparin is cleared from the body through its rapid metabolism in endothelial cells, by the liver, and also through slower renal clearance. Endothelial metabolism is through a zero order mechanism as heparin is bound by surface receptors on endothelial cells and macrophages, internalized and depolymerized into smaller oligosaccharides and renal clearance is first order mechanism. The molecular weight of a heparin, *i.e.,* UFH, LMWH or ULMWH, impacts its route of clearance and can preclude the use of a particular heparin in patients with renal failure. Heparin is administrated by *intravenously* or *subcutaneously* (*subcutaneous* bioavailability depends on a heparin's molecular weight) and the level of heparin in the circulation is monitored by the activated partial thromboplastin time assay.

6.2. Heparan sulfate

HS is a heparin-related polysaccharide localized on the surface of cells, including endothelial cells, which can contain and AT pentasaccharide binding site and exhibit anticoagulant properties. HS differs from heparin with respect to its level of sulfation and fine structure. HS contains about 0.6. sulfo groups per disaccharide repeating unit compared to heparins 2.6. sulfo groups. Less than 40% of the uronic acid residues in HS are iduronic compared to up to 90% in heparin. HS is also more polydisperse than heparin with molecular weights ranging from 10-70 kDa. HS generally has only about 10% of heparins anticoagulant activity. HS, found on the surface of endothelial cells throughout the body, is responsible for a variety of functions from inflammatory responses, regulating levels of anticoagulant activity within blood vessels, and in cell-cell communication (33).

6.3. Low molecular weight heparin

LMWH consists of smaller fragmented heparin molecules prepared through the controlled chemical or enzymatic depolymerization of UFH (Figure 4B). The depolymerization method in the production process affects generated LMWH's properties. Commonly used methods include oxidation, deamination and β-elimination. Oxidation processes generate polysaccharide molecules with both even and odd numbers of residues. Deaminative methods produce terminal anhydromannitol residues at the reducing end. Elimination methods result in the formation of an unsaturated uronic residue at the non-reducing end. More than ten LMWHs have been clinically used and they display similar biological properties. Because of their subtle structural differences, LMWHs are not clinically interchangeable. Most LMWHs have an average molecular weight between 4-5 kDa (1, 29), a longer plasma half-life, better bioavailability at low doses, as well as a more predictable dose response characteristic than UFH. This allows outpatient *subcutaneous* treatment with LMWH instead of inpatient *intravenous* administration of UFH. LMWHs have a reduced ability to inactivate thrombin as only 25% to 50% of LMWH species contain the 18 saccharide units
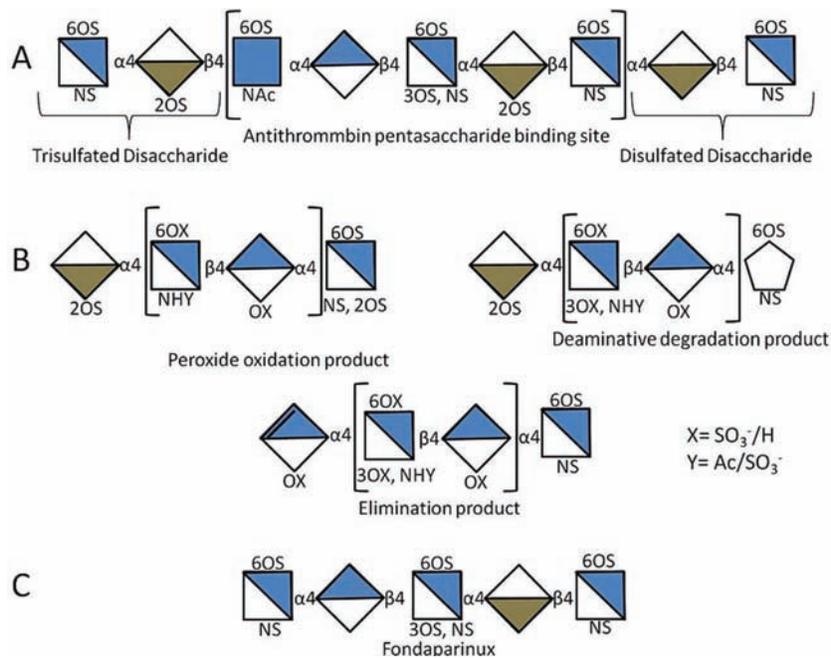
1378

Figure 4. Structure of unfractionated heparin (UFH), low molecular weight heparin (LMWH) and ultra low molecular weight heparin (ULMWH). The brackets shown indicate multiple copies of the domains. A) Major domains in UFH are labeled. There are typically a combined number of 20 to 50 copies of both trisulfated and disulfated domains in UFH. B) LMWHs synthesized using different methods. Three major methods and typical structures with reducing and non-reducing ends are shown. Approximately 7-10 of the domains, shown in brackets, are present in each LMWH. C) AT binding domain of the ULMWH, fondaparinux. The symbols used are defined in Figure 5.
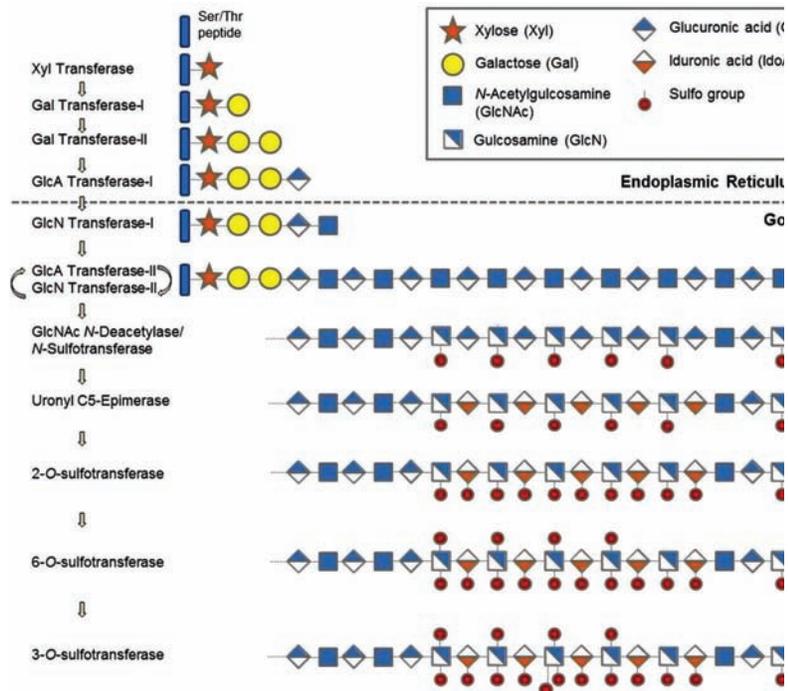
Figure 5. Biosynthetic pathway of heparin and heparan sulfate proteoglycan (HSPG). The glycosaminoglycan-protein linkage region is assembled by several glycosyltransferases. Repeating disaccharide unit is then elongated by GlcA and GlcN transferases. Next, various modifications, such as *N*-deacetylation and *N*-sulfonation of glucosamine, conversion of GlcA to IdoA, and *O*-sulfonations, take place through the actions of the specific enzymes shown.

1379

Table 1. Some of heparin's biological roles and related heparin binding proteins

Biological role	Binding protein	References
Coagulation pathway	Factors IIa (thrombin), IXa, and Xa	(45, 46, 49)
	Antithrombin (AT)	
	Protein C inhibitor	
Inflammation and angiogenesis	Platelet growth factor 4	
	Interleukin 8	
	Stromal-derived factor 1a	
	Neutrophil elastase	
Growth factor signaling	Fibroblast growth factors (FGFs)	
	Fibroblast growth factor receptors (FGFRs)	
	Endothelial growth factors (EGFs)	
	Platelet derived growth factors (PDGFs)	
Pathogen proteins	Human immunodeficiency virus (HIV)-1 envelop protein	
	Herpes simplex virus (HSV) envelop proteins	
	Hepatitis viruses (B, C and E)	

required to form a ternary complex with AT and thrombin. Despite this limitation, LMWHs still inactivate factor Xa as well as heparin, since factor Xa inactivation only requires a pentasaccharide. LMWHs also show low non-specific binding to macrophages, endothelial cells, platelets, osteoblasts, platelet factor 4 (PF4), and nonspecific binding to plasma proteins ((1, 29, 34-36) reducing many of the problems associated with heparin like shorter plasma half-lives, heparin induced thrombocytopenia (HIT), and osteoporosis ((1, 29, 37).

Since LMWHs contains chains smaller than UFHs and larger than ULMWHs it shows intermediate activities. The longer chains in a LMWH can capitalize on UFH characteristics, binding to stabilin-2 a scavenger receptor on liver endothelial cells is responsible for internalizing UFHs, or shorter chains can capitalize on ULMWHs characteristics, having excellent *subcutaneous* bioavailability. In the case of kidney failure, renal clearance is blocked or greatly reduced so the alternative routes for heparin clearance become more important. The liver plays a critical role in heparin clearance (7, 38). Liver clearance is believed to involve the stabilin-2-receptor (7). This receptor requires GAG chains larger than decasaccharides in length for binding and clearance. In UFH, most of the chains satisfy this chain size requirement In LMWH many of chains are of insufficient size for liver clearance and in ULMWH none of the chains are sufficient in size for liver clearance. Protamine, an FDA-approved drug used to

neutralize UFH, does not completely neutralize LMWHs heparins, so bleeding effects are much more likely. While the shorter sized LMWH chains pose a problem for using protamine as an antidote, they offer an advantage by reducing LMWH binding to platelet factor 4 reducing the risk of the HIT side effect (39).

6.4. Ultra low molecular weight heparin

ULMWHs, such as fondaparinux (Figure 4C), are even smaller heparin chains, many being homogenous compounds, ranging in size from 1.5.-3.5. kDa (40, 41). The advantages of ULMHs include a higher degree of bioavailability, longer plasma half-lives, lower bleeding risk, lower risk of osteoporosis, and penetration of the blood brain barrier (40, 42).

ULMWHs are pure Factro Xa inhibitors, having high anti-Xa activity but no anti-IIa activity. A phase I study implied that larger safety margins with respect to bleeding risk (43). Although these ULMWHs have some significant benefits such as no substantial binding to PF4, their drawbacks include high cost and inability to be removed by other means than renal clearance.

7. HEPARIN'S BIOLOGICAL ROLES

Heparin's major biological role is the regulation of coagulation system. Moreover, heparin and HS have a wide range of biological roles related to inflammation, angiogenesis, growth factors, developmental process, and various disease processes (44-46) (Table 1). Heparin is found in the intracellular vesicles in mast cells while the less sulfated HS, is ubiquitously distributed on various cell surfaces and in the extracellular matrix of most animal tissues. Heparin-protein interactions have been energetically investigated over the past 25 years. More than 400 of heparin/HS-binding-proteins are known and this accumulated knowledge and computational technology opened up systematic investigations into the HS-protein interactome (47, 48).

7.1. Inflammation and angiogenesis

Heparin and HS can bind to chemokines, which are a group of cytokine-like proteins involved with inflammation and angiogenesis (44). Chemokines have various functions including leucocyte degranulation and migration, selective recruitment and activation of cells, and angiogenesis promotion. On the surface of endothelial cells, HS enhances the local accumulation of chemokines and chemokine binding to G-protein-coupled receptors (44). In inflammation, PF4 is also associated with heparin and HS. One of the most serious adverse effects of clinically administered heparin is a rapid loss of platelets, resulting in HIT.

7.2. Growth factor signaling

Heparin binds to multiple families of growth factors, including fibroblast growth factors (FGFs),

endothelial growth factors (EGFs), and platelet derived growth factors (PDGFs). The 23 members of the FGF family are involved in developmental and physiological processes, including cellular proliferation and differentiation, morphogenesis, and angiogenesis. Heparin and HS activate signal transduction. HS proteoglycans on the cell's surface are required for the high affinity binding of FGFs to their family of seven fibroblast growth factor receptors (FGFRs). HS mediates the activity of FGF by inducing the dimerization FGF·FGFR, resulting in a signal transduction (50). The crystal structure revealed how heparin intimately binds to both FGF and FGFR (51, 52). Recent study showed that HS proteoglycan is responsible for accumulation of Factor XIIa, which acts as a growth factor by expressing pro-mitogenic activities (53).

7.3. Developmental process

HS participates in developmental processes. Studies in *Drosophila* indicated that HS proteoglycan is absolutely necessary in morphogen signaling pathway involving Wnt and Hedgehog (54-57). In mice, several studies demonstrated the importance of heparin/HS biosynthetic enzymes in development. A mutation in the sulfotransferase, 2-*O*-sulfotransferase (*OST*), leads to multiple abnormalities (58), while a deficiency in the glycosyltransferases Ext1 or Ext2 show embryonic lethality or exostoses, respectively (59, 60). Deficiencies in the secreted 6-*O*-endosulfatases, Sulf1 and Sulf2, display that these enzymes, while redundant, are essential for the survival of neonatal mice (61).

7.4. Various disease processes

Heparin and HS are related to a variety of disease processes. Thrombin, the key factor in the coagulation pathway, plays a role in cancer progression. Thus, the antitumor effects of anticoagulants have been investigated (62). A meta-analysis of clinical trials of heparin's antitumor activity has shown that among anticoagulants, LMWH in particular, improved the survival period of cancer patients, but increased the risks for bleeding complications (63). The remaining important questions for exploiting heparin as a cancer therapeutic includes the optimal types of cancer for its use, the use of non-anticoagulant heparin, the safety of heparin's long-term use, the influence of cancer stage, and duration of heparin treatment. In addition, the complicated mechanism of heparin's antitumor activity remains to be elucidated.

Heparin and HS have also been proposed as therapeutic agents for the treatment of Alzheimer's disease. The precise pathological role of HS-proteoglycans in Alzheimer's disease remains elusive, however, possible protective mechanisms include reduction of amyloid β-peptide (Aβ) generation, prevention of Aβ aggregation and deposition, attenuation of Aβ's toxic effects and acceleration of Aβ removal (64).

Heparin and HS also function as pathogen receptors (49). Since HS is found on the external surface of the cells, a viral coat protein may bind HS and help the virus invade. The interaction of heparin or HS with human immunodeficiency virus (HIV) and herpes simplex virus (HSV) have been attracted much attention.

8. HEPARIN'S THERAPEUTIC APPLICATIONS

Heparin is a complicated drug to use therapeutically. Its primary use is as an essential component of extracorporeal therapy to maintain blood flow in kidney dialysis and heart-lung oxygenation. It has its uses as a drug that can be used to treat and/or prevent deep vein thrombosis, pulmonary embolism, ischemic complications of unstable angina and other diseases related to anticoagulation. It is also used in general medical procedures like surgery and implantation. Furthermore, heparins' anti-inflammatory effect has been investigated to treat allergic asthma, allergic rhinitis and similar diseases.

UFH is mainly administrated *intravenously* while LMWH and ULMWH are mainly administrated *subcutaneously*. Other routes of heparin administration that have been explored include oral, intranasal, inhalation and even transdermal but its low bioavailability by these routes generally precludes these routes.

8.1. Heparin's anti-thrombotic and anti-embolytic therapeutic applications

With deep vein thrombosis and pulmonary embolism there are various dosing requirements depending on the severity of illness, the heparin being administered, and other preexisting medical problems. For the initial treatment of if there is intermediate risk of pulmonary embolism, *intravenous* or *subcutaneous* UFH, or *subcutaneous* LMWH heparin is administered over the first 5 to 10 days (14, 65, 66). The dosing is generally 170-200 IU/kg subcutaneously for LMWH and 230-300 IU/kg for UFH (14, 66, 67). These can be given as one dose or split into two smaller doses twice daily. After this initial period the patient can be transitioned to VKAs or a newer oral anticoagulant. This treatment normally continues for three months or longer to ensure that the risk has been reduced (14, 65). Risk is determined based on an individual patient's chance of recurrence and their bleeding risk. In high-risk situations where pulmonary embolism has been triggered by shock or hypertension the treatment parameters can be different. The initial treatment is an immediate *intravenous* bolus of UFH, then with thrombolytic therapy, surgical or catheter pulmonary embolectomy, followed by the same three-month treatment used in lower risk patients (66).

Special cases of thrombolic pulmonary embolism, such as in patients with cancer or pregnant, have to be treated differently (14, 65, 66). LMWHs are

highly recommended in pregnant patients as they can be *subcutaneously* administered twice a day, forgoing the use of VKAs. The twice-daily administration of LMWHs balances the peaks and dips in the plasma levels of LMWH (14). The use of VKAs are contraindicated by pregnancy as there are side effects including teratogenic effects in the first trimester, and fetal or neonatal intracranial bleeding in the third trimester since VKAs can cross the blood brain barrier (14, 66). VKAs can be used after pregnancy even to breast feeding mothers (66). In cancer patients the initial treatment time is extended 3-6 months with LMWH or VKAs but can continue indefinitely if the cancer is not cured since venous thromboembolism is 4-times more likely in cancer patients. If the cancer is cured or is in remission treatment is normally continued for 6 months. Heparin has also been shown to exhibit anti-neoplastic properties (68). This is largely due to heparins anti-angiogenic properties, which help to inhibit tumor formation, since tumor growth beyond a size of 1 mm^3 is dependent on angiogenesis (68).

Ischemic complications of acute coronary syndrome, which includes myocardial infarction and unstable angina, can be prevented with heparin. Heparin is not the primary source of treatment and is normally used in conjunction with aspirin to inhibit platelet activation and prevent the growth of plaques or a vasodialator, like nitroglycerine, to widen the blood vessels. Both heparin and LMWH have been shown to reduce the recurrence rate of angina and myocardial infarction (69, 70). Despite the minor advantages in reduced angina recurrence with UFH, compared to LMWH, UFH results in a greater increase in the risk of HIT (71). Heparin also dampens the coagulation reactions to levels similar to patients with stable coronary artery disease (70). Similarly the thromboembolic complications associated with atrial fibrillation can also be treated and prevented with heparin, reducing the risk of stroke (72, 73).

8.2. Heparin's anti-inflammatory therapeutic applications

In addition to the *intravenous* and *subcutaneous* routes of heparin administration, heparin has also been used intranasaly. The intranasal effects of heparin, while not related to anticoagulation, are still important. Heparin administered intranasaly has been shown to reduce inflammation (74-77). Heparin inhibits changes in nasal airway pressure, leukocyte infiltration, eosinophil cell migration and eosinophil cationic proteins (75, 77). These properties along with inhibition of mast cell-endothelial cell interaction and the reduction of methacholine hypersensitivity also show potential for heparin to treat diseases related to allergic responses like asthma, allergic rhinitis and many more (75, 77). The dose for this type of anti-inflammatory response can be as much as 1000 IU/kg with lower doses 300 IU/kg not exhibiting the same airway response for asthma patients (74, 75). Despite the uses of large doses and repeated doses

the clotting parameters were unchanged (76), reducing the risk of adverse side effects like bleeding. Similarly heparin is much milder than steroids potentially making it a viable alternative to current asthma treatment (74).

9. ADVERSE REACTIONS

Heparin's most well-known adverse reactions are hemorrhage, HIT, osteoporosis, general hypersensitivity reactions, and elevations of aminotransferase levels (78-80).

9.1. Hemorrhage

Since heparins work as anticoagulants, bleeding or hemorrhage complication is expected. Bleeding, or hemorrhage is one of the major adverse reactions (>2%) of heparins. Hemorrhage site includes adrenal gland, ovary, retroperitoneal area, but hemorrhage can occur virtually anywhere in the patient. The highest risk of bleeding reported for UFH is in women over 60 years of age (78) and for patients with cardiovascular, hematologic and gastrointestinal diseases and those with a hereditary AT deficiency.

Neurological impairment resulted from spinal or epidural hematomas may occur. Indeed, one ULMWH medical drug label states that "Epidural or spinal hematomas may occur in patients who are anticoagulated with LMWH, heparinoids, or fondaparinux sodium and are receiving neuraxial anesthesia or undergoing spinal puncture. These hematomas may result in long-term or permanent paralysis" (80).

Clinical trials of the LMWH Lovenox® (enoxaparin sodium) showed that both Lovenox and UFH have the similar rate of major bleeding events (79). In addition, another set of clinical trials of the ULMWH, Arixtra® (fondaparinux sodium), reported that the rates of major and minor bleeding between the ULMWH Arixtra and LMWHs (enoxaparin sodium or dalteparin sodium) are similar (80).

9.2. Heparin-induced thrombocytopenia

HIT is a serious antibody-associated reaction resulting in abnormal and irreversible aggregation of platelets, leading to thromboembolic events and potential death. HIT can occur several weeks after the discontinuation of heparin treatment. The antibody that triggers HIT reacts with a complex formed between UFH (or LMWH) and a partially unfolded conformation of the chemokine PF4 (39). Interaction with the platelet monocyte Fc receptors leads to pro-coagulant factor release and thrombin generation (81). However, the precise mechanistic details of developing the heparin-PF4 immune response and subsequent HIT remain elusive.

Platelet count and pre-test clinical scoring systems are used in the diagnosis of HIT. Recently, the

HIT expert probability score was developed (82). This score focuses on 8 clinical features and each feature has a point range from -3 to +3. Laboratory tests can then be used to confirm the presence of PF4/heparin antibodies.

According to a meta-analysis, LMWH had statistically significant lower risk of HIT over UFH (P <.001), and the absolute risk for HIT with UFH and LMWH, determined by inverse variance weighted average, are 2.6.% and 0.2.%, respectively (83). In this meta-analysis, HIT was defined as a "decrease in platelets greater than 50% or to less than 100 x 10^9/L and positive laboratory HIT assay". It is noteworthy that while patients treated with heparin frequently demonstrated thrombocytopenia, HIT accounts for only a small fraction of these cases. The same meta-analysis showed that the total events of HIT for UFH and LMWH are 31/1255 patients and 1/1255 patients, respectively, while the total events of thrombocytopenia for UFH and LMWH are 238/3758 and 152/3758, respectively (83).

UFH and LMWH exhibit the similar risk of thrombocytopenia. In clinical trials, moderate thrombocytopenia (platelet counts between 50,000 to 100,000/mm^3) occurred at 1.2. % in UFH, at 1.3. % in LMWH and at 0.7.% in placebo. Severe thrombocytopenia (platelet counts less than 50,000/mm^3) occurred at 0.2. % in UFH, at 0.1. % in LMWH and at 0.4.% in placebo (79). As for ULMWH, another set of clinical trials reported that the rate of moderate thrombocytopenia in patients given Arixtra® occurred 0.5.% and severe thrombocytopenia occurred at 0.0.4% (80).

The definition of HIT or thrombocytopenia varies in clinical studies, and sometimes is not clearly delineated. Another limitation is that many studies evaluate the risk of thrombocytopenia but not the risk of HIT. It is often difficult to compare the rates of adverse reactions for different drugs since clinical trials are often conducted under different conditions.

9.3. Osteoporosis

Long-term use of heparin can cause osteoporosis and increases fracture risk. This is because long-term use leads to reductions in bone-mineral density. The reason of UFH-induced osteoporosis is that UFH inhibits osteoblast differentiation and its function, which prevents bone formation (84). In addition, UFH accelerates bone resorption by reducing osteoclast differentiation controlling factor. The risk of osteoporosis associated with LMWH's has not yet been evaluated well because there is a scarcity of long-term data. It is possible to speculate that ULMWH may be better than UFH and show a reduced risk of osteoporosis, as fondaparinux does not inhibit human osteoblast cell proliferation *in vitro* ((85, 86). However, appropriate clinical studies are needed to verify this hypothesis.

9.4. Others

Both UFH and LMWH can cause an increase in aspartate (AST (SGOT)) and alanine (ALT (SGPT)) aminotransferase levels, but to levels expected to be asymptomatic. The rates of these elevations are reported that 5.9.% or 6.1. % of patients with UFH or Lovenox, respectively (79). Other adverse reactions of heparin include local irritation, hypersensitivity and delayed transient alopecia.

10. FUTURE PROSPECTS

For the foreseeable future heparins will continue to serve crucial roles in anticoagulant therapy needed in modern medicine and remain largely unthreatened from competition from other types of anticoagulants. Thus, the future of heparins relies less on the improvement of their properties and competition than on the heparin's continued availability in sufficient quantities in high quality at reasonable costs. Currently, most of the heparin approved for use in the U.S. and worldwide is prepared from the intestinal mucosa of Chinese pigs. Most of the pigs raised worldwide are already used in the production of heparin. As modern medical procedures (i.e., hemodialysis, open-heart surgery, etc.,) become increasingly needed and increasingly available in second-world and third-world countries the demand for heparin-based products will outstrip their supply.

10.1. Risks associated with porcine and bovine derived heparins

Due to the pivotal role of heparin and LMWH in the anticoagulant market there are concerns about sourcing, processing and the quality of heparin active pharmaceutical ingredient (API). Porcine intestinal heparin produced in China accounts for more than 50% of the heparin API worldwide (5). This single source raises major concerns (5, 30, 87). First, sourcing of heparin API from a single species could lead to shortages if a disease (i.e., porcine reproductive and respiratory disease syndrome (also called as pig blue ear disease) (88) or porcine epidemic diarrhea virus (89), etc.,) decreases the number of animals available from which to prepare heparin API (87). Second, without a domestic supply, there could be a severe shortage of heparin API in the US since China controls a majority of the worldwide market. Moreover, low regulatory control in China's food and drug industry is, although the heparin supply chain has been safe over the years, believed to be partly responsible for the 2007 contamination crisis involving the adulteration of Chinese porcine intestinal heparin with oversulfated chondroitin sulfate leading to deaths in the Americas, Europe, and Asia (2, 5, 30). Oversulfated chondroitin sulfate tightly binds to FXIIa enhancing the production of vasoactive bradykinin causing severe hypotensive effects (2). One solution to these problems is the introduction of new sources of heparin API. The FDA has shown interest in reintroducing bovine heparin

to diversify the market but there would need to be a method of mitigating the risk of contamination (5). Thus, further investigation is required to determine differences in structure, composition, activity and risks associated with cows or other animal sources.

10.2. Comparison of porcine and bovine derived heparins

The structure and activity of both bovine intestinal and bovine lung heparins have been under intensive investigation and these bovine heparins exhibit different structure and activity than porcine intestinal heparin. Bovine intestinal heparin is generally less sulfated and more heterogeneous than porcine heparin (90). Furthermore bovine intestinal heparin has a lower molecular weight and is more polydisperse than porcine heparin (90). These results show a larger inherent variability in the bovine intestinal heparin physically and chemically. Porcine intestinal heparin displays a lower glucuronic acid content and higher GlcNS3S6S than bovine intestinal heparin suggesting that they undergo different levels of biosynthetic modification (90). Porcine intestinal heparin also shows significantly higher activity than bovine intestinal heparin (37, 90). Bovine intestinal heparin requires twice the dose of porcine intestinal heparin to obtain the same antithrombotic effect, however, the bleeding risks between the two are comparable at similar doses (90). Bovine intestinal heparin also requires higher doses of the antidote, protamine, in order to be neutralized (90). Bovine lung heparin is also distinctly different from porcine intestinal heparin, having higher levels of *N*-sulfo and *O*-sulfo groups, a lower average molecular weight and reduced anticoagulant activity (30). While comparison studies on heparins derived from different animal species and tissues continue, it is becoming clear that these are not equivalent drugs and will require different monographs, and will not be easily interchanged by physicians administering these anticoagulants.

Different production issues also come into play for heparin API derived from different species and tissues (91). Since the processes used for isolation and purification are different, process impurities might be encountered. Moreover, cows are susceptible to "mad cow disease" or bovine spongiform encephalopathy (BSE) that can cause Creutzfeldt-Jakob disease (CJD) in humans (5). Bovine lung heparin was once used in the U.S. but was voluntarily withdrawn from the U.S. market following an outbreak BSE and CJD in Europe in the 1990s (5). New requirements for the slaughtering of cattle may be required if a bovine sourced heparin API were to be reintroduced into the U.S. market. Other food animal sources might also possible but each new source will undoubtedly encounter similar problems.

10.3. Bioengineered heparin

Bioengineered heparin, made from non-animal source materials, has been proposed to address the current issues surrounding the supply and quality of heparin API. Bioengineered heparin is a synthetic heparin, relying on chemoenzymatic synthesis, and designed to be equivalent (a generic version) to animal-based heparin API (91, 92). Despite the clear advantages of a bioengineered heparin API (i.e. elimination of virus/prion impurities, better controlled process, independence from sourcing from a single species or country) the challenges are numerous. These include: a complicated multi-step process to match heparin's complex structure and heterogeneity in chain length and sulfation patterns; the large (multi-ton) quantities of heparin required; the relatively low cost of heparin API ($15-20/g); and development costs and regulatory hurdles.

The scheme currently proposed for the preparation of bioengineered heparin can be divided into three parts, up-stream, mid-stream and down-stream (91, 92). The upstream portion of the scheme uses a fermentation of *E. coli* K5 strain to prepare the heparin's polysaccharide backbone, heparosan. The midstream portion of the scheme involves the chemical conversions of *N*-acetyl heparosan into an *N*-acetyl, *N*-sulfo heparosan of the appropriate molecular weight and composition. The downstream portion of the scheme involves a group of enzymatic modifications, C5-epimerization, 2-*O*-sulfation, 6-*O*-sulfation, and 3-*O*-sulfation to afford a heparin's chemical structure (Figure 6). These reactions mimic heparin's biosynthetic pathway occurring within the Golgi but without using any animal sourced materials.

Our laboratory is actively developing a process to prepare bioengineered heparin. Improvements in the up-stream portion of the scheme include increase yields of crude heparosan of up to 17 g/L (93, 94). Metabolic engineering is also being investigated to enhance heparosan biosynthesis (94, 95). The mid-stream chemical process step parameters have been statistically examined, using response surface method, to enhance the control of the reaction conditions to obtain the *N*-acetyl, *N*-sulfo heparosan intermediate having the desired structural characteristics (96). The down-stream portion of the scheme has been improved through the high-cell density cultivation of the *E. coli* to express larger amounts of the biosynthetic enzymes (97-99), the removal of endotoxins, associated with *E. coli* produced heparosan and biosynthetic enzymes have been examined (100), as have the covalent immobilization of these enzymes (101), and the preliminary study of reduction of enzymatic process steps (102).

Future challenges of bioengineered heparin include successful synthesis of bioengineered heparin which is physicochemically and biologically equivalent to heparin API produced from porcine intestine, process development to produce bioengineered heparin with a commercially feasible method and affordable cost, and
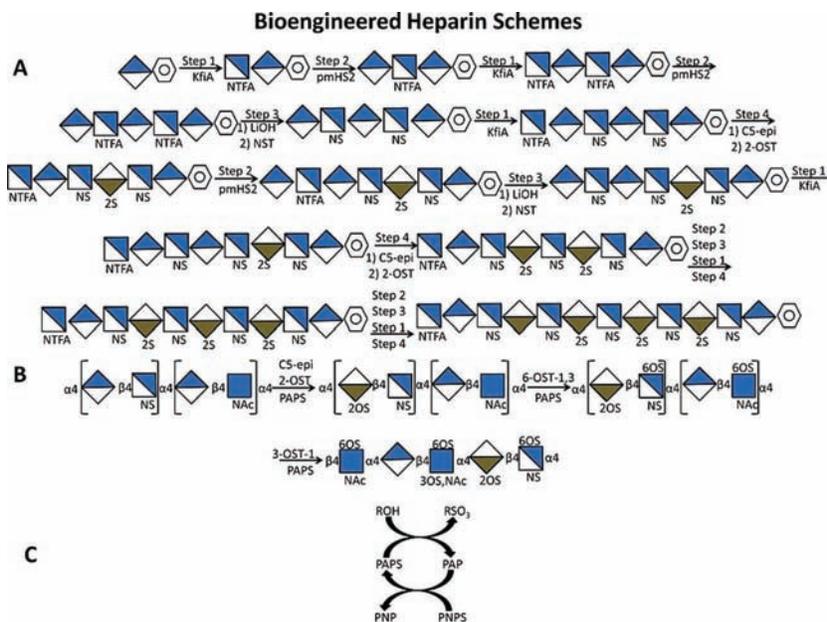
Figure 6. Chemoenzymatic synthesis of heparin. A) Homogenous low molecular weight heparin. B) Enzymatic portion of the bioengineered heparin scheme. C) Cofactor recycling where 3'-phosphoadenosine-5'-phosphosulfate (PAPS) is generated from 3'-phosphoadenosine-5'-phosphate (PAP) using aryl sulfotransferase IV and *p*-nitrophennol sulfate (PNPS) as a sacrificial sulfo group donor affording *p*-nitrophennol (PNP). The symbols used are defined in Figure 5.

a scale-up to more than kilogram batch with high quality (e.g. purity > 99.5% and no unknown impurity exceeding 0.1.% (103)). The regeneration of 3'-phosphoadenosine-5'-phosphosulfate (PAPS), which is an expensive sulfo donor for the enzymatic *O*-sulfation, is a typical approach to reduce the cost of material (Figure 6) (91).

10.4. Synthetic heparin oligosaccharides

Heparin oligosaccharide synthesis has also received significant attention. By using a chemoenzymatic approach, more than 30 heparin/HS oligosaccharide with various chain lengths and sulfation patterns have been synthesized (104). Three recent examples are described here. In the first example, a homogenous heptasaccharide was synthesized in 10 enzymatic steps starting from a simple disaccharide with 43% recovery yield (105). The heptasaccharide had a similar *in vitro* anti-Xa activity and pharmacokinetic profiles in rabbits to that of fondaparinux (ULMWH), a chemically synthesized homogenous pentasaccharide, which is currently a clinically used drug. In the second study, a new LMWH, a homogenous dodecasaccharide, with better pharmaceutical profiles than current clinically used LMWH, was chemoenzymatically synthesized (7) (Figure 6). Current clinically used LMWHs have a number of major drawbacks, as stated above. These are not completely neutralized with FDA-approved antidote, protamine, while UFH can be completely neutralized and these LMWHs can only be at reduced doses in renal-impaired patients, since LMWH is partially excreted through kidney. The chemoenzymatically synthesized

dodecasaccharide, prepared in 22 steps in an overall yield of 10%, showed protamine reversible activity. In addition, this compound has been metabolized in liver in mouse model. In a final example, highly purified heparin-oligosaccharides having up to 21 saccharide residues have been synthesized (106). Interestingly, this study suggests that the minimum length for a heparin to possess anti-IIa activity is 19 saccharide residues (molecular weight ~ 3850).

Purely chemical approaches have also been demonstrated to synthesize heparin oligosaccharides. These long and elaborate chemical syntheses require highly specialized techniques, making the resulting products very expensive. It is possible to synthesize heparin oligosacchardies containing tailor-made unnatural saccharide residues, which are difficult to synthesize with an enzymatic approach due to a high level of enzyme substrate specificity. A recent study, for example, utilized iterative combination of three tetrasaccharide modules to chemically synthesize a dodecasaccharide heparin-like molecule (107). Study of heparin oligosaccharide synthesis providing the tools to develop new heparin-based oligosaccharide therapeutics and improves our understanding of the structure and function relationships of heparin.

11. CONCLUSIONS

Heparin-based anticoagulants are an essential component of modern medicine. Despite the longevity

of heparin as a drug, the prospects for its future use are quite good. Some improvements of heparin-based therapeutics are still needed and will undoubtedly transpire in the next decade. A more immediate concern is meeting the world's needs for safe, high quality and relatively inexpensive sources of this critical life-saving drug.

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Abbreviations: UFH: unfractionated heparin; LMWH: low-molecular-weight heparin; HSPG: heparan sulfate proteoglycan; vWF: Von Willebrand factor; GP: glycoprotein; ADP: adenosine diphosphate; TXA2: thromboxane A2; TF: tissue factor; AT: antithrombin; HS: heparan sulfate; DXI: direct factor Xa inhibitor; DTI: direct thrombin inhibitor; VKA: vitamin K antagonist; t-PA: tissue plasminogen activator; ECMO: extracorporeal membrane oxygenators; DIC: disseminated intravascular coagulation; ULMWH: ultra-low-molecular weight heparin; FDA: Food and Drug Administration; GAG: glycosaminoglycan; PF4: platelet factor 4; HIT: heparin induced thrombocytopenia; FGF: fibroblast growth factor; EGF: endothelial growth factor; PDGF: platelet derived growth factors; FGFR: fibroblast growth factor receptors; OST: *O*-sulfotransferase; HIV: human immunodeficiency virus; HSV: herpes simplex virus; API: active pharmaceutical ingredient; BSE: bovine spongiform encephalopathy; CJD: Creutzfeldt-Jakob disease

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