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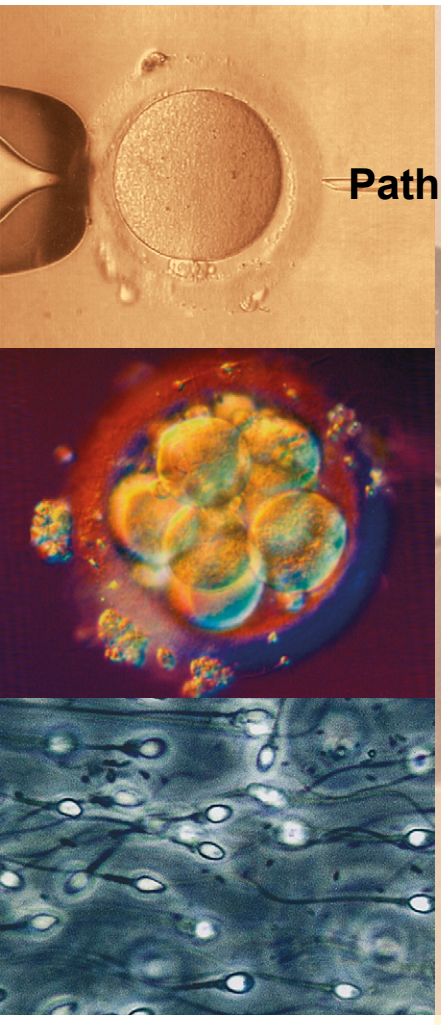

Heparin and Related Substances: Physiology and Pathophysiology in General and in Human Reproduction - Part 1

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Heparin and Related Substances: Physiology and Pathophysiology in General and in Human Reproduction – Part 1*

W. Würfel

Although heparin is usually associated with blood clotting and thrombosis prevention, its anti-coagulatory properties play an extremely significant role in clinical routine. However, it appears to have little importance in rheological homeostatis, particularly since heparin and related substances can also be found in simpler organisms without clotting mechanisms. In fact, heparin and heparan sulfate glycans are proteoglycans that have a long history in the development of species. Accordingly, their primary effects are electrostatic, not receptor-mediated, and they exhibit binding via their pronounced electronegativity. They exist in free form and as membrane-bound molecules. In physiological terms, heparin and heparinoids are immunological co-factors involved in inflammation and wound healing; they play significant roles in embryo development and organogenesis, serve as "homing factors," and are involved at various points in carcinogenesis. They show diverse interactions with cytokines, growth factors and other mediators that are important in the implantation process and its control and continue to be so throughout pregnancy. It can be assumed that reproduction is a key focus of physiological functions; this would explain why heparin is so effective above and beyond its anti-coagulatory effects, e.g. in treating anti-phospholipid syndrome. To date no clear confirmation of the existence of a heparin deficiency syndrome has been possible, although initial results from studies concerning the general administration of heparin during pregnancy point in this direction. **J Reproduktionsmed Endokrinol_Online 2016; 13 (2): 38–48.**

Key words: heparin, heparinoids, heparan sulfate glycans, history, measurement, human reproduction, rheology, endometrium, embryonic and fetal development, immunology, antiphospolipid syndrome, cancer

Introduction

Heparin is used routinely as a "blood thinner," and heparin's anti-coagulatory properties are doubtless its most prominent characteristic [1]. However, heparin and heparan sulfate glycans can also be found – in some cases, in high concentrations – in organisms without a blood clotting system [1], such as fresh-water clams [2], prawns [3], and other invertebrates [1]. This indicates on the one hand that the anti-coagulatory properties of heparin (or related substances) reflect only part of their physiological significance [4, 5], while also proving that this family of substances has an extremely long phylogenetic history [4, 5].

History of the Discovery of Heparin

Jay McLean (1890–1957), a 4th-semester student at John Hopkins University under Professor William Howell, had been assigned the task of extracting a stable thrombophilic substance based on cephalin prepared by Howell from rabbit brains [6, 7]. He used procedures already described by Erlandsen in 1907 for isolating cuorin (from ox heart) [8], and by Baskoff for identifying heparophosphatides (from horse liver) [9]. However, the ether-based extraction performed by McLean in 1916 resulted in the isolation not of a thromboplastic, but an anti-coagulant substance, initially believed by McLean to be anti-thrombin [10]. Admittedly, in his memoirs he claims to have soon realized the substance was a heparophosphatide and cites this as the reason for its later name of heparin [6]. The name was first used in 1918, by which time McLean had left the university for financial reasons [11]; his work was continued under William Howell, primarily by Emmett Holt jr. [12]. However, the fat-soluble substance extracted from canine liver by Holt and named "heparin" naturally had a different biochemical makeup from McLean's heparophosphatide. In 1922 the the two scientists and their working group described a water-soluble polysaccharide [11], presenting a more advanced version in 1925 [13]. Clinical tests were performed at institutions including the Mayo Clinic and revealed frequent side-effects such as headache, fever and nausea [14]. Yet heparin was commercially available from 1924, albeit without any significant medical role. In Canada, a group of scientists which formed around Charles Best from 1929 [15] published an improved procedure for isolating heparin from horse liver in 1933 (Arthur Charles und David Scott, 1933 [16–18]). This heparin, produced by Connaught Laboratories in Toronto, had significantly fewer side-effects, although still proving relatively toxic in individual cases [15]. Not until 1937 was a crystalline heparin produced, virtually free from side-effects and linked with the name of Gordon Murray [19, 20]. Its molecular structure was identified in 1935 by Eric Jorpes from the Karolinska Institute in Sweden, who had visited the Connaught Laboratories around 1929 [21]. One year later, the company Vitrum AB launched the first heparin for IV use, which was still relatively toxic [22]. The development of heparin preparations for human use that were largely free from side-effects took place in parallel in Canada and Sweden.

Jay McLean later also resumed his study of heparin, focusing on its clinical use in cases such as venous occlusion and endocarditis [23, 24].

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Molecular Structure

Heparin and its related substances such as heparan sulfate are proteoglycans, or "protein-carbohydrate hormones" (proteo-polysaccharides). These polysaccharides form linear chains in a polydisperse structure; the main constituents are pyranosyl-uronic acid (L-iduronic acid + D-glucoronic acid) + 2-amino glucosamine (Fig. 1), with variable acetyl or sulfo groups [25].

Heparan sulfates show far higher variability than heparin itself, with dominating glucoronic acid and reduced sulfo groups [26].

For this reason the level of sulfation is a popular method for distinguishing between heparin and heparan sulfates; however, no precise biochemical distinction has ever been successfully made.

Heparan sulfate proteoglycans (HSPG) are classified into three families based on their biochemical and/or physiological characteristics:
- Membrane-spanning HSPGs (e. g. syndecan [27], betaglycan [28] and CD 44 [29])
- Glycophosphatidylinositol-anchored HSPGs (e.g. glypicans [30])
- ECM (extracellular matrix) proteoglycans (e.g. agrin [31], perlecan [32] and collagen XVII [33]), which may be soluble or transmembrane (such as collagen XVII)

See also Figure 2.

Heparin and heparan sulfate glycans both belong to the family of glycosaminoglycans (GAGs) and have the highest electronegative charge known of any physiological molecules in our organism [4]: heparin has approx. 2.7 negatively charged groups per disaccharide (sulfo and carboxyl groups) whereas HSPGs have fewer than two. Accordingly, ion binding and not receptor binding is the focus of interaction with other molecules, and thus also of mediation of the effect. This makes clear that heparin/HSPG activity is strongly dependent on molecule size.

For example, a minimum of 17–18 saccharide groups are required for sufficient bioactivity response to thrombin (coagulant factor II). This does not apply to, say, ultra-low-molecular-weight (ulmw) heparins, so that their "general" bioactivity is lower than that of native heparin. However, the anticoagulation effect of ulmw heparins is only selective and this is often delivered via pentasaccharide, the "core ligand" (see below).

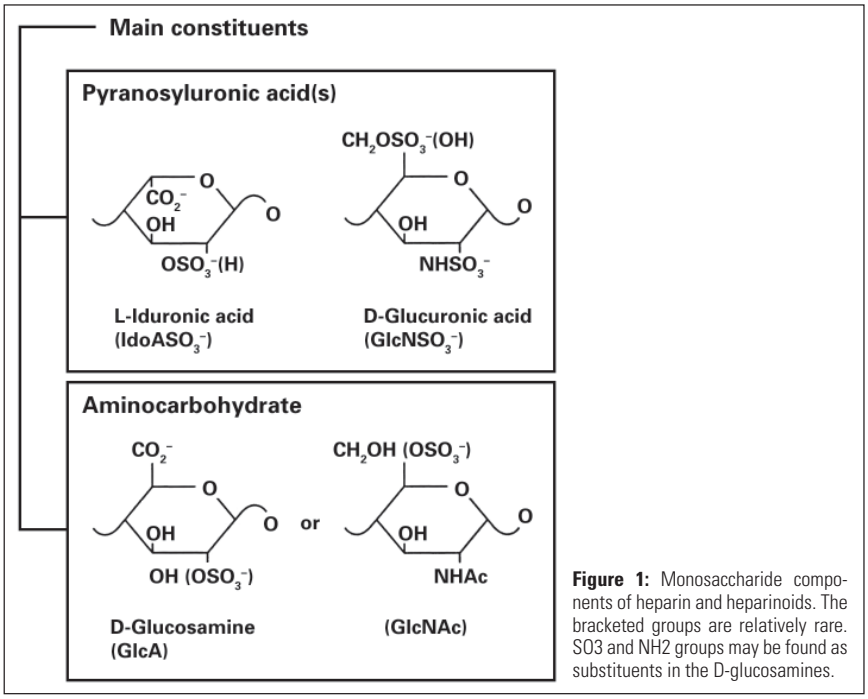

Figure 1: Monosaccharide components of heparin and heparinoids. The bracketed groups are relatively rare. SO3 and NH2 groups may be found as substituents in the D-glucosamines.

The high electronegative charge results in high binding affinity which extends to all proteins, particularly:
- Growth factors [35, 36]
- Chemokines and cytokines [37, 38]
- Lipoproteins [39]
- Receptor (protein) complexes [40]
- Proteases/esterases and their inhibitors [41, 42]
- Enzymes [43, 44]
- Viral coat proteins [45, 46]

An overview is given in Table 1.

As already mentioned, a type of ligand/receptor-mediated interaction exists, based on that of L-iduronic acid and its steric plasticity, creating a definable pentagonal structure: GlcNAc/NS(6S) – GlcA-GlcNS(3S,6S)-IdoA(2S)-GlcNS(6S) [48]. It is named DEFGH (the letters are historical abbreviations of the active groups or molecular positions in this pentasaccharide) [49], and is also known as high-affinity heparin (HAH). This pentasaccharide is essential for clinically significant interaction with activated coagulation factor Xa.

Where highly selective Xa suppression is desired, this pentasaccharide is actually all that is needed – the rationale behind the develop of the synthetic "heparin" Fondaparinux (Arixtra®) [50].

Only around 30% of naturally occurring heparins function using HAH, while around 70% use ion binding [50].

Synthesis of Heparin and Heparan Sulfates

Heparin is present in humans in the liver (approx. 25 IU/100 mg), lungs, and muscular tissue, and at lower levels also in the intestine (intestinal mucous membrane), spleen and thymus gland [25]. Similar conclusions were reached by Charles and Scott in 1933 [17], who also found that heparin is virtually undetectable in serum – contrary to Howell's assumptions [12]. Synthesis can be clearly detected in neutrophilous granulocytes and – especially – in mastocytes, i.e. immune system cells, and in endothelial cells [25, 51].

Synthesis of heparan sulfates can be proven for an extremely large number of cell types, which is only logical given their importance for membranes and the extracellular matrix (ECM) [51].

Intracellular synthesis occurs in the Golgi apparatus. The initial step is synthesis of a tetrasaccharide comprising glucuronic acid, 2 molecules of galactose and a molecule of xylose bonded to a core protein [52]. When chain formation is

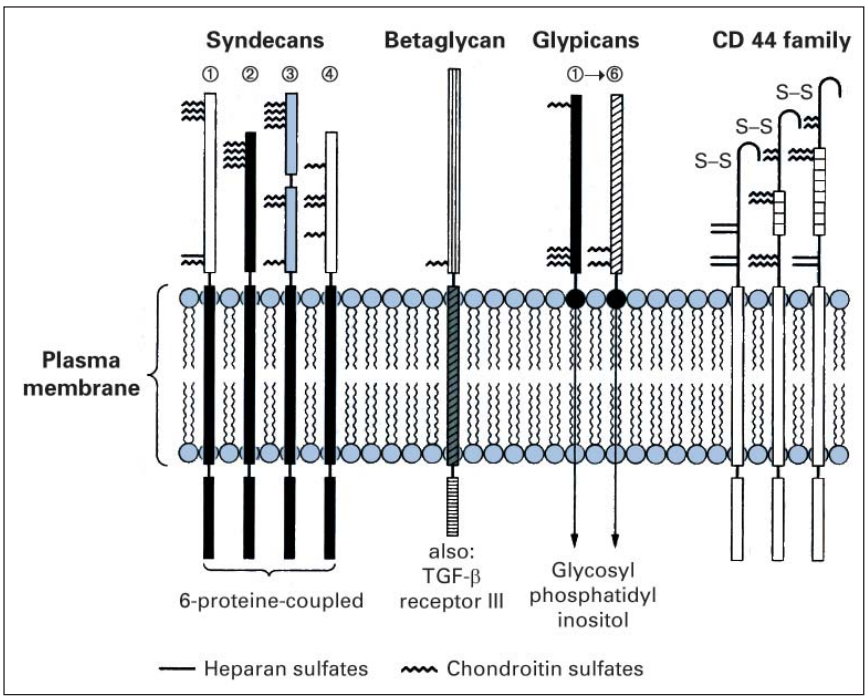

Figure 2: The diagram shows transmembrane heparan sulfates each bound to protein complexes. Collagen XVII, which also has a transmembrane form, is not shown. Mod. from [4].

initiated, further saccharides are gradually added to form a polymer. In a third stage, the chain is modified, e.g. by sulfation or epimerization (into iduronic acid) [52].

The synthesis paths of heparin and heparan sulfates are thus virtually identical, making it harder to provide evidence of types of tissue where heparin synthesis does and does not take place. One theory, certainly worthy of serious consideration, states that synthesis takes place only in immunocompetent cells and in the endothelium (see below).

Unfractionated heparin (uf heparin) has a molecular weight of approx. 12–40 kilodaltons (kDa), with an average of approx. 14 kDa [50]. Uf heparins for clinical use are generally produced from bovine lung or porcine (intestinal) mucous membrane; biological activity of both organisms is similar, although the mean molecular weight of bovine heparins is slightly higher [53].

Low-molecular-weight heparin (lmw heparin), i.e. "fractionated" heparin, is produced from uf heparin (preferably porcine) by means of chemical or enzymatic depolymerization (heparinases, see below) and various fractionation methods [54]. Uf heparin already contains around 20–30% lmw heparins [53]. Low-molecular-weight heparins have better bioavailability and longer-lasting effects, and also cause fewer undesirable side-effects – presumably because they undergo far less (unspecific) binding with proteins, tissues and, primarily, blood cells (principally HIT-2, see below) [55]. Anticoagulation activity generates 80–120 anti-Xa units (i.e. U/mg or 35–45 anti-IIa U/mg [55]).

It is the uf heparins that show varying pharmacological and anti-coagulatory properties depending on their source material and production method. Anti-coagulatory units (IU) were defined to guarantee comparability of anti-thrombotic effects and express the in-vitro effect (5th International Standard for Unfractionated Heparin, NIBSC-Code 97/578). According to the standard, parenterally applied heparin must show specific anticoagulatory activity of 150 IU/mg.

By contrast, anti-Xa activity is used as the basis for measuring effectiveness of lmw heparins (aXa-IU/mg); anti-IIa activity (aIIa-IU/mg) and the ratio of aXa/aIIa must also be stated [56].

A definition of biological activity is difficult, even from a historical perspective. The "Howell unit" was the first attempt, defining the quantity of uf heparin necessary to prevent coagulation of 1 ml of feline blood for 24 h at 0 degrees, i.e. approx. 0.002 mg [57]. The United States Pharmacopoeia Unit (USP Unit) defines it differently, as the quantity of heparin necessary to cause only 50% of coagulation in 1 ml of ovine plasma in 1 h at 37 degrees Celsius [57].

Biodegradation of Heparin and Heparan sulfates

Heparan sulfates are broken down in the lysosomes. At present the assumption is a single heparanase (Hpa-1); the coded gene is located at chromosome 4 (q22) (approx. 40–50 kDa) [58]. A number of splicing variations exist [59]. Evidence of the presence of Hpa-1 is practically confined to lymphoid tissue, so that its effects can be assumed to be largely extracellular, e.g. in inflammation processes or during embryonal implantation [60]. Heparanase activity is also shown by CTAP-III (connective tissue activating peptide), present in thrombocytes [61], and by Hpa-2, shown to be present in non-lymphoid tissue [62]. The extent to which these are independent biodegradation pathways is the subject of scientific debate.

Numerous other enzymes are involved in biodegradation, including sulfatases, an iduronidase and a glucuronidase [60].

Hpa-1 is overexpressed in some human malignomas [63], with the degree of overexpression correlating to the negativity of the prognosis [64]. One reason could be that Hpa-1 biodegrades the heparan sulfates in basal membranes and thus promotes invasion of the malignoma [65].

A distinction must be made between heparanases and heparinases [66]; the latter were first identified in Pedobacter heparinus (previously Flavobacterium heparinum) and other sources [67]. 3 different heparinases – I, II and III (synonyms: heparin lyases or heparitinases). They are able to break down polymers at different points [66, 67].

In humans, heparin is primarily broken down in the liver (lysosomes), but also in the kidney, in a process described as uroheparinase [67]. 20–30 % of heparin is excreted unaltered; the degradation products of heparin and heparan sulfates are excreted in the urine.

Coagulation

The anti-coagulatory effect of heparin is primarily based on activation of antithrombin III (AT III) [68], part of the family of serine protease inhibitors (serpins). They include
- heparin cofactor II (HC II),
- plasminogen activator inhibitor 1 (PAI-1),
- protease nexin-1 (PN1) and
- protein-C inhibitor (PCI, also known as PAI-3).

These serpins can bind to heparin or heparin-like glycosamin glycanes (GAG) [69] and this influence coagulation, although activation of AT III is the dominant factor in the effect of heparin.

Antithrombin III is an inhibitor of activated factor X (Xa) and activated thrombin (IIa) [69]. In addition, a significantly lower effect is exercised via HC II, which, however, only inhibits thrombin [70]. Although antithrombin III and HC II have very similar structures [71], AT III only binds to heparin, while HC II also binds to further heparinoid, i.e. GAGs, such as dermatan sulfate [72].

Antithrombin III inhibits further serine proteases including factor IXa, as well as factors XIa and XIIa and kallikrein. AT III has no effect on factor V and factor VIII [69].

Electropositive domains known as exosites mediate the electrostatic bond between heparin and antithrombin III [73]. They show concentrations of the aminoacids arginine and lysine [74]. The conformation changes upon binding with heparin, forming a complex that has higher potential for coagulation factor inhibition than antithrombin alone [71]. Relatively long-chain heparins are necessary to generate a sufficiently inhibitory effect, which also applies to HC II [75]. However, serpin activation by the pentasaccharide (HAH) is sufficient for the inhibition of factor Xa alone [76], forming the basis for the consideration of fondaparinux as already described (see above).

TFPI (tissue factor pathway inhibitor) drives a different mechanism of action of heparin [77]. This inhibitor is primarily synthesized in the vascular endothelium [78] and inhibits factors Xa and VIIa in a two-stage reversible reaction [79]. Like other GaGs, heparin can release TFPI [80]. Around one-third of heparin's anticoagulatory effect is determined by TFPI [81], causing higher TFPI levels to show in serum after heparin treatment [77, 81].

Heparin is known to trigger forms of thrombocytopenia, namely heparin-induced Type 1 and Type 2 thrombocytopenia (HIT-1 and HIT-2). HIT-2 can take on life-threatening forms by inducing thrombosis and embolism [82] (Fig. 2).

The pathophysiological focus here is the increased release of platelet factor 4 (PF-4) from thrombocytes and subsequent formation of a PF-4/heparin complex [82]. HAH plays a particularly significant role in platelet activation, as does the density of the receptor status on the thrombocytes [83]. A major role is played by heparin's high electronegativity, while PF-4 shows high electropositivity [84]. This electrostatic bond also explains why such complex formation is found more frequently in long-chain heparins (uf) than short-chain heparins (nm) or even pentasaccharide alone [82].

A decisive element in the further pathophysiological cascade is the formation of antibodies (IgG) against the PF-4/heparin complex, albeit virtually exclusively directed against PF-4 [85]. Once such antibodies form, they in turn activate the thrombocytes. This increases the release of PF-4, which can antagonize heparin and bind HSPGs to cell membranes [84, 86].

Differential diagnosis of both HITs is clinically significant as the consequences for treatment differ (Tab. 2). HIT-2 occurs far more rarely in fractionated, i.e. low-molecular heparins [88, 89], and is extremely unusual during pregnancy; accordingly, there is only one publication on the subject [89].

Protamine is a mixture of strongly alkaline peptides and has a discrete anti-coagulatory effect. However, formation of a heparin complex is essential to trigger this effect. Upon heparin complex formation it inhibits heparin-induced anti-Xa effect completely, and anti-IIa effect partially. Fondoparinux remains unaffected [90].

Protamines are produced from the sperm or roe of some salmonids. The neutral water-soluble salts such as protamine sulfate and protamine hydrochloride are used [90]. The effect develops rapidly (5-15 min). Given the slight anti-coagulatory effect, overdosing may cause haemorrhage [91].

No essential physiological significance of heparin for coagulation has yet been proved [92, 93], despite its frequent use as an anti-coagulant. Heparins are cur-

Table 1: Summary of the main interactions of heparin and HPSG. Mod. from [47].

Growth factors
- FGF (fibroblast growth factor)
- HB-EGF (heparin-binding epidermal growth factor)
- EGF (epidermal growth factor)
- IGF-2 (insulin-like growth factor)
- TGF-β (transforming growth factor)
- Hepatocyte growth factor
- PGF-4 (platelet growth factor)
- VEGF (vaso-endothelial growth factor)
- G(M)-CSF (granulocyte (macrophage) colony stimulating factor)

Proteases/esterases and their inhibitors
- Antithrombin (serpins), primarily III
- HC II
- Protein C inhibitor
- PAI (plasminogen activator inhibitor)
- Complement/complement inhibitors
- Nexin-1
- TFPI (tissue factor pathway inhibitor)
- Secretory leukocyte protease inhibitor

Chemokines, further cytokines
- IL-6, IL-8 (GP-130-cytokines
- IFN-γ
- SDF-1a (stromal-derived factor)

Receptor (protein) complexes
- EGF receptor

Enzymes
- Factors Xa, IIa (thrombin) IXa
- Neutrophil elastase
- Kathepsin G
- Superoxide dismutase

Viral envelope proteins
- HIV envelope protein GB-120
- HSV envelope proteins B (gB), gC and gD
- Dengue virus envelope protein

Lipoproteins
- Apolipoprotein E
- Lipid-binding proteins

Further proteins
- Malaria proteins
- Endotoxin
- Annexin V

Table 2: Heparin-induced thrombocytopenias (HIT). Mod. from [87, 88].

	HIT-1	HIT-2
Cause	Direct thrombocyte activation by heparin	Formation of antibodies (et al.), PF-4 activation
Start	1–2 days after 1st dose of heparin	5–14 days after 1st dose of heparin, or earlier if dose administered previously
Thrombocytes	Rare < 100,000/ml	Decline > 50% of starting value
Frequency	Approx. 10%	Approx. 1%, rarer in pregnancy
Clinical	Generally asymptomatic	Possible thromboembolic vascular occlusions, embolism
Treatment	None	Discontinue immediately, switch to alternative anticoagulant where applicable

rently assumed to be non-essential for physiological hemostasis [94]. The same appears to apply to HSPGs, which are generally membrane-bound and assumed to have a local effect [95]. Given the diverse and, in some cases, widely differing (co-) effects, the term "multivalent biomodulators" is applied to heparin and GaGs [96].

Immune System

Significant levels of heparin synthesis takes place in mastocytes. Synthesized heparin is stored in granules in the mastocytes and, together with histamine, is their most important (exocrine) synthesized product [96, 97]. Synthesis has also been shown to occur in neutrophile granulocytes [92, 93].

Mastocytes are found in high concentrations near blood and lymph vessels, along nerve cells, and particularly in mucous membranes and skin [93].

An often-repeated theory is that heparin synthesis only exists in mastocytes and endothelial cells and that the heparin content of e.g. lung or liver tissue is derived from these cells [98]. This is, however, not the case, as mastocytes are virtually absent from mucous membranes in various intestinal sections where heparin is found in relatively high concentrations [100].

The complement system, comprising approximately 25 different proteins [100], is an important partner for the effect of heparin. The main task of the complement system is opsonization, i.e. interaction with the surface of pathogens to enable phagocytes to destroy them. In addition, the complement system can trigger general inflammatory reactions, may develop chemokine effects triggered by individual fragments, and may also attack bacterial envelopes [100, 101].

The complement system can be activated in three ways [101]:
– the classic method,
– the lectin method,
– the alternative method.

The classic method focuses on activation of glycoproteins C1-C9 by IgG or IgM antibodies, while IgA, IgE and IgD antibodies are ineffective. Another possibility is activation by direct binding of C1q to pathogen surfaces or to collagen or CRP [102].

In the lectin method, mannose-binding lectin (MBL) binds to mannose or glycans on the pathogen surface and thus activates proteases MASP-1 to -3, triggering the same cascade reaction as the classic method [100, 102].

The alternative method, also known as the properidin method, is activated by degradation of relatively unstable factor C3 into C3a and C3b. C3a has a chemotactic effect as an inflammatory anaphylatoxin. With other factors such as factor B, Cb3 can form the C3 convertase of the alternative method and trigger cascade reactions in the presence of pathogenic surfaces [101].

Heparin leads to partial inhibition of the complement system and its activation in both the classic activation method [103] and the properidin method [104]. The focus is on C3, which is of central importance in all activation paths [104] even though other complement factors [105, 106] such as C2, C4 or HI are the target of the inflammation.

In addition, binding activity of specific proteins involved, e.g. C4 binding protein (decreases) or vitronectin (increases) is modulated [107, 108].

Individual factors of the complement system are also known to be activated. However, the inhibitory effects predominate overall, so that the effect of heparin on the complement system can best be characterized as immune-suppressive, as was identified as early as 1929 [109].

Formation of free radicals or reactive oxygen species (ROS) is also inhibited [110, 111], as is synthesis of pro-inflammatory cytokines in leukocytes, primarily neutrophil leukocytes [112], as well as in mononuclear cells and macrophages. The most important effect is inhibition of TNFa synthesis and release; in addition, heparin is a potent antagonist of the endotoxin [113, 114]. A further noteworthy effect is the inhibition of migration in eosinophil granulocytes [115].

Heparin therefore has anti-inflammatory properties, as has been shown in clinical studies. Positive therapeutic effects are found for e.g. rheumatoid arthritis [116], allergic bronchial asthma, allergic rhinitis [117], ulcerating colitis and Crohn's disease [118].

Heparin's somewhat general anti-inflammatory properties are countered by specific virostatic, bacteriostatic and even bacteriocidal effects caused by heparin's powerful negative charge. Heparin therefore binds to many proteins, including pathogenic proteins such as HIV envelope protein [119], the envelope protein of the herpes simplex virus (HSV) [120] and the envelope protein of the Dengue virus [121]; in the latter, the HAH complex evidently plays an important part in binding. There is now good evidence for heparin's anti-malarial activity [122, 123].

In this context HSPGs must be evaluated differently; for example, syndecan-3, a membrane-bound HSPG (see below), has a supporting effect in binding and cell transmission of the HI virus (HIV) and filoviridae [124, 125]. However, this need not result in aggravation of the disease, as it is perfectly possible that improved intracellular absorption of the viruses actually helps to eliminate them more rapidly [126].

Mastocytes play a major role in the bacteriostatic and bacteriocidal effect of heparin. They are shown to occur in many organs in contact with the external environment – such as lungs and dermis, where they occur in proximity to blood vessels, nerves and hair follicles – often in high concentrations [overview in 127]. Mastocyte activation is effected by bacteria or bacterial excretory products via specific receptors, e.g. CD 85 or toll-like receptors (TLR) 2, 4, 6, 7, 9) or inflammation mediators such as complement factors, endothelin-1 and neurotensin.

Mastocytes release various cytokines (e.g. TNFα), enzymes and proteases (e.g. chymase, tryptase or carboxypeptidase) and/or chemokines, but primarily histamine and heparin. Heparin in particular establishes communication with other immunocompetent cells and thus influences differentiation of dendritic cells from monocytes [128].

Mastocytes are therefore extremely important for combating bacterial and parasitical infections (pruritis) and are closely involved in the innate immune system (cf. TLRs, see above). Heparin and histamine promote circulation in inflamed areas by vasodilation and anti-coagulation (flushing).

Mastocytes are also able to limit inflammatory reaction once triggered. This is useful given that proliferating and prolonged inflammation has a destructive potential that is hazardous to the organism. Heparin also appears to be extremely significant here [cf. 127].

Heparin thus possesses a pronounced immunomodulating effect.

It develops intrinsic effects in warding off infections, and communicates with various cells involved in specific immunodefence, e.g. dendritic cells. Together with other substances such as histamine, heparin ensures good local perfusion in the initiation and maintenance of inflammation; however, given its general anti-inflammatory properties it also plays a role in containing inflammation.

Cell Proliferation and Cellular Differentiation

HSPGs (and, to a lesser extent, heparin) in particular interact with a variety of growth factors and hence modulate their effect on cell growth, cell differentiation, and thus cell development [129]. The majority of studies in existence focus on FGF (fibroblast growth factor) and VEGF (vascular endothelial growth factor [130].

In their inactive forms, both these growth factors can be identified on the surface of endothelial cells or in the extra-cellular matrix, where they are bound to HSPGs [131]. This bond is broken by physiological or pathological stimulus, whereupon the growth factors form complexes with the HSPGs; these complexes significantly increase the binding affinity of the growth factors to their receptor [132].

This was particularly clearly shown for FGF-2. To date 22 different FGF variants have been identified, which differ – in some cases, considerably – in terms of their activation and receptor affinity [133].

The HAH complex with a minimum of one iduronic acid molecule in the 2-O position is critically important for this binding [134]. However, longer-chain HS fragments are necessary to initiate contact with the receptor [135]. This explains why some growth factor-HS complexes have an activating effect and others are inhibitory [135]. The same applies to heparin binding and the biological activity it triggers [136], for which reason the effects of uf heparins and fractionated or short-chain heparins differ (see above).

Similar mechanisms have been shown for further growth factors [overview in 137], e.g.
- members of the EGF (epidermal growth factor) family,
- PDGF (platelet-derived growth factor),
- TGF-β (transforming growth factor) 1 and 2 and binding protein,
- HGF (hepatocellular growth factor),
- IGF-2 (insulin-like growth factor) and IGF binding proteins 3 and 5,
- G(M)-CSF (granulocyte-macrophage colony-stimulating factor) and
- heparanases.

They are essential for the breakdown of heparin and HSPGs (see above). However, heparin and some HS fragments also have an intrinsic effect on these enzymes [138], whereby increasing concentrations of heparin result in inhibition of heparanase activity [139]. This means that heparin bioactivity does not increase in a linear fashion, but exponentially from a specific concentration.

Direct influence on cell growth thus exists. Growth inhibition of smooth muscle cells has been shown both in culture medium [140] and directly on the endothelial wall [141, 142].

The fact that heparin binds specifically to smooth muscle cells has given rise to the question of whether a specific heparin receptor exists [142, 143]. It is assumed that no such receptor exists and that in fact, heparin and various HSPG fragments are capable of activating intracellular signal transduction in a similar way to extra-cellular activation (of growth factors, proteases, cytokines etc.).

Proven interactions include the following:
- Suppression of serum and glucocorticoid-regulated kinase (SGK), an early gene response to cell proliferation [144],
- Selective regulation of tyrosine kinase, a key protein in e.g. angiogenesis [145],
- Inhibition of mitogen-activated protein kinases [146],
- Inhibition of calcium/calmodulin-regulated protein kinase II [147].

Here too, the structure of the heparin or HPSGs is critically important; many biological effects can only be shown for longer-chain (generally unfractionated) heparins and heparinoids, not for their short-chain counterparts [148]. In turn, contrasting effects can accordingly be observed, i.e. activation instead of inhibition and vice versa [149].

This modulating influence on cell growth and cell differentiation shows that heparin plays an important role here. This is proven for e.g. cell repair processes with cicatricial build-up [150], embryo implantation followed by pregnancy [4, 151] and malignant infiltration [152, 153].

The important role of numerous membrane-bound HSPGs, such as syndecane [154], glypicane [155] and perlecan [156], has also been shown for these processes.

Membranous Heparan Sulfate Glycans

Membranous HPSGs have a different physiological significance than soluble HPSGs.

In general, it may be assumed that membrane-bound structures target cell interaction and regulation of substrate metabolism between extra-cellular and intracellular space and vice versa. In addition, it can be assumed that membrane-bound molecules extending far into extracellular space have an affinity for various extra-cellular ligands.

The following groups or molecules are distinctly defined:
- Syndecans
- Glypicans
- Betaglycans
- CD44

Syndecans

Syndecans are transmembranous HPSGs anchored with a CoR protein and associated with chondroitin sulfate chains (see Fig. 2). Four members of this HSPG family are currently known (1–4), specified in their sequence of cloning [157]. While the transmembranous and intracellular part is extremely similar throughout all species, the extracellular part varies considerably [158].

Distribution in tissue varies, in some cases widely:
- Syndecan-1 is primarily found in epithelial tissues, but also in mesodermal tissue e.g. skeletal muscle [157, 159].
- Syndecan-2 primarily occurs in mesenchymal cells/tissues, e.g. fibroblasts and hepatocytes [160].
- Syndecan-3 is primarily found in nerve and muscle tissue [161].
- Syndecan-4 is found the most frequently in the organism and occurs in epithelial and endothelial cells, e.g. fibroblasts, myocytes, chondrocytes and nerve cells [157, 162].

Syndecans bind to various substances, particularly growth factors (see above) and primarily FGF-2, but also TGF-β (syndecan-2) [163], EGF, VEGF, and HBF [157]. Their actual function is that of a co-receptor (see: FGF bindung), principally for g-protein coupled receptors; a further function is cell adhesion (primarily syndecan-1), with noteworthy interactions with integrin and fibronectin [164].

Syndecans are also important during phylogenesis [165]. Their presence can first be shown from the 16-32-cell stage of the preimplantation embryo, when they are found on all cells of the inner cell mass (ICM) of the blastocyst and they are among those responsible for cell adherence [166]. They are also important in advanced embryo development, particularly in development of epithelial tissue.

The same applies to neurogenesis, primarily with respect to syndecan-2 and syndecan-3 [167], i.e. development of neurons (from neural stem cells) and neural synapses [168]. Syndecans also play a role in muscular development, particularly syndecan-4, which partly develops its effect through growth factors FGF-2 and TGF-β [169]. Myogenic satellite cells, found between the basal membrane and the sarcolemma, are crucial in this context. They are activated in proliferation processes including repair. This results in expression of syndecan-3 and -4 [170] at all stages of life, including embryos [171].

Glypicans

Glypicans are not transmembranous HPSGs, but are anchored to the cell surface by the GPI (glycosylphosphoinositol) anchor [172].

Six different glypicans (1–6) have been identified in vertebrates, with widely varying expression in tissues:
- Glypican-1 is primarily found in the brain, kidneys and skeletal musculature [173].
- Glypican-2 is important for the development of the nervous system [174].
- Glypican-3 is primarily found in mesodermal tissue and is strongly expressed during intestinal development [175].
- Glypican-4 primarily occurs in blood vessels [176].
- Glypican-5 occurs in brain and kidney tissue [177].
- Glypican-6 is the most frequently found, particularly in adult ovarian tissue, in fetal and adult kidneys, in smooth muscle tissue, in intestinal mesenchymal cells, in the lungs and in the teeth [178].

Similarly to syndecans, glypicans play an important role in regulating cell growth, e.g. in inducing cell apoptosis [179].

Although many aspects of the different interactions are still unclear, regulation of IGF-2 by glypicans, primarily glypican-3, appears to be important [173, 175, 178]. This is demonstrated by e.g. mutations in glypican-3 that can lead to excessive cell growth [180]. Here Simpson-Golabi-Behmel syndrome (SGBS) should be mentioned, characterized by features including pre- and post-natal overgrowth [181]. In this respect it is very similar to Beckwith-Wiedemann syndrome, and indeed both syndromes involve over-expression of IGF-2. This forms the basis for the theory that this syndrome is also caused by glypican-3 insufficiency (resulting from polymorphism) [180].

Syndecan-1 and glypican-1 have a synergetic effect on musculature by means of activation of FGF-2, resulting in increased cell proliferation with simultaneous inhibition of differentiation [173].

Like many other growth factors, FGF-2 is also involved in neurogenesis, primarily where stem cell differentiation is concerned [182]. Glypicans are also involved here; the precise nature of the interactions is unclear [182]. High expression of glypican-4 in the ventricular zone of the developing brain has been proven, particularly in neural cells with stem cell properties. After completion of differentiation, however, this expression recedes to the point of negligibility [183], although other glypicans (e.g. -1, -2, -5) show high expression in differentiated and post-mitotic neurons [174, 177].

Beta Glycan

Unlike alpha glycans, beta glycan (see Fig. 2) is an HSPG which has transmembrane localization, a CoR protein and an extra-cellular domain.

Beta glycan is basically a co-receptor for members of the TGF-β superfamily [184], particularly TGF-β2. Its effect does not result from its own intrinsic activity but from increased receptor affinity for TGF-β at both its receptors (TβRI and TβRII) [185]. For this reason, beta glycan is also known as Tβ-receptor-III (TβRIII) [186].

The CoR protein shows particularly high affinity for members of the TGF-β superfamily; HS side chains can also bind alkaline FGF (βFGF) [185].

Beta glycan also exists in soluble form in serum and the ECM, albeit in low concentrations. Unlike the membrane-bound beta glycans, this soluble type has an inhibitory effect on the TGF-β family [187].

CD44

CD44 is a glycoprotein occurring on cell surfaces. It exists in various forms that all originate from a single gene, [188] and are thus splicing variants (CD44v and the longer form of CD44E [on some epithelial cells]) [189]. The various (iso-) forms show differing concentrations of heparan, chondroitin and keratan sulfate [188, 189].

The physiological function of the CD44 family is primarily cell-to-cell adhesion. They can thus bind collagens and osteopontin, interact with metalloproteinases (MMP) and in some cases bind growth factors, albeit with low affinity [189, 190].

HCELL (e-selectin/l-selectin ligand of hematopoetic cells) is a sialated and glycosylated variant with functions including a "homing receptor" for bone marrow, i.e. (re-)directs hematopoetic and mesenchymal stem cells there [191]. It also appears to be important in carcinomas [192], particularly types of leukemia, in which it was first shown to occur on blast cells [193]. (Over-) expression on endometrial cells, particularly in foci of endometriosis, has also been shown. This higher expression is designed to promote peritoneal adherence [194].

Pericellular HPSGs

Unlike the HPSGs previously described, which are primarily limited to cell surfaces, pericellular HSPGs do not have CoR protein, are not anchored to cell membrane and are thus mainly found in the ECM, often in close proximity to cells or their membranes ("pericellular").

They include perlecan, agrin and collagen XVIII.

Perlecan

Perlecan takes its name from its appearance under an electron microscope, where it resembles a string of pearls.

Perlecan is a relatively widespread HPSG and has been identified in virtually all basal membranes [195]. It forms reticular structures with a profound filtering effect (strongly negative charge), e.g. in the kidneys.

Perlecan is coded by the HSPG2 gene. It comprises a core protein with approx. 660 KDa, has a high negative charge from residual sulfate, and has five domains which differ widely in some respects [196–198]:

- Domain I has HSPG chains – a domain for sperm protein enterokinase.
- Domain II contains receptors including one LDL receptor.
- Domain III has close similarity to the short arm of laminin A.
- Domain IV contains neural cell adhesion molecules and binds to nidogenes, fibronectin and heparin.
- Like domain IV, domain V also has molecular arrangements which correspond to EGF and bind to nidogenes, fibulin-2 and heparin.

Perlecan is crucial to normal embryonic growth [199]. Analyses of knock-out mice have shown that perlecan deficiency causes abnormal brain development and relatively premature mortality from skeletal dysplasia [200].

Development of the embryonic myocardium is a similar case [201]. Perlecan deficiency causes holes to form in the myocardium [201].

In both developing embryos and adults, perlecan deficiency prevents correct localization of acetylcholinesterase at neuromuscular junctions [202] yet without affecting actual acetylcholinesterase synthesis [203]. It can therefore be assumed that perlecan plays an important role in localizing and concentrating this vital neurotransmitter [204].

In combination with syndecan-1, perlecan anchors the lipoprotein lipase which splits triglycerides from chylomicrons. Given this mechanism, both HSPGs are involved in the lipid transport of chylomicrons to the epithelium [159, 199].

Mutations in the HSPG2 gene (1p36) are the cause of Schwartz-Jampel syndrome (chondrodystrophic myotonia) [205], dyssegmental dysplasia, Silverman-Handmaker type [206] and probably also Roland-Debuquois syndrome [205, 206]. These overall extremely rare, but severe disorders differ in their level of perlecan synthesis, which is virtually absent in dyssegmental dysplasia but relatively high in Roland-Debuquois syndrome. The different perlecan synthesis rates would also correlate with the different clinical manifestations and their severity.

Agrin

Agrin is a larger proteoglycan which, like syndecan and glypican, contains chondroitin and has three different binding sites for heparan sulfate [207]. It is coded by the AGRN gene [208].

Agrin derives its name from the synthesis or clustering of the acetylcholinesterase receptor (AChR) and aggregation of AChR during synaptogenesis, and from the importance of this proteoglycan in these processes [209, 210]. On the one hand, agrin inhibits cell overgrowth in the development of the nervous system, both peripheral and central (e.g. hippocampus, retina) [210]; on the other, agrin deficiency in knock-out mice impairs synapse development extensively or completely [211].

The same evidently applies to the MuSK receptor (receptor for muscle-specific kinase); agrin also binds to it and is thus responsible for (re-)phosphorylation [212]. Further muscle proteins to which agrin binds include laminin and dystroglycan.

Collagen XVIII

Collagens (from the Greek for "glue-producing") are structural proteins primarily found in the ECM. They are composed of α-helical peptide chains (600–3000 amino acids) which are, unusually, left-handed and join to form a right-handed triple helix or superhelix ("coiled coil").

29 types of collagen are currently known (I-XXIX), classified as fibrillar, reticulate, facit and short-chain, as well as collagens with anchoring fibrils and transmembrane collagens [213].

Collagen XVIII is a further HPSG for which 8 different glycosylation variations have been described.

Collagen XVIII is primarily found in endothelial and epithelial basal membrane

[214]. Individual glycosylation variations show anti-angiogenetic and anti-tumorous properties [215], primarily endostatin [215]. This is a split product of collagen XVIII with molecular weight of approx. 20 kDa, which is also endogenically effective as an angiogenesis inhibitor (198). Clinical studies have shown benefits from combining recombinant (rh) endostatin with chemotherapeutic agents in treatment of various types of malignoma [217, 218].

Conflict of Interest

The authors have no conflict of interest to disclose.

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