

Heparin Derivatives as Angiogenesis Inhibitors

M. Presta*, D. Leali, H. Stabile, R. Ronca, M. Camozzi, L. Coco, E. Moroni, §S. Liekens, and M. Rusnati

Unit of General Pathology and Immunology, Department of Biomedical Science and Biotechnology, School of Medicine, University of Brescia, 25123 Brescia, Italy. §Present address: Department Cell Biology, New York University Medical Center, New York, NY, USA

Abstract: Angiogenesis is the process of generating new capillary blood vessels. Uncontrolled endothelial cell proliferation is observed in tumor neovascularization and in angioproliferative diseases. Tumors cannot grow as a mass above few mm³ unless a new blood supply is induced. It derives that the control of the neovascularization process may affect tumor growth and may represent a novel approach to tumor therapy.

Angiogenesis is controlled by a balance between proangiogenic and antiangiogenic factors. The angiogenic switch represents the net result of the activity of angiogenic stimulators and inhibitors, suggesting that counteracting even a single major angiogenic factor could shift the balance towards inhibition.

Heparan sulfate proteoglycans are involved in the modulation of the neovascularization that takes place in different physiological and pathological conditions. This modulation occurs through the interaction with angiogenic growth factors or with negative regulators of angiogenesis. Thus, the study of the biochemical bases of this interaction may help to design glycosaminoglycan analogs endowed with angiostatic properties.

The purpose of this review is to provide an overview of the structure/function of heparan sulfate proteoglycans in endothelial cells and to summarize the angiostatic properties of synthetic heparin-like compounds, chemically modified heparins, and biotechnological heparins.

Key Words: angiogenesis, drug, endothelium, FGF, growth factors, heparin, proteoglycans, tumor.

INTRODUCTION

Glycosaminoglycans (GAGs) are negatively charged polysaccharides composed of repeating disaccharide units. GAGs are normally found as proteoglycans (PGs) composed of one or more polysaccharide chains attached to a core protein. PGs are present in almost all the cell types where they can be found in soluble forms, in the extracellular matrix (ECM), associated with the plasma membrane, or segregated into intracellular granules [1].

In the last years the studies about GAGs and PGs have increased dramatically leading to the comprehension of their biosynthesis and structure, together with the demonstration of the involvement of GAGs and PGs in various physiological processes. The biological functions of GAGs and PGs are highly diversified, ranging from relatively simple mechanical support functions to more intricate effects on various cellular processes such as cell adhesion, proliferation and differentiation. These effects are due to the ability of PGs to act as "receptors" for adhesion molecules and free molecules such as growth factors, cytokines, and a variety of enzymes including proteases and coagulation enzymes.

A particular class of PGs, namely the heparan sulfate PGs (HSPGs), have been demonstrated to be involved in the modulation of the neovascularization that takes place in different physiological and pathological conditions [2]. This modulation occurs through the interaction of HSPGs with angiogenic growth factors or with negative regulators of angiogenesis (Table 1), suggesting that the study of the biochemical bases of protein/HSPG interaction may help to design synthetic GAG analogs endowed with angiostatic properties.

The purpose of this review is to provide an overview of the structure/function of HSPGs in endothelial cells and to summarize the angiostatic properties of heparin-derivatives.

HEPARAN SULFATE PROTEOGLYCANS

Cell Association of HSPGs

Typical concentrations of HSPGs on the cell surface are in the range of 10⁵-10⁶ molecules/cell as measured in various cell culture systems.

HSPGs can link to the plasma membrane through a hydrophobic transmembrane domain of their core protein or through a glycosyl-phosphatidylinositol (GPI) anchor covalently bound to the core protein. Also, HSPGs can interact with the cell by non-covalent linkage to different cell-surface macromolecules [3]. Interestingly, interaction of

*Address correspondence to this author at the General Pathology and Immunology, School of Medicine, via Valsabbina 19, 25123 Brescia, Italy; Tel: ++39-0303717311; Fax: ++39-0303701157; E-mail: presta@med.unibs.it

free sulfated GAGs or soluble HSPGs with the cell surface can lead to intracellular signaling and modulation of gene expression [4, 5].

Table 1. Heparin-Binding Proangiogenic and Antiangiogenic Proteins

Fibroblast growth factors (FGFs)
Vascular endothelial growth factor (VEGF)
Placenta growth factor (PlGF)
Heparin-binding EGF-like growth factor
Hepatocyte growth factor (HGF)
Angiogenin
Transforming growth factor- (TGF-)
Interferon- (IFN-)
Platelet-derived growth factor (PDGF)
Pleiotrophin
Midkine
Platelet factor-4 (PF-4)
Interleukin-8 (IL-8)
Macrophage inflammatory protein-1 (MIP-1)
Interferon- -inducible protein-10 (IP-10)
HIV-Tat transactivating factor
Thrombospondin
Endostatin

Transmembrane HSPGs are glypicans, cerebroglycan, betaglycan, CD44, and the members of the syndecan family: syndecan 1, fibroglycan (syndecan 2), N-syndecan (syndecan 3) and ryudocan (syndecan 4). Glypicans and cerebroglycan are typical GPI-anchored HSPGs. Syndecans and betaglycan are typical transmembrane HSPGs characterized by a core protein composed of an extracellular domain, a single membrane-spanning domain and a short cytoplasmic domain that can interact with the cytoskeleton. Extracellular domain contains the consensus sequences for glycosylation and a conserved putative proteolytic cleavage site. Four tyrosine residues are highly conserved in the C-terminus of all the members of the syndecan family and one of them fits a consensus sequence for tyrosine phosphorylation [6]. Tyrosine phosphorylation of the intracellular domain of syndecan-1 by cytoplasmic tyrosine kinases has been described in intact cells [7], thus supporting the hypothesis of HSPG involvement in signal transduction. Indeed, recent observations have defined a syndecan-regulated receptor signaling [8] and a cross-talk between syndecan 4 and fibroblast growth factor (FGF) signaling [9].

Perlecan is a typical peripheral membrane HSPG that interacts with the cell surface through its core protein [10]. The cell-adhesion motif Arg-Gly-Asp within the core protein of perlecan binds integrins α_1 or α_3 present on endothelial

cell surface [11]. However, HSPGs may associate with the cell surface and/or ECM also through their GAG-chain, as demonstrated by the observation that half of the total content of HSPGs in endothelial cells can be released after incubation with soluble heparin [12].

HSPGs exist also in soluble form following their mobilization from the cell surface. Transmembrane HSPGs are released after proteolytic digestion of their core protein [13]. HSPGs bound to the cell surface *via* their GAG-chain can be mobilized by free GAGs by a simple law of mass action [12] or by enzymatic digestion of their polysaccharidic backbone [14]. GPI-anchored HSPGs can be released by action of endogenous phospholipase [15].

Finally, it is important to recall that cell-associated HSPGs can be internalized *via* endocytosis and metabolized in the lysosomal compartment [1]. In some cell types oligosaccharides originated during intracellular degradation appear to be delivered specifically to the nucleus [16, 17].

HS Chain Synthesis

The biosynthesis of HS chains in PGs is a process that leads to the production of molecules characterized by great structural heterogeneity with respect to the size of the polysaccharide chain, the ratio of iduronic (IdoA) to glucuronic acid (GlcA) units, and the amount and distribution of sulfate groups along the carbohydrate backbone. The biosynthesis of heparin/HS can be conveniently separated into three steps: i) addition of the linkage region to the core protein; ii) chain elongation; iii) chain modifications (see [1, 18] for a detailed description of the process). All the polymer modifications are incomplete *in vivo*. In other words, not all the sugar residues that are potential substrates for the various enzymes are transformed into their relative products. Since 2-O- and 6-O-sulfation occur only after C5 epimerization that, in turn, needs the preceding N-deacetylation/N-sulfation reaction, the distribution of 2-O- and 6-O-sulfate groups is restricted to N-sulfated regions. This partial modification process is the biosynthetic basis for the structural heterogeneity of heparin/HS. The regulation of the chain modification process leads to cell- or organ-specific HS structures that may allow a fine modulation of the biological functions of HSPGs.

As stated above, the final structure of heparin/HS depends upon the incompleteness of the reactions that occur during the biosynthetic process. The modification process is more complete in heparin whose structure is largely accounted for by regular trisulfated disaccharide sequences made up of alternating, -1,4-linked residues of 2-O-sulfated L-IdoA (IdoA2) and N,6-disulfated D-glucosamine (GlcNS6S). These regular sequences are occasionally interrupted by nonsulfated uronic acids (either GlcA or IdoA) and by undersulfated hexosamines (GlcNS, GlcNAc, GlcNAc6S). 3-O-sulfated glucosamines (GlcNS3S or GlcNS3S6S) are minor but important constituents of heparin, since they are part of a pentasaccharidic sequence of the binding site for antithrombin, which is essential for the expression of significant anticoagulant activity [19]. In contrast, the modifications that occur during the biosynthesis

of HS are less extensive, leading to HS molecules characterized by lower IdoA content and a lower overall degree of O-sulfation and resulting in high heterogeneity of distribution of the sulfate groups along the chain. Eventually, disaccharides containing GlcNAc or GlcNS may form clusters ranging from 2 to 20 adjacent GlcNAc-containing disaccharides and from 2 to 10 adjacent GlcNS-containing disaccharides. However, about 20-30% of the chain contains alternate GlcNAc- and GlcNS-disaccharides units [20].

Endothelium and HSPGs

HSPGs are necessary for the structural and functional integrity of the endothelium. HSPGs present at the basal site of blood vessels act as matrix receptors by interacting with a variety of basement membrane proteins. Moreover, basal HSPGs are responsible for the charge selectivity of filtration in endothelium [21] and inhibit smooth muscle cell proliferation and migration [22]. HSPGs are also present at the luminal surface of the endothelium [23] where they are involved in the binding and internalization of lipoprotein lipase [24]. Moreover, luminal HSPGs play a major role in determining the anticoagulative properties of the vessel surface by binding to proteases of the intrinsic coagulation cascade, thrombin, and protease inhibitors, including antithrombin III [25]. Finally, endothelial cell-surface HSPGs act as co-receptors for a wide spectrum of angiogenic growth factors and inhibitors [2].

Both macrovascular and microvascular endothelial cells synthesize HSPGs. Different HSPGs have been identified in endothelial cells in culture where they account for the majority of extracellular sulfated GAGs. Endothelial HSPGs may be found at intracellular level, associated with plasma membrane and ECM, or in a soluble form. Syndecan 1 is the most represented HSPG in microvascular endothelial cells [26]. It is mainly stored inside the cell, a small portion being present at the basal surface [27]. Syndecan 4 is also expressed on the surface of endothelial cells [28] while perlecan is abundant in endothelial ECM [29]. Finally, a variety of poorly characterized soluble HSPGs with molecular weight spanning from 28 to 800 kD have been isolated from cultured media of endothelial cells [29, 30]. The observation that the levels of HSPGs in endothelial cells derived from the microvasculature, where the angiogenic process takes place, are 10-15 times higher than those found in macrovascular endothelial cells [31] is in keeping with the role played by endothelial HSPGs in the modulation of angiogenesis. Indeed, depletion of HSPGs from endothelial cell surface inhibits neovascularization [32]. Conversely, recent observations have shown that cell surface expression and secretion of heparanase promote tumor growth and vascularization [33].

The HSPG-dependent regulation of angiogenesis is due, at least in part, to their capacity to bind to and modulate the activity of angiogenic growth factors (see below), and contrasting effects can be obtained depending on the types of HSPGs and/or on the experimental conditions adopted. For instance, purified perlecan enhances angiogenesis induced by FGF2 while other purified HSPGs are ineffective [29]. Glypican augments the binding of FGF1 and FGF2 to human

tyrosine kinase FGF receptor-1 (FGFR-1) in a cell free-system and replaces heparin in supporting FGF2-induced cellular proliferation of HS-negative cells expressing FGFR-1 [34].

Overexpression of syndecan 1 on the surface of NIH 3T3 cells inhibits FGF2-induced cell proliferation [35]. In contrast, syndecan 1 stimulates FGF2-mediated cell growth when immobilized to matrix [36]. However, soluble syndecan, glypican, and fibroglycan block the restoration of FGFR binding induced by heparin/HS in cell mutants deficient in cell surface HSPGs [37]. Also, soluble heparin/HS inhibit the binding of FGF2 to FGFRs and HSPGs present on the surface of endothelial cells [38]. These data suggest that negative effects on angiogenesis may be exerted by the binding of angiogenic growth factors to soluble HSPGs or GAGs rather than to cell-associated HSPGs.

Besides their capacity to modulate receptor binding, HSPGs may modulate angiogenesis also by protecting angiogenic growth factors from heat [39] and proteolytic degradation [38] and by affecting their radius of diffusion [40]. Finally, HSPGs present in the ECM may act as a reservoir for angiogenic growth factors that will reach higher local concentrations and will sustain the long-term stimulation of endothelial cells [14, 41].

Given the above considerations, the expression of endothelial HSPGs during the angiogenic process must be tightly enforced. This kind of control may take place at different levels: i) quantitative and qualitative differential expression of the various HSPG species; ii) modulation of the composition of GAG chains; iii) digestion of cell-associated GAGs by degrading enzymes; iv) induction of HSPG mobilization from cell surface. It is interesting to note that angiogenic growth factors and cytokines can effectively operate some of the above described controls. For instance, FGF2 and transforming growth factor-1 (TGF-1) increase the expression of HSPGs, in particular of syndecan 1, in 3T3 fibroblasts [42, 43]. Moreover, HS complexed with FGF2 is protected from degradation by chinese hamster ovary cell heparanase [44]. A similar action may take place also in endothelial cells. On the other hand, the levels of endothelial HSPGs decrease significantly in growing microvessels of the rabbit eye [45, 46] and of chick chorioallantoic membrane [47]. Accordingly, total HSPG content decreases in sprouting endothelial cells *in vitro* [48]. Concomitantly, a relative increase in soluble, low molecular weight HSPGs occurs in endothelial cells during migration and sprouting, reflecting an enhanced HSPG turnover [30, 48]. Accordingly, FGF2 increases the amount of soluble, FGF2-binding HSPG species in the conditioned medium of cultured endothelial cells as the consequence of an increased proteolytic plasmin-dependent activity [13]. Other authors have reported a decrease of HSPG content in migrating endothelial cells concomitant with an increase of chondroitin sulfate and dermatan sulfate PGs [30]. Accumulation of chondroitin sulfate can be obtained also by stimulation of endothelial cells by different interleukins (ILs) [49]. Finally, it has been demonstrated that FGF2 modulates the expression and processing of biglycan in migrating endothelial cells [50]. These observations point to the existence of an accurate, mutual control between growth factors and HSPGs in

endothelium that may be of particular relevance during the angiogenic process.

INTERACTION OF ANGIOGENIC GROWTH FACTORS WITH HSPGS

An Overview

The biological functions of HSPGs relay on their capacity to bind different molecules including extracellular matrix proteins, enzymes, and protease inhibitors [1, 51]. Of major interest for the aim of this review is the capacity of HSPGs to bind several growth factors, cytokines, and chemokines involved in the angiogenesis process, thus affecting their biological activity.

HSPGs modulate the biological activity of heparin-binding growth factors and cytokines through different mechanisms. i) The optional binding of the growth factor to soluble, ECM-associated or cell-surface HSPGs results in a fine control of the bioavailability of the protein. This is the case for TGF- β that binds betaglycan [52], a cell-associated PG, and decorin [53], which is present in the ECM, and for FGF2 that binds basement membrane perlecan as well as cell-membrane syndecans [28, 34]. ii) The association with HSPGs stabilizes the growth factor and protects it from proteolytic degradation [38, 54]. iii) HSPGs modulate the access of growth factors to specific signaling receptors by different mechanisms (see below). iv) HSPGs can control the intracellular fate of the growth factor [55]. Finally, the possibility exists that: v) transmembrane HSPGs themselves may transduce an intracellular signal [8]; vi) HSPGs may activate an intracellular transduction signal by interacting directly with growth factor receptors [56, 57].

Whatever the mechanism(s) of regulation of growth factor activity by HSPGs, it is interesting to note that the binding of the same growth factor to different HSPGs may have different biological consequences. This is the case for syndecan [58], betaglycan [59], and perlecan [29], all able to bind FGF2 but with different effects. Indeed, syndecan inhibits the mitogenic activity of FGF2 [35] whereas perlecan promotes FGF2-induced cell proliferation and angiogenesis [29].

Conversely, modifications of HSPG composition can regulate the sensitivity of the cell to different growth factors. This may be of particular relevance when the spatial and temporal control of the activity of different growth factors must be tightly enforced. This possibility is exemplified by the shift in cell-surface HSPG properties from a FGF2-binding to an FGF1-binding phenotype in murine neuronal cells during embryonic development [60].

The modality by which the various HSPGs “discriminate” among the several heparin-binding growth factors is based on their different core proteins, the high heterogeneity of GAG-chain composition, and on the possibility that both the protein moiety and GAG-chains may interact with different growth factors. For instance, betaglycan can exist as a “nude” core protein and the presence and composition of the GAG-chains of this HSPG can be regulated in response

to FGF2 [10]. FGF2 itself binds the GAG-chain of betaglycan while the core protein can interact with TGF- β [59]. Also, the number and fine structure of HS chains in syndecan 1 vary in different tissues and in relation to cell differentiation [6]. Similarly, HS chains of perlecan purified from different sources vary in their capacity to interact with FGF2 and FGFRs [61]. Finally, different sulfated groups and distinct oligosaccharide sequences of the GAG-chain are responsible for the binding to different growth factors [62].

In conclusion, HSPGs are characterized by a structural variability that appears to be highly regulated and that offers virtually unlimited possibilities for selective interactions with different growth factors, cytokines, and chemokines.

HSPGs Mediate the Binding of Angiogenic Growth Factors to Tyrosine-Kinase Receptors

Growth factors induce an angiogenic response in target endothelial cells by binding to cognate cell-surface tyrosine kinase (TK) receptors [63]. The interaction of heparin-binding growth factors to TK receptors is modulated by HSPGs. For instance, the interaction of FGF2 or of the heparin-binding vascular endothelial growth factor-165 (VEGF₁₆₅) isoform to TK receptors is strongly reduced in cells made HSPG-deficient by treatment with heparinase or chlorate [64, 65]. However, controversial results exist about the absolute requirement for HSPGs in FGF2/receptor interaction. Yayon *et al.* [66] reported that HS-deficient CHO cells transfected with FGFR-1 do not bind FGF2 unless heparin or HS are added to the cell culture medium. Accordingly, stable expression of perlecan antisense cDNA in mouse fibroblasts and human melanoma cells causes a dramatic reduction of the capacity of transfected cells to interact and to proliferate in response to FGF2. FGF2-binding capacity and responsiveness can be recovered by addition of soluble heparin to the cell culture [67]. In contrast, Roghani *et al.* [68] have shown that FGFRs expressed in CHO cell mutants or myeloid cells retain the capacity to bind FGF2 also in the absence of heparin. In these experimental conditions, heparin induces a three-fold increase in the affinity of the growth factor for its receptor.

Controversial results were obtained also in cell-free systems. Ornitz *et al.* [69] showed that heparin represents an absolute requirement for cell-free binding of FGF2 to a soluble form of the extracellular portion of FGFR-1, whereas Roghani *et al.* [68] reported that heparin is not necessary for the binding of FGF2 to soluble FGFR. In agreement with these latter results, the formation of the FGF2/FGFR complex in solution occurs also in the absence of heparin and it is enhanced by the GAG [70]. Similar results were obtained for the interaction of VEGF₁₆₅ with soluble KDR/*flk-1* receptor [71].

Interestingly, HSPGs are required also for receptor interaction of VEGF₁₂₁, a VEGF isoform lacking heparin binding ability [72]. This latter observation, as well as the capacity of heparin to induce FGF2/FGFR interaction in HS-deficient cells, can be interpreted on the basis of the capacity of GAGs to form ternary complexes by interacting with both ligand and receptor proteins [70, 73, 74]. Indeed, a heparin-

binding domain has been identified in the NH₂-terminus of IgG-like domain II of FGFR-1 [75]. Analysis of the crystal structure of heparin/FGF/FGFR complexes has confirmed this hypothesis [76, 77]. The puzzling observation that heparin itself can activate FGFR in the absence of the growth factor [57] further increases the complexity of the ternary interaction among GAGs, growth factors, and TK receptors.

From the above considerations it derives that: 1) heparin-related molecules can be used to modulate the biological activity of heparin-binding angiogenic growth factors and cognate TK receptors; 2) the structural requirements of heparin necessary to bind the growth factor and the TK receptor or to affect their mutual interaction may be different; 3) heparin-related molecules with different structures able to differently affect the biological activities of angiogenic growth factors can be designed.

The identification of the structural requirements of heparin responsible for its interaction with FGF2 and FGFR have been investigated by several laboratories with different experimental models. The results indicate that size [78, 79] and degree of sulfation [37, 74, 80] are critical for the capacity of heparin to induce FGF2/FGFR interaction. Heparin requires both 2-O-sulfate and 6-O-sulfate groups, as well as N-sulfate groups, to promote the binding of FGF2 to soluble FGFR-1 [81]. Thus, the binding of heparin/HS to FGF2, which does not require 6-O-sulfate groups, is not sufficient to induce FGF2 interaction with FGFR. Accordingly, unmodified heparin, but not 6-O-desulfated heparin, protects FGFR-1 from trypsin digestion [81]. These data support the hypothesis that HSPGs modulate the binding of FGF2 to FGFR through the formation of a ternary complex in which the GAG chain interacts with FGF2 *via* 2-O-sulfate and N-sulfate groups while 6-O-sulfate groups are required for its interaction with FGFR [74]. The analysis of the crystal structure of FGFR-2 ectodomain bound to FGF1 and heparin decasaccharide is in keeping with this hypothesis [77].

A common theme among growth factors interacting with TK receptors is the involvement of ligand-induced receptor dimerization in receptor activation [82]. Recently, several crystallography studies of FGF/FGFR complexes regarding FGF1 and FGF2 bound to FGFR-1 or FGFR-2 have revealed assemblages of two ligand molecules bound to two receptor molecules [83-85]. However, crystal structures of HS/FGF/FGFR ternary complexes revealed markedly different geometrics [76, 77]. A single molecule of heparin/HS may bind several molecules of FGF2 [86], suggesting that GAGs induce oligomerization of angiogenic growth factors. Indeed, heparin induces dimerization of FGF2 in a cell free system [86]. It has been demonstrated that the dimerization and activation of FGFR catalyzed by heparin-dependent oligomerization of FGF1 is required to induce a mitogenic response [87]. Heparin has been hypothesized to play a similar role also for FGF2 [88] and hepatocyte growth factor (HGF) [89]. Recent observations have demonstrated that FGF2 molecules self-associate through specific interactions in a sequential fashion, leading to the formation of bioactive FGF2 dimers, and that heparin can serve as a platform to stabilize the intermolecular FGF2 interactions, thus favoring receptor dimerization [90].

HEPARIN-LIKE COMPOUNDS AND HEPARIN DERIVATIVES AS ANGIOGENESIS INHIBITORS

The capacity of various angiogenic factors to bind heparin/HS indicates that molecules able to interfere with this interaction may act as angiogenesis inhibitors. The ability of low molecular weight heparin fragments administered systemically to reduce the angiogenic activity of FGF2 and VEGF support this hypothesis [91-93]. Several heparin-like anionic molecules and heparin derivatives have been developed as possible candidate drugs. Here, the antiangiogenic activity of polysulfated/polysulfonated compounds, chemically modified heparins, and biotechnological heparins will be described.

Polysulfated/Polysulfonated Compounds as Angiogenesis Inhibitors

Suramin

Suramin is a polysulfonated naphthylurea (Fig. (1)) originally developed for the treatment of trypanosomiasis and onchocerciasis. More recently, suramin has been employed in patients unresponsive to conventional chemotherapy and anti-tumor activity has been reported in the treatment of adrenocortical carcinoma and prostate carcinoma [94]. However, a limitation on the clinical use of suramin is represented by the serious toxic side-effects consequent to the administration of the high doses of the molecule required to achieve anti-tumor activity. For instance, *in vivo* administration of suramin, that shows a plasma half-life equal to 30-50 days [95], dramatically increases tissue GAGs, leading to mucopolysaccharidosis-like pathologic conditions [96], and elevates the concentration of circulating HS and dermatan sulfate, thus inducing coagulopathy [94].

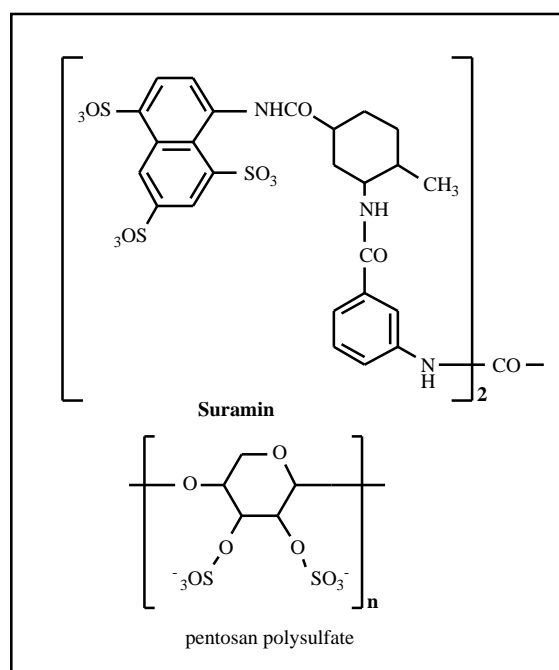


Fig. (1). Chemical structure of suramin and pentosan polysulfate.

As an angiogenesis inhibitor, suramin has been demonstrated to inhibit the activity exerted by FGFs and VEGF on cultured endothelial cells by preventing their interaction with cell-surface HSPGs and TK receptors and to block their angiogenic activity in different animal models [97]. This is due, at least in part, to the capacity of suramin to bind to the heparin-binding region of the growth factor *via* one or more of its sulfonate groups [98, 99]. Indeed, suramin is ineffective against angiogenesis elicited by non-heparin binding growth factors [97]. Accordingly, suramin is able to mimic heparin/HS for the capacity to protect FGF2 from trypsin digestion. Interestingly, the same capacity is observed for the related polysulfonated compound trypan blue [86]. In order to improve the therapeutic ratio of this class of compounds, various polysulfonated naphthylureas structurally related to suramin have been investigated for the capacity to inhibit the activity exerted by FGF2 on cultured endothelial cells and in a rat sponge angiogenesis assay [100, 101]. Also, a series of sulfonated distamycin A derivatives structurally related to suramin have been developed [98]. The results demonstrate that the number of sulfonate groups and modifications of the backbone of the molecule significantly affect the activity of these compounds. In particular, an extended multiple ring structure with at least two aromatic groups intervening between the two terminal naphthyl rings confers to suramin derivatives a reduced toxicity without affecting, or even improving, their FGF2 antagonist capacity.

Pentosan Polysulfate

Pentosan polysulfate is a polymer of xylose hydrogen sulfate and contains two sulfate groups per carbohydrate monomer (Fig. (1)). It binds FGFs as well as other heparin-binding growth factors [102, 103] and it has been shown to interact also with the heparin-binding site of FGFR-1 [104]. It inhibits the growth of SW13 adrenocortical cells transfected with FGF4 [105] and of gastric cancer cell lines overexpressing the angiogenic factor midkine [106]. Also, it suppresses the tumorigenicity of MCF-7 breast carcinoma cells transfected with FGF1 or FGF4 [107]. Even though the contribution of a possible angiostatic effect of pentosan polysulfate was not investigated in these studies, independent observations have shown that cultured endothelium is sensitive to inhibition by pentosan polysulfate. Interestingly, microvascular endothelium appears to be more sensitive to inhibition by pentosan polysulfate and suramin derivatives than large-vessel endothelium [100]. Also, some observations have suggested that pentosan polysulfate may be more effective in inhibiting FGF4-dependent than FGF2-dependent cell proliferation. If confirmed, these findings support previous observations about different structural requirements in FGF4-heparin interaction when compared to FGF2-heparin interaction [74]. Pentosan polysulfate has been found to inhibit the growth of Kaposi's sarcoma-derived spindle cells *in vitro* [108] and the activity of extracellular HIV-Tat protein [109]. As observed for suramin, pentosan polysulfate is also a potent anti-HIV agent *in vitro* [110]. These observations suggested that pentosan polysulfate might be worth exploring as a potential agent for the treatment of Kaposi's sarcoma. A trial in patients with HIV-related Kaposi's sarcoma has shown that the maximally tolerated dose of pentosan polysulfate given by continuous

venous infusion is 3 mg/kg per day. No patient had an objective clinical anti-tumor response to either systemic or intralesional pentosan polysulfate administration; however, three patients had stable Kaposi's sarcoma for 3-27 weeks. Dose-limiting toxic effects were characterized by anticoagulation and thrombocytopenia and were reversible [111, 112].

Sulfonic Acid Polymers

The sulfonic acid polymers PAMPS [poly(2-acrylamido-2-methyl-1-propanesulfonic acid)], PAS [poly(anethole-sulfonic acid)], and PSS [poly(4-styrenesulfonic acid)] are more potent inhibitors of neovascularization than suramin and pentosan polysulfate in the chick embryo chorioallantoic membrane (CAM) assay [113]. Also, these sulfonic acid polymers exert an anti-angiogenic effect in the *in vitro* rat aorta-ring assay and inhibit FGF2-induced human umbilical vein endothelial cell proliferation. Interestingly, a significant correlation was found between the angiostatic activity of these compounds in the CAM assay *in vivo* and their capacity to inhibit the FGF2-induced mitogenic response *in vitro*, thus suggesting that FGF2 is a target for sulfonic acid polymers [113]. Accordingly, PAMPS, PAS, and PSS inhibit FGF2 binding to HSPGs and FGFRs in endothelial cells. They also abrogate the formation of the HSPG/FGF2/FGFR ternary complex, as evidenced by their capacity to prevent FGF2-mediated cell-cell attachment of FGFR-1-overexpressing, HSPG-deficient CHO cells to wild-type HSPG-bearing cells (Fig. (2)) [114]. Direct interaction of the polysulfonates with FGF2 was demonstrated by their ability to protect the growth factor from proteolytic cleavage. Accordingly, molecular modeling, based on the crystal structure of the interaction of FGF2 with a heparin hexamer, showed the feasibility of docking PAMPS into the heparin-binding domain of FGF2 (Fig. (3A)). In agreement with their FGF2-binding capacity, PSS, PAS, and PAMPS inhibited FGF2-induced cell proliferation in endothelial cells of different origin. The anti-proliferative activity of these compounds was associated with the abrogation of FGF2-induced tyrosine phosphorylation of FGFR-1. Moreover, the polysulfonates PSS and PAS inhibited FGF2-induced activation of mitogen-activated protein kinase-1/2 (ERK_{1/2}), involved in FGF2 signal transduction [114]. *In vivo*, sulfonic acid polymers inhibit the angiogenic response triggered by a FGF2 implant in the avascular rabbit cornea (Table 2) and the growth of rat hemangiosarcoma xenografts in nude mice (Fig. (3B,C)).

Tab. 2. Inhibitory Effect of PAMPS and PAS on FGF2-Mediated Angiogenesis in the Rabbit Cornea

Implant	Area of neovascularization ^a	p value
FGF2 (650 ng)	16.2 ± 3.3 mm ²	--
FGF2 + PAMPS (100 µg)	8.7 ± 3.8 mm ²	< 0.05
FGF2 + PAS (100 µg)	10.8 ± 2.3 mm ²	< 0.05

^a The area of corneal neovascularization was determined 8 days after implantation by measuring the vessel length (L) from the limbus and the number of clock hours (C) of limbus involved. A formula was used to determine the area of a circular band segment: $C/12 \times 3.1416 [r^2 - (r-L)^2]$, where $r = 6$ mm, the measured radius of the rabbit cornea.

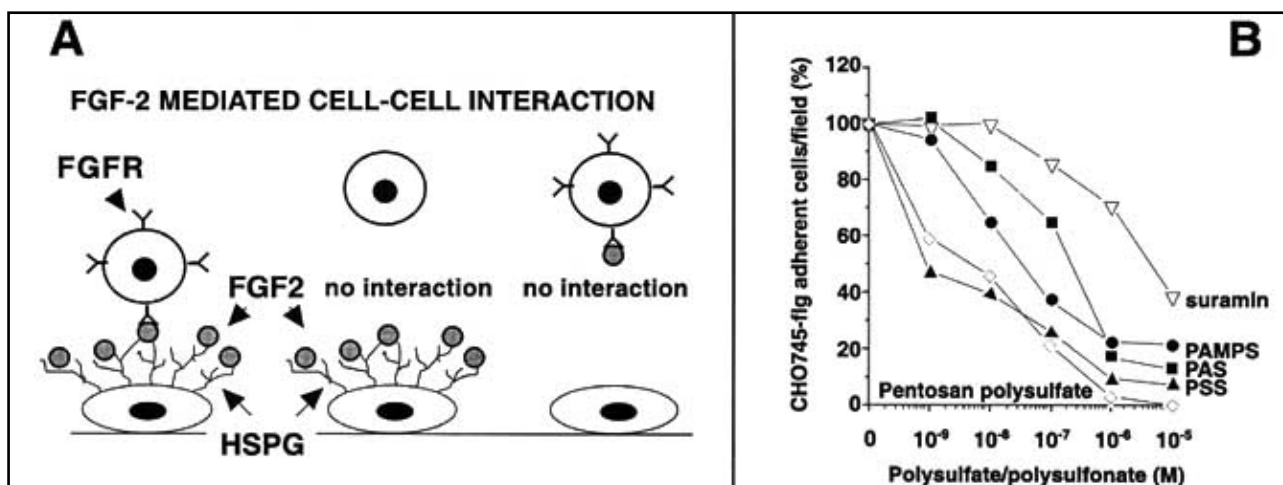


Fig. (2). Polysulfated/polysulfonated compounds inhibit the formation of the HSPG/FGF2/FGFR ternary complex. A) Schematic representation of the experimental model. FGF2 mediates the interaction of FGFR1-bearing cells with HSPGs of the cell monolayer (*left*). No interaction occurs in the absence of FGFR1 (*center*) or of HSPGs (*right*). B) Various polysulfated/polysulfonated compounds prevent FGF2-mediated cell-cell interaction with different potency.

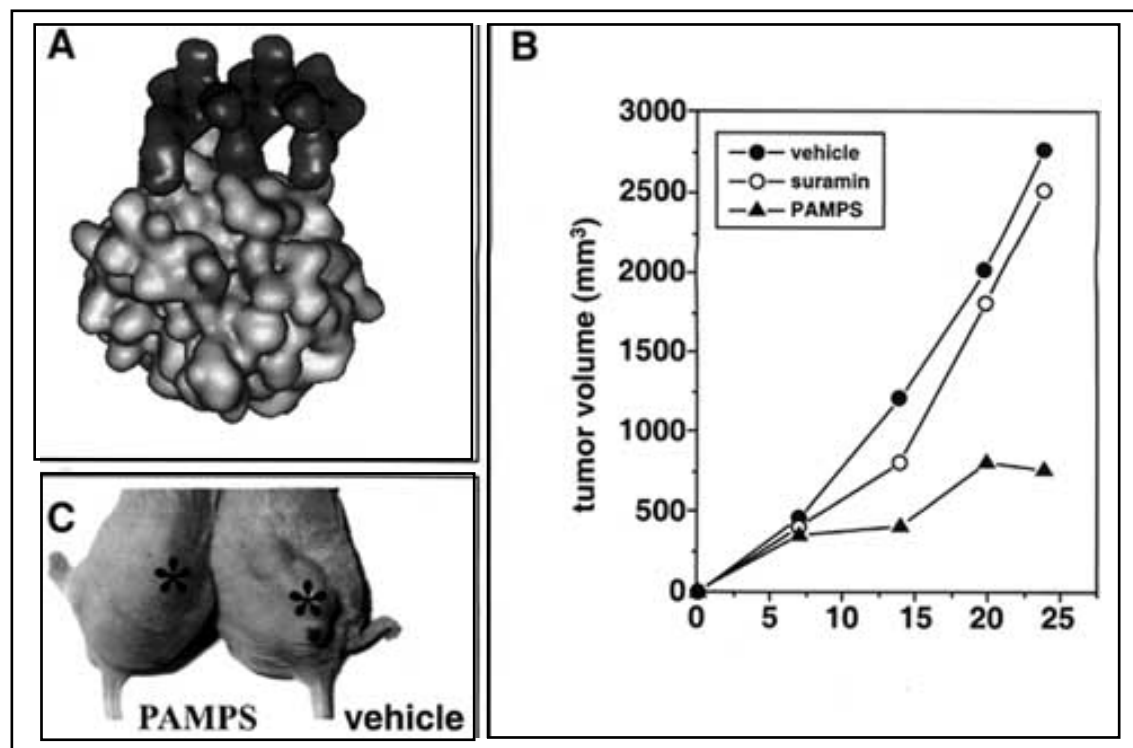


Fig. (3). Antitumor activity of PAMPS. A) Computer modeling of PAMPS/FGF2 interaction. Molecular surfaces of PAMPS (dark gray) and FGF2 (light gray) were generated by the WebLab Viewer software. B) Nude mice were injected s.c. with rat hemangiosarcoma cells. Then, vehicle, suramin, or PAMPS were administered from day 7 *via* intratumor injection at 30 mg/kg body wt/day. C) Animals were sacrificed 25 days after cell injection and photographed.

Miscellanea

Besides the molecules described above, various polyanionic compounds have been described as potential angiostatic drugs. A non-comprehensive list includes: chemically sulfated malto-oligosaccharides [115]; sulfated

chitin derivatives [116]; β -cyclodextrin tetradecasulfate in combination with angiostatic steroids [117]; heparin-steroid conjugates [118]; the nonsulfated polyanionic compounds triphenylmethane derivative aurintricarboxylic acid [119] and poly-4-hydroxyphenoxy acetic acid (RG-13577) [120].

Chemically Modified Heparins as Angiogenesis Inhibitors

As stated above, low molecular weight heparin fragments reduce the angiogenic activity of FGF2 and VEGF [91-93], indicating that size modifications can confer angiostatic properties to the GAG chain. Also, selective 6-O-desulfation, but not 2-O-desulfation, confers angiostatic capacity to full length heparin [121]. In the latter case, the angiostatic activity of 6-O-desulfated heparin appears to be due, at least in part, to the capacity of the GAG chain to interact with FGF2 but not to FGFR, thus preventing the formation of the bioactive HSPG/FGF2/FGFR ternary complex [121].

The structure and minimal size of heparin (and HS) chains able to bind to FGFs (especially to FGF2) has been extensively investigated using both natural fragments [122-124] and synthetic oligosaccharides [125, 126]. Whereas oligosaccharides as small as tetrasaccharide bind to FGF2 [127, 128], oligosaccharide chains longer than octa-decasaccharides are necessary for mitogenic activity [121, 127, 129]. Also some undersulfated heparin oligosaccharides (missing, i.e., 6-O-sulfate groups on aminosugar residues) are able to bind to FGF2 [122-124].

The X-ray structures of tetrasaccharide and hexasaccharide/FGF2 complexes indicate that the minimum FGF2-binding heparin structure consists of a N-sulfate group (NS) and a 2-O-sulfate group (2S) on contiguous GlcN and IdoA residues, respectively [130]. Molecular models clearly show NS/2S pairs on both sides of a heparin helix, irrespective of the conformation of the IdoA2S residues [131]. As shown by molecular modeling [129, 132] and X-ray diffraction studies [76, 83, 84], relatively long arrays of NS/2S pairs are necessary for binding more than one FGF2 molecule and formation of signal-transducing heparin/FGF/FGFR complexes.

Attempts have been made to investigate the possibility to modify the structure of the heparin chain to confer it the ability to bind FGF2 with a 1:1 molar ratio, thus hampering FGF2 dimerization and formation of FGF2/FGFR complexes. To this purpose, sulfation gaps along the regular heparin sequences were generated by selectively removing 2S groups to reach a ratio of about 1:1 between sulfated and nonsulfated uronic acid residues. Then, in order to disrupt the original helical chain conformation, the C(2)-C(3) bonds of all nonsulfated uronic acid residues were split, generating flexible joints along the heparin chains while minimizing cleavage of glycosidic bonds [133]. The novel heparin derivative (Fig. (4)) retained the FGF2-binding ability of the parent heparin. However, it was a poor inducer of FGF2 dimerization, inhibited better than heparin the FGF2-induced growth of endothelial cells, and was antiangiogenic in the CAM assay. Since the splitting reaction also occurred at the level of the essential glucuronic acid residue of the active site for antithrombin, the heparin derivative was no longer anticoagulant [133].

Biotechnological Heparins as Angiogenesis Inhibitors

The capsular K5 polysaccharide from *Escherichia coli* has the same structure [4)-D-GlcA-(1-4)-D-GlcNAc-1(1-)]_n as the heparin precursor N-acetyl heparosan [134]. Previous studies had shown the possibility to generate K5 derivatives by chemical sulfation in N- and/or O-positions [135]. Also, enzymatic conversion of D-GlcA to L-IdoA residues in K5 can be performed, thus generating heparin/HS-like sequences by *in vitro* chemical/enzymatic modifications of the bacterial polysaccharide [136].

Recently, the FGF2 antagonist and anti-angiogenic activity of novel sulfated derivatives of the *Escherichia coli*

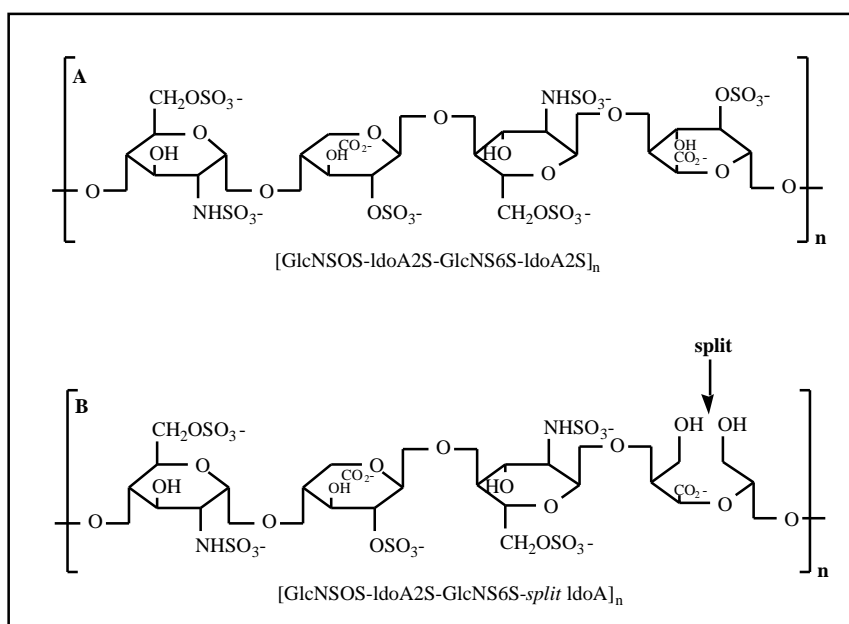


Fig. (4). Prevalent sequences in regular regions of heparin (A) and in chemically-modified 50% 2-O-desulfated and glycol-split heparin (B).

K5 polysaccharide has been reported [137]. K5 polysaccharide was chemically sulfated in N-position, in O-position after N-deacetylation, or in both. O-sulfated and N,O-sulfated K5 derivatives with low and high degree of sulfation competed with immobilized heparin for the binding to ^{125}I -FGF2 with different potency. Accordingly, they abrogated the formation of the HSPG/FGF2/FGFR ternary complex, as evidenced by their capacity to prevent FGF2-mediated cell-cell attachment of FGFR1-overexpressing HSPG-deficient CHO cells to wild-type CHO cells. They also inhibited ^{125}I -FGF2 binding to HSPGs and FGFRs in FGFR1-overexpressing, HSPG-bearing CHO cells and in endothelial cells. Also, K5 derivatives inhibited with different potency FGF2-mediated cell proliferation in endothelial cells of different origin. In all these assays, the N-sulfated K5 derivative and unmodified K5 were poorly effective. Among all the derivatives tested, only highly O-sulfated and N,O-sulfated K5 derivatives prevented the sprouting of FGF2-transfected endothelial cells in 3D fibrin gel and "spontaneous angiogenesis *in vitro*" on Matrigel. *In vivo*, the highly N,O-sulfated K5 derivative exerted a potent anti-angiogenic activity when tested in the chick embryo CAM assay.

These data indicate that both the degree of sulfation and charge distribution modulate the biological activity of sulfated K5 derivatives. Indeed, highly O-sulfated and N,O-sulfated K5 derivatives (with a $\text{SO}_3^-/\text{COO}^-$ equal approximately to 3.8 for both compounds) were equally effective on endothelial cells in most of the *in vitro* assays whereas their low sulfated counterparts were poorly effective. The highly O-sulfated K5 derivative consists of the virtually homogeneous repeat of GlcA2S3S-GlcNAc3S6S disaccharide units whereas 70% of the highly N,O-sulfated K5 derivative sequence is represented by GlcA2S3S-GlcNS6S disaccharide units (Fig. (5)). This suggests that the degree of sulfation of K5 derivatives, rather than their charge distribution, may be a major determinant for their FGF2-antagonist activity. Also, N-sulfation represents an absolute requirement for the angiostatic activity of K5 derivatives that must be sulfated also in O position(s). Moreover, the data indicate that the almost complete N-sulfation and 6-O-sulfation of the Glc residues is not sufficient to confer an angiostatic capacity to K5. Thus, sulfation in 2-O- and/or 3-O-position in GlcA residues and/or in 3-O-position in Glc residues are also required. In agreement with these observations, previous findings had shown a limited capacity of non-sulfated K5 to affect angiogenesis in the CAM assay when compared to HS-derived oligosaccharides [138].

Even though the identification of the minimal sulfation pattern required for a full angiostatic activity in K5 derivatives will require further investigation, the data demonstrate the possibility to generate FGF2 antagonists endowed with anti-angiogenic activity by specific chemical sulfation of bacterial K5 polysaccharide. In particular, the highly N,O-sulfated K5 derivative was effective *in vitro* and *in vivo* on endothelial cells of murine, bovine, avian, and human origin. Also, the highly N,O-sulfated K5 derivative is endowed with a low anti-coagulant activity when compared to heparin [137]. This compound may provide the basis for the design of novel biotechnological heparins with angiostatic properties and possible therapeutic implications.

CONCLUDING REMARKS

The consequences of the interaction of angiogenic growth factors with heparin/HS in endothelium can result either in the inhibition or in the enhancement of their biological activity. Generally speaking, the binding of the growth factor to cell-associated HSPGs causes its storage in the ECM with consequent increase in local concentration, prolonged half-life, and decrease in the radius of diffusion. This will favor growth factor oligomerization, TK receptor interaction and signaling. Conversely, the binding of the growth factor to soluble HSPGs may antagonize all or part of these effects, resulting in a potential angiostatic action. Exceptions can be represented by heparin/HS-derived oligosaccharides whose size and sequence allow a ternary interaction among soluble GAG, growth factor, and TK receptor. Thus, the biological activity of angiogenic growth factors on endothelial cells is controlled by a complex interplay among free and cell-associated heparin/HS. In this scenario, natural and synthetic heparin-related angiostatic compounds play their pharmacological action.

These considerations point to the importance of the accurate definition of the molecular bases of protein-heparin/HS interaction for the design of molecules endowed with angiogenic agonist or antagonist activity. Specific oligosaccharide sequences appear to be involved in the interaction with different growth factors and their receptors [62, 139]. For instance, the minimal FGF2-binding sequence in HS has been identified as a pentasaccharide which contains the disaccharide units IdoA2S-GlcNS or IdoA2S-GlcNS6S [122] whereas high affinity FGF1/HS interaction occurs *via* a -IdoA2S-GlcNS6S-IdoA2S- motif [124].

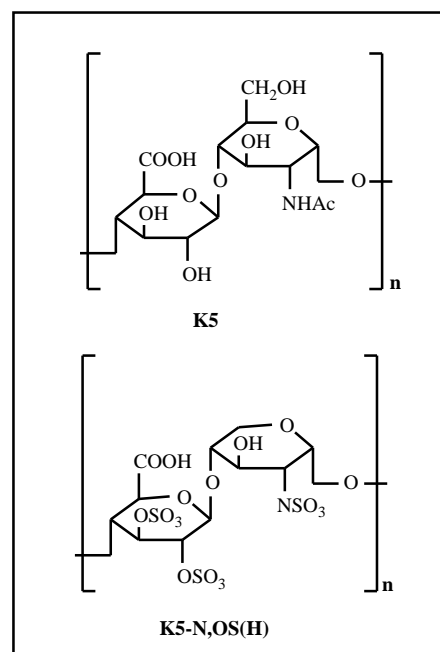


Fig. (5). Chemical structure of the E.coli K5 polysaccharide and of the prevalent sequence (approx. 70%) of the antiangiogenic highly N,O-sulfated K5 derivative.

Accordingly, binding studies involving chemically modified heparins or HS preparations have shown that 2-O-sulfate and N-sulfate groups are important for FGF2 interaction. However, FGF1 and FGF4 differ distinctly from each other and from FGF2 in their interaction with selectively O-desulfated heparins, 6-O-sulfate groups being required in addition to 2-O-sulfate groups for GAG interaction [74, 140]. HIV-Tat protein [141], the longer spliced variant of platelet-derived growth factor A [142], and FGF8 [143] require 2-O-sulfate, 6-O-sulfate and N-sulfate groups for optimal interaction with heparin. HGF interacts mainly with 6-O-sulfate groups of GlcNS residues while N-sulfates and IdoA2S units play a limited role [144]. In contrast, N-sulfate groups are critically important for the interaction of the GAG with midkine [145]. Finally, heparin/HS subpopulations with chemokine-binding selectivity exist [146] with IL-8 interacting preferentially with the 6-O-sulfate and 2-O-sulfate groups of heparin [147].

The data indicate that distinct structural requirements are necessary for the interaction of heparin/HS with different growth factors. Even though these specific binding sequences may be hidden in heparin due to its high degree of sulfation, the high heterogeneity in HS structure allows a more refined tailoring of selective binding regions that may influence the biological activity and bioavailability of heparin/HS-binding growth factors. Thus, the specific tailoring of molecules with selective action towards defined heparin-binding growth factors can be envisaged. To this respect, the synthetic angiostatic compounds described above are quite "non-selective", being able to affect the activity of a variety of growth factors. The lack of selectivity may be responsible, at least in part, for the side effects of these compounds, including their anticoagulant activity. In contrast, controlled chemical and/or enzymatic modifications of bacterial K5 polysaccharide [136, 137] may allow the synthesis of non-anticoagulant biotechnological heparins endowed with increased specificity.

New methods are emerging for the modeling of carbohydrate interaction with protein combining site [148], heparin/HS sequencing [149], and the generation of biosynthetic oligosaccharides libraries [150]. These techniques will help to optimize the characterization of the chemical structure of saccharide species and to guide chemical/enzymatic engineering toward pharmacological analogues with wider therapeutic window and increased specificity of action.

ACKNOWLEDGEMENTS

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro, National Research Council (Target Project on Biotechnology), Ministero dell'Istruzione, dell'Università e della Ricerca (Cofin 2000 and Centro di Eccellenza "IDET"), and Consorzio Italiano Biotecnologie to M.P.

ABBREVIATIONS

CAM = Chorioallantoic membrane
GAG = Glycosaminoglycan

ECM = Extracellular matrix
FGF = Fibroblast growth factor
FGFR = Tyrosine kinase FGF receptor
GlcA = Glucuronic acid
GlcNS6S = N,6-disulfated D-glucosamine
GPI = Glycosyl-phosphatidylinositol
HGF = Hepatocyte growth factor
HS = Heparan sulfate
HSPG = Heparan sulfate proteoglycan
IdoA = Iduronic acid
IdoA2 = 2-O-sulfated L-IdoA
IL = Interleukin
NS = N-sulfate group
PAMPS = Poly(2-acrylamido-2-methyl-1-propanesulfonic acid)
PAS = Poly(anetholesulfonic acid)
PG = Proteoglycan
PSS = Poly(4-styrenesulfonic acid)
2S = 2-O-sulfate group
TGF = Transforming growth factor
TK = Tyrosine kinase
VEGF = Vascular endothelial growth factor

REFERENCES

References 151-153 are related articles recently published in *Current Pharmaceutical Design*.

- [1] Lindahl, U.; Lidholt, K.; Spillmann, D.; Kjellen, L. *Thromb. Res.*, **1994**, *75*, 1-32.
- [2] Iozzo, R. V.; San Antonio, J. D. *J. Clin. Invest.*, **2001**, *108*, 349-55.
- [3] Poole, A. R. *Biochem. J.*, **1986**, *236*, 1-14.
- [4] Wrenshall, L. E.; Cerra, F. B.; Singh, R. K.; Platt, J. L. *J. Immunol.*, **1995**, *154*, 871-80.
- [5] Weiser, M. C.; Grieshaber, N. A.; Schwartz, P. E.; Majack, R. A. *Mol. Biol. Cell*, **1997**, *8*, 999-1011.
- [6] Woods, A. *J. Clin. Invest.*, **2001**, *107*, 935-41.
- [7] Reiland, J.; Ott, V. L.; Lebakken, C. S.; Yeaman, C.; McCarthy, J.; Rapraeger, A. C. *Biochem. J.*, **1996**, *319* (Pt 1), 39-47.

- [8] Rapraeger, A. C. *J. Cell Biol.*, **2000**, *149*, 995-8.
- [9] Horowitz, A.; Tkachenko, E.; Simons, M. *J. Cell Biol.*, **2002**, *157*, 715-25.
- [10] Lopez-Casillas, F.; Cheifetz, S.; Doody, J.; Andres, J. L.; Lane, W. S.; Massague, J. *Cell*, **1991**, *67*, 785-95.
- [11] Hayashi, K.; Madri, J. A.; Yurchenco, P. D. *J. Cell Biol.*, **1992**, *119*, 945-59.
- [12] Lowe-Krentz, L. J.; Thompson, K.; Patton, W. A., 2nd. *Mol. Cell Biochem.*, **1992**, *109*, 51-60.
- [13] Saksela, O.; Rifkin, D. B. *J. Cell Biol.*, **1990**, *110*, 767-75.
- [14] Bashkin, P.; Doctrow, S.; Klagsbrun, M.; Svahn, C. M.; Folkman, J.; Vlodavsky, I. *Biochemistry*, **1989**, *28*, 1737-43.
- [15] Brunner, G.; Gabrilove, J.; Rifkin, D. B.; Wilson, E. L. *J. Cell Biol.*, **1991**, *114*, 1275-83.
- [16] Fedarko, N. S.; Conrad, H. E. *J. Cell Biol.*, **1986**, *102*, 587-99.
- [17] Ishihara, M.; Fedarko, N. S.; Conrad, H. E. *J. Biol. Chem.*, **1986**, *261*, 13575-80.
- [18] Sugahara, K.; Kitagawa, H. *Curr. Opin. Struct. Biol.*, **2000**, *10*, 518-27.
- [19] Casu, B.; Lindahl, U. *Adv. Carbohydr Chem. Biochem.*, **2001**, *57*, 159-206.
- [20] Esko, J. D.; Lindahl, U. *J. Clin. Invest.*, **2001**, *108*, 169-73.
- [21] Kanwar, Y. S.; Farquhar, M. G. *Proc. Natl. Acad. Sci. USA*, **1979**, *76*, 1303-7.
- [22] Castellot, J. J., Jr.; Addonizio, M. L.; Rosenberg, R.; Karnovsky, M. J. *J. Cell Biol.*, **1981**, *90*, 372-9.
- [23] Marcum, J. A.; McKenney, J. B.; Rosenberg, R. D. *J. Clin. Invest.*, **1984**, *74*, 341-50.
- [24] Williams, M. P.; Streeter, H. B.; Wusteman, F. S.; Cryer, A. *Biochim. Biophys. Acta*, **1983**, *756*, 83-91.
- [25] Danielsson, A.; Raub, E.; Lindahl, U.; Bjork, I. *J. Biol. Chem.*, **1986**, *261*, 15467-73.
- [26] Kojima, T.; Shworak, N. W.; Rosenberg, R. D. *J. Biol. Chem.*, **1992**, *267*, 4870-7.
- [27] Bernfield, M.; Kokenyesi, R.; Kato, M.; Hinkes, M. T.; Spring, J.; Gallo, R. L.; Lose, E. *J. Annu. Rev. Cell Biol.*, **1992**, *8*, 365-93.
- [28] Kojima, T.; Katsumi, A.; Yamazaki, T.; Muramatsu, T.; Nagasaka, T.; Ohsumi, K.; Saito, H. *J. Biol. Chem.*, **1996**, *271*, 5914-20.
- [29] Aviezer, D.; Hecht, D.; Safran, M.; Eisinger, M.; David, G.; Yayon, A. *Cell*, **1994**, *79*, 1005-13.
- [30] Kinsella, M. G.; Wight, T. N. *Biochemistry*, **1988**, *27*, 2136-44.
- [31] Marcum, J. A.; Rosenberg, R. D. *Biochem. Biophys. Res. Commun.*, **1985**, *126*, 365-72.
- [32] Sasisekharan, R.; Moses, M. A.; Nugent, M. A.; Cooney, C. L.; Langer, R. *Proc. Natl. Acad. Sci. U S A*, **1994**, *91*, 1524-8.
- [33] Goldshmidt, O.; Zcharia, E.; Abramovitch, R.; Metzger, S.; Aingorn, H.; Friedmann, Y.; Schirmacher, V.; Mitrani, E.; Vlodavsky, I. *Proc. Natl. Acad. Sci. USA*, **2002**, *99*, 10031-6.
- [34] Bonneh-Barkay, D.; Shlissel, M.; Berman, B.; Shaoul, E.; Admon, A.; Vlodavsky, I.; Carey, D. J.; Asundi, V. K.; Reich-Slotky, R.; Ron, D. *J. Biol. Chem.*, **1997**, *272*, 12415-21.
- [35] Mali, M.; Elenius, K.; Miettinen, H. M.; Jalkanen, M. *J. Biol. Chem.*, **1993**, *268*, 24215-22.
- [36] Salmivirta, M.; Heino, J.; Jalkanen, M. *J. Biol. Chem.*, **1992**, *267*, 17606-10.
- [37] Aviezer, D.; Levy, E.; Safran, M.; Svahn, C.; Buddecke, E.; Schmidt, A.; David, G.; Vlodavsky, I.; Yayon, A. *J. Biol. Chem.*, **1994**, *269*, 114-21.
- [38] Coltrini, D.; Rusnati, M.; Zoppetti, G.; Oreste, P.; Grazioli, G.; Naggi, A.; Presta, M. *Biochem. J.*, **1994**, *303* (Pt 2), 583-90.
- [39] Gospodarowicz, D.; Cheng, J. *J. Cell Physiol.*, **1986**, *128*, 475-84.
- [40] Flaumenhaft, R.; Moscatelli, D.; Rifkin, D. B. *J. Cell Biol.*, **1990**, *111*, 1651-9.
- [41] Presta, M.; Maier, J. A.; Rusnati, M.; Ragnotti, G. *J. Cell Physiol.*, **1989**, *140*, 68-74.
- [42] Elenius, K.; Maatta, A.; Salmivirta, M.; Jalkanen, M. *J. Biol. Chem.*, **1992**, *267*, 6435-41.
- [43] Nugent, M. A.; Edelman, E. R. *J. Biol. Chem.*, **1992**, *267*, 21256-64.
- [44] Tumova, S.; Bame, K. J. *J. Biol. Chem.*, **1997**, *272*, 9078-85.
- [45] Ausprunk, D. H.; Boudreau, C. L.; Nelson, D. A. *Am. J. Pathol.*, **1981**, *103*, 367-75.
- [46] Ausprunk, D. H.; Boudreau, C. L.; Nelson, D. A. *Am. J. Pathol.*, **1981**, *103*, 353-66.
- [47] Ausprunk, D. H. *Dev. Biol.*, **1982**, *90*, 79-90.
- [48] Oohira, A.; Wight, T. N.; Bornstein, P. *J. Biol. Chem.*, **1983**, *258*, 2014-21.
- [49] Montesano, R.; Mossaz, A.; Ryser, J. E.; Orci, L.; Vassalli, P. *J. Cell Biol.*, **1984**, *99*, 1706-15.
- [50] Kinsella, M. G.; Tsoi, C. K.; Jarvelainen, H. T.; Wight, T. N. *J. Biol. Chem.*, **1997**, *272*, 318-25.
- [51] Iozzo, R. V. *J. Clin. Invest.*, **2001**, *108*, 165-7.
- [52] Massague, J. *Cell*, **1992**, *69*, 1067-70.
- [53] Yamaguchi, Y.; Mann, D. M.; Ruoslahti, E. *Nature*, **1990**, *346*, 281-4.
- [54] Saksela, O.; Moscatelli, D.; Sommer, A.; Rifkin, D. B. *J. Cell Biol.*, **1988**, *107*, 743-51.

- [55] Rusnati, M.; Urbinati, C.; Presta, M. *J. Cell Physiol.*, **1993**, *154*, 152-61.
- [56] Revis-Gupta, S.; Abdel-Ghany, M.; Koland, J.; Racker, E. *Proc. Natl. Acad. Sci. USA*, **1991**, *88*, 5954-8.
- [57] Gao, G.; Goldfarb, M. *EMBO J.*, **1995**, *14*, 2183-90.
- [58] Chernousov, M. A.; Carey, D. J. *J. Biol. Chem.*, **1993**, *268*, 16810-4.
- [59] Andres, J. L.; DeFalcis, D.; Noda, M.; Massague, J. *J. Biol. Chem.*, **1992**, *267*, 5927-30.
- [60] Nurcombe, V.; Ford, M. D.; Wildschut, J. A.; Bartlett, P. F. *Science*, **1993**, *260*, 103-6.
- [61] Knox, S.; Merry, C.; Stringer, S.; Melrose, J.; Whitelock, J. *J. Biol. Chem.*, **2002**, *277*, 14657-65.
- [62] Gallagher, J. T. *J. Clin. Invest.*, **2001**, *108*, 357-61.
- [63] Mustonen, T.; Alitalo, K. *J. Cell Biol.*, **1995**, *129*, 895-8.
- [64] Rapraeger, A. C.; Krufka, A.; Olwin, B. B. *Science*, **1991**, *252*, 1705-8.
- [65] Gitay-Goren, H.; Soker, S.; Vlodavsky, I.; Neufeld, G. *J. Biol. Chem.*, **1992**, *267*, 6093-8.
- [66] Yayon, A.; Klagsbrun, M.; Esko, J. D.; Leder, P.; Ornitz, D. *M. Cell*, **1991**, *64*, 841-8.
- [67] Aviezer, D.; Iozzo, R. V.; Noonan, D. M.; Yayon, A. *Mol. Cell Biol.*, **1997**, *17*, 1938-46.
- [68] Roghani, M.; Mansukhani, A.; Dell'Era, P.; Bellosta, P.; Basilico, C.; Rifkin, D. B.; Moscatelli, D. *J. Biol. Chem.*, **1994**, *269*, 3976-84.
- [69] Ornitz, D. M.; Yayon, A.; Flanagan, J. G.; Svahn, C. M.; Levi, E.; Leder, P. *Mol. Cell Biol.*, **1992**, *12*, 240-7.
- [70] Rusnati, M.; Coltrini, D.; Caccia, P.; Dell'Era, P.; Zopetti, G.; Oreste, P.; Valsasina, B.; Presta, M. *Biochem. Biophys. Res. Commun.*, **1994**, *203*, 450-8.
- [71] Tessler, S.; Rockwell, P.; Hicklin, D.; Cohen, T.; Levi, B. Z.; Witte, L.; Lemischka, I. R.; Neufeld, G. *J. Biol. Chem.*, **1994**, *269*, 12456-61.
- [72] Cohen, T.; Gitay-Goren, H.; Sharon, R.; Shibuya, M.; Halaban, R.; Levi, B. Z.; Neufeld, G. *J. Biol. Chem.*, **1995**, *270*, 11322-6.
- [73] Turnbull, J. E.; Gallagher, J. T. *Biochem. Soc. Trans.*, **1993**, *21*, 477-82.
- [74] Guimond, S.; Maccarana, M.; Olwin, B. B.; Lindahl, U.; Rapraeger, A. C. *J. Biol. Chem.*, **1993**, *268*, 23906-14.
- [75] Kan, M.; Wang, F.; Xu, J.; Crabb, J. W.; Hou, J.; McKeehan, W. L. *Science*, **1993**, *259*, 1918-21.
- [76] Schlessinger, J.; Plotnikov, A. N.; Ibrahimi, O. A.; Eliseenkova, A. V.; Yeh, B. K.; Yayon, A.; Linhardt, R. J.; Mohammadi, M. *Mol. Cell*, **2000**, *6*, 743-50.
- [77] Pellegrini, L.; Burke, D. F.; von Delft, F.; Mulloy, B.; Blundell, T. L. *Nature*, **2000**, *407*, 1029-34.
- [78] Ishihara, M.; Tyrrell, D. J.; Stauber, G. B.; Brown, S.; Cousens, L. S.; Stack, R. J. *J. Biol. Chem.*, **1993**, *268*, 4675-83.
- [79] Tyrrell, D. J.; Ishihara, M.; Rao, N.; Horne, A.; Kiefer, M. C.; Stauber, G. B.; Lam, L. H.; Stack, R. J. *J. Biol. Chem.*, **1993**, *268*, 4684-9.
- [80] Walker, A.; Turnbull, J. E.; Gallagher, J. T. *J. Biol. Chem.*, **1994**, *269*, 931-5.
- [81] Rusnati, M.; Presta, M. *Int. J. Clin. Lab. Res.*, **1996**, *26*, 15-23.
- [82] Ullrich, A.; Schlessinger, J. *Cell*, **1990**, *61*, 203-12.
- [83] Plotnikov, A. N.; Schlessinger, J.; Hubbard, S. R.; Mohammadi, M. *Cell*, **1999**, *98*, 641-50.
- [84] Plotnikov, A. N.; Hubbard, S. R.; Schlessinger, J.; Mohammadi, M. *Cell*, **2000**, *101*, 413-24.
- [85] Stauber, D. J.; DiGabriele, A. D.; Hendrickson, W. A. *Proc. Natl. Acad. Sci. U S A*, **2000**, *97*, 49-54.
- [86] Coltrini, D.; Rusnati, M.; Zopetti, G.; Oreste, P.; Isacchi, A.; Caccia, P.; Bergonzoni, L.; Presta, M. *Eur. J. Biochem.*, **1993**, *214*, 51-8.
- [87] Spivak-Kroizman, T.; Lemmon, M. A.; Dikic, I.; Ladbury, J. E.; Pinchasi, D.; Huang, J.; Jaye, M.; Crumley, G.; Schlessinger, J.; Lax, I. *Cell*, **1994**, *79*, 1015-24.
- [88] Klagsbrun, M.; Baird, A. *Cell*, **1991**, *67*, 229-31.
- [89] Sakata, H.; Stahl, S. J.; Taylor, W. G.; Rosenberg, J. M.; Sakaguchi, K.; Wingfield, P. T.; Rubin, J. S. *J. Biol. Chem.*, **1997**, *272*, 9457-63.
- [90] Kwan, C. P.; Venkataraman, G.; Shriver, Z.; Raman, R.; Liu, D.; Qi, Y.; Varticovski, L.; Sasisekharan, R. *J. Biol. Chem.*, **2001**, *276*, 23421-9.
- [91] Norrby, K. *Int. J. Exp. Pathol.*, **2000**, *81*, 191-8.
- [92] Norrby, K.; Ostergaard, P. *Int. J. Microcirc. Clin. Exp.*, **1997**, *17*, 314-21.
- [93] Norrby, K.; Ostergaard, P. *Int. J. Microcirc. Clin. Exp.*, **1996**, *16*, 8-15.
- [94] Hawkins, M. J. *Curr. Opin. Oncol.*, **1995**, *7*, 90-3.
- [95] Collins, J. M.; Klecker, R. W., Jr.; Yarchoan, R.; Lane, H. C.; Fauci, A. S.; Redfield, R. R.; Broder, S.; Myers, C. E. *J. Clin. Pharmacol.*, **1986**, *26*, 22-6.
- [96] Constantopoulos, G.; Rees, S.; Cragg, B. G.; Barranger, J. A.; Brady, R. O. *Am. J. Pathol.*, **1983**, *113*, 266-8.
- [97] Fan, T. P.; Jaggar, R.; Bicknell, R. *Trends Pharmacol. Sci.*, **1995**, *16*, 57-66.
- [98] Ciomei, M.; Pastori, W.; Mariani, M.; Sola, F.; Grandi, M.; Mongelli, N. *Biochem. Pharmacol.*, **1994**, *47*, 295-302.
- [99] Middaugh, C. R.; Mach, H.; Burke, C. J.; Volkin, D. B.; Dabora, J. M.; Tsai, P. K.; Bruner, M. W.; Ryan, J. A.; Marfia, K. E. *Biochemistry*, **1992**, *31*, 9016-24.
- [100] Braddock, P. S.; Hu, D. E.; Fan, T. P.; Stratford, I. J.; Harris, A. L.; Bicknell, R. *Br. J. Cancer*, **1994**, *69*, 890-8.
- [101] Takano, S.; Gately, S.; Neville, M. E.; Herblin, W. F.; Gross, J. L.; Engelhard, H.; Perricone, M.; Eidsvoog, K.; Brem, S. *Cancer Res.*, **1994**, *54*, 2654-60.

- [102] Zugmaier, G.; Lippman, M. E.; Wellstein, A. *J. Natl. Cancer Inst.*, **1992**, *84*, 1716-24.
- [103] Belford, D. A.; Hendry, I. A.; Parish, C. R. *J. Cell Physiol.*, **1993**, *157*, 184-9.
- [104] Pantoliano, M. W.; Horlick, R. A.; Springer, B. A.; Van Dyk, D. E.; Tobery, T.; Wetmore, D. R.; Lear, J. D.; Nahapetian, A. T.; Bradley, J. D.; Sisk, W. P. *Biochemistry*, **1994**, *33*, 10229-48.
- [105] Wellstein, A.; Zugmaier, G.; Califano, J. A., 3rd; Kern, F.; Paik, S.; Lippman, M. E. *J. Natl. Cancer Inst.*, **1991**, *83*, 716-20.
- [106] Rha, S. Y.; Noh, S. H.; Kwak, H. J.; Wellstein, A.; Kim, J. H.; Roh, J. K.; Min, J. S.; Kim, B. S.; Chung, H. C. *Cancer Lett.*, **1997**, *118*, 37-46.
- [107] McLeskey, S. W.; Zhang, L.; Trock, B. J.; Kharbanda, S.; Liu, Y.; Gottardis, M. M.; Lippman, M. E.; Kern, F. G. *Br. J. Cancer*, **1996**, *73*, 1053-62.
- [108] Nakamura, S.; Sakurada, S.; Salahuddin, S. Z.; Osada, Y.; Tanaka, N. G.; Sakamoto, N.; Sekiguchi, M.; Gallo, R. C. *Science*, **1992**, *255*, 1437-40.
- [109] Rusnati, M.; Urbinati, C.; Caputo, A.; Possati, L.; Lortat-Jacob, H.; Giacca, M.; Ribatti, D.; Presta, M. *J. Biol. Chem.*, **2001**, *276*, 22420-5.
- [110] Baba, M.; Nakajima, M.; Schols, D.; Pauwels, R.; Balzarini, J.; De Clercq, E. *Antiviral Res.*, **1988**, *9*, 335-43.
- [111] Pluda, J. M.; Shay, L. E.; Foli, A.; Tannenbaum, S.; Cohen, P. J.; Goldspiel, B. R.; Adamo, D.; Cooper, M. R.; Broder, S.; Yarchoan, R. *J. Natl. Cancer Inst.*, **1993**, *85*, 1585-92.
- [112] Parker, B. W.; Swain, S. M.; Zugmaier, G.; DeLap, R. L.; Lippman, M. E.; Wellstein, A. *J. Natl. Cancer Inst.*, **1993**, *85*, 1068-73.
- [113] Liekens, S.; Neyts, J.; Degreve, B.; De Clercq, E. *Oncol. Res.*, **1997**, *9*, 173-81.
- [114] Liekens, S.; Leali, D.; Neyts, J.; Esnouf, R.; Rusnati, M.; Dell'Era, P.; Maudgal, P. C.; De Clercq, E.; Presta, M. *Mol. Pharmacol.*, **1999**, *56*, 204-13.
- [115] Foxall, C.; Wei, Z.; Schaefer, M. E.; Casabonne, M.; Fugedi, P.; Peto, C.; Castellet, J. J., Jr.; Brandley, B. K. *J. Cell Physiol.*, **1996**, *168*, 657-67.
- [116] Murata, J.; Saiki, I.; Makabe, T.; Tsuta, Y.; Tokura, S.; Azuma, I. *Cancer Res.*, **1991**, *51*, 22-6.
- [117] Li, W. W.; Casey, R.; Gonzalez, E. M.; Folkman, J. *Invest. Ophthalmol. Vis. Sci.*, **1991**, *32*, 2898-905.
- [118] Thorpe, P. E.; Derbyshire, E. J.; Andrade, S. P.; Press, N.; Knowles, P. P.; King, S.; Watson, G. J.; Yang, Y. C.; Rao-Bette, M. *Cancer Res.*, **1993**, *53*, 3000-7.
- [119] Gagliardi, A. R.; Collins, D. C. *AntiCancer Res.*, **1994**, *14*, 475-9.
- [120] Miao, H. Q.; Ornitz, D. M.; Aingorn, E.; Ben-Sasson, S. A.; Vlodayvsky, I. *J. Clin. Invest.*, **1997**, *99*, 1565-75.
- [121] Lundin, L.; Larsson, H.; Kreuger, J.; Kanda, S.; Lindahl, U.; Salmivirta, M.; Claesson-Welsh, L. *J. Biol. Chem.*, **2000**, *275*, 24653-60.
- [122] Maccarana, M.; Casu, B.; Lindahl, U. *J. Biol. Chem.*, **1993**, *268*, 23898-905.
- [123] Turnbull, J. E.; Fernig, D. G.; Ke, Y.; Wilkinson, M. C.; Gallagher, J. T. *J. Biol. Chem.*, **1992**, *267*, 10337-41.
- [124] Kreuger, J.; Salmivirta, M.; Sturiale, L.; Gimenez-Gallego, G.; Lindahl, U. *J. Biol. Chem.*, **2001**, *276*, 30744-52.
- [125] Ornitz, D. M.; Herr, A. B.; Nilsson, M.; Westman, J.; Svahn, C. M.; Waksman, G. *Science*, **1995**, *268*, 432-6.
- [126] Kovensky, J.; Duchaussoy, P.; Bono, F.; Salmivirta, M.; Sizun, P.; Herbert, J. M.; Petitou, M.; Sinay, P. *Bioorg. Med. Chem.*, **1999**, *7*, 1567-80.
- [127] Moy, F. J.; Safran, M.; Seddon, A. P.; Kitchen, D.; Bohlen, P.; Aviezer, D.; Yayon, A.; Powers, R. *Biochemistry*, **1997**, *36*, 4782-91.
- [128] Waksman, G.; Herr, A. B. *Nat. Struct. Biol.*, **1998**, *5*, 527-30.
- [129] Mulloy, B.; Linhardt, R. J. *Curr. Opin. Struct. Biol.*, **2001**, *11*, 623-8.
- [130] Faham, S.; Hileman, R. E.; Fromm, J. R.; Linhardt, R. J.; Rees, D. C. *Science*, **1996**, *271*, 1116-20.
- [131] Mulloy, B.; Forster, M. J.; Jones, C.; Davies, D. B. *Biochem. J.*, **1993**, *293* (Pt 3), 849-58.
- [132] Venkataraman, G.; Sasisekharan, V.; Herr, A. B.; Ornitz, D. M.; Waksman, G.; Cooney, C. L.; Langer, R.; Sasisekharan, R. *Proc. Natl. Acad. Sci. U S A*, **1996**, *93*, 845-50.
- [133] Casu, B.; Guerrini, M.; Naggi, A.; Perez, M.; Torri, G.; Ribatti, D.; Carminati, P.; Giannini, G.; Penco, S.; Pisano, C.; Belleri, M.; Rusnati, M.; Presta, M. *Biochemistry*, **2002**, *41*, 10519-28.
- [134] Vann, W. F.; Schmidt, M. A.; Jann, B.; Jann, K. *Eur. J. Biochem.*, **1981**, *116*, 359-64.
- [135] Casu, B.; Grazioli, G.; Razi, N.; Guerrini, M.; Naggi, A.; Torri, G.; Oreste, P.; Tursi, F.; Zoppetti, G.; Lindahl, U. *Carbohydr. Res.*, **1994**, *263*, 271-84.
- [136] Naggi, A.; Torri, G.; Casu, B.; Oreste, P.; Zoppetti, G.; Li, J. P.; Lindahl, U. *Semin. Thromb. Hemost.*, **2001**, *27*, 437-43.
- [137] Leali, D.; Belleri, M.; Urbinati, C.; Coltrini, D.; Oreste, P.; Zoppetti, G.; Ribatti, D.; Rusnati, M.; Presta, M. *J. Biol. Chem.*, **2001**, *276*, 37900-8.
- [138] Hahnenberger, R.; Jakobson, A. M.; Ansari, A.; Wehler, T.; Svahn, C. M.; Lindahl, U. *Glycobiology*, **1993**, *3*, 567-73.
- [139] Powell, A. K.; Fernig, D. G.; Turnbull, J. E. *J. Biol. Chem.*, **2002**, *277*, 28554-63.
- [140] Bellosta, P.; Iwahori, A.; Plotnikov, A. N.; Eliseenkova, A. V.; Basilico, C.; Mohammadi, M. *Mol. Cell Biol.*, **2001**, *21*, 5946-57.
- [141] Rusnati, M.; Coltrini, D.; Oreste, P.; Zoppetti, G.; Albin, A.; Noonan, D.; d'Adda di Fagagna, F.; Giacca, M.; Presta, M. *J. Biol. Chem.*, **1997**, *272*, 11313-20.
- [142] Feyzi, E.; Lustig, F.; Fager, G.; Spillmann, D.; Lindahl, U.; Salmivirta, M. *J. Biol. Chem.*, **1997**, *272*, 5518-24.

- [143] Loo, B. M.; Salmivirta, M. *J. Biol. Chem.*, **2002**, 277, 32616-23.
- [144] Lyon, M.; Gallagher, J. T. *Biochem. Soc. Trans.*, **1994**, 22, 365-70.
- [145] Kaneda, N.; Talukder, A. H.; Ishihara, M.; Hara, S.; Yoshida, K.; Muramatsu, T. *Biochem. Biophys. Res. Commun.*, **1996**, 220, 108-12.
- [146] Witt, D. P.; Lander, A. D. *Curr. Biol.*, **1994**, 4, 394-400.
- [147] Spillmann, D.; Witt, D.; Lindahl, U. *J. Biol. Chem.*, **1998**, 273, 15487-93.
- [148] Peters, T.; Pinto, B. M. *Curr. Opin. Struct. Biol.*, **1996**, 6, 710-20.
- [149] Venkataraman, G.; Shriver, Z.; Raman, R.; Sasisekharan, R. *Science*, **1999**, 286, 537-42.
- [150] Jemth, P.; Kreuger, J.; Kusche-Gullberg, M.; Sturiale, L.; Gimenez-Gallego, G.; Lindahl, U. *J. Biol. Chem.*, **2002**, 277, 30567-73.
- [151] Brandes, A. A.; Basso, U.; Pasetto, L. M. and Ermani, M. *Curr. Pharm. Des.*, **2001**, 7(16), 1553-80.
- [152] Gordeladze, J. O.; Reseland, J. E. and Drevon, C. A. *Curr. Pharm. Des.*, **2001**, 7(4), 275-90.
- [153] MacDonald, K. P. and Hill, G. R. *Curr. Pharm. Des.*, **2002**, 8(5), 395-403.