Preclinical and clinical evidence of nephro- and cardiovascular protective effects of glycosaminoglycans

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Abstract

Despite advances in pharmacological treatment, diabetic nephropathy is still the leading cause of end-stage renal disease and an important cause of morbidity and mortality in diabetics. Glycosaminoglycans are long, unbranched mucopolysaccharides that play an important role in establishing a charge-selective barrier that restricts the passage of negatively charged molecules, such as albumin and other proteins, at the level of the glomerular basal membrane. Their loss is associated with loss of selectivity and proteinuria. Extensive preclinical evidence and some clinical trials suggest that glycosaminoglycans replacement is associated with improvement of glomerular selectivity and of proteinuria. Sulodexide could also have some other effects, potentially useful to reduce the renal damage and the cardiovascular disease associated with proteinuria, such as improvement of haemorheological and blood lipid parameters, an endothelium protective effect and anti-inflammatory action. This review will discuss the evidence supporting the potential nephroprotective effects of sulodexide and other glycosaminoglycans.

Key words: glycosaminoglycans, cardiovascular diseases, proteinuria, diabetic nephropathy, sulodexide.

Introduction

Despite advances in dialysis techniques, pharmacological treatment, and patient rehabilitation programmes, mortality and morbidity rates of end-stage renal disease (ESRD) are still high and mainly related to cardiovascular diseases (CVD) [1, 2]. Diabetic nephropathy is still the leading cause of ESRD [3] and an important cause of morbidity and mortality in patients with either type 1 or type 2 diabetes mellitus, both directly and as a risk factor for cardiovascular disease [4]. The microangiopathic complications of diabetes increase with longer duration of diabetes and worse glycaemic control [5]. In fact, the presence of albuminuria or proteinuria is a well known risk factor for coronary heart disease [6, 7]. Although recent evidence shows that an early multi-pharmacological approach is able to slow the progression of diabetic nephropathy to ESRD [8], the disease rarely stops and slightly regresses just in a few selected and optimally treated patients [9]. So, a better understanding of the pathophysiology of chronic kidney disease is needed in order to develop new efficacious treatments for this pandemic disease.

The aim of this review is to evaluate the available literature data supporting the possible role of glycosaminoglycans (GAGs) in renovascular pathology and their possible usefulness in the treatment of patients affected by diabetic nephropathy.

Role of glycosaminoglycans in renal physiology

The glomerular filtration barrier consists of fenestrated glomerular endothelium, podocyte foot processes interconnected by slit diaphragms, and intervening glomerular basement membrane (GBM). Its characterization as both a size and charge-selective barrier emerged from studies conducted decades ago. Podocyte cytoskeleton and its connections with specific glycans and proteins constitute the basis of slit diaphragm and cell-extracellular matrix interactions. Anionic sites in GBM consist of GAGs rich in heparin sulphate (HS) and their removal by enzymatic digestion resulted in increased permeability [10-12].

Glycosaminoglycans are long, unbranched mucopolysaccharides that consist of repeating disaccharide units. Apart from hyaluronan, which is uniquely synthesized without a protein core and is "spun out" by enzymes at cell surfaces directly into the extracellular space, the other GAGs are usually added to protein cores in the Golgi apparatus to yield proteoglycans [13].

Glomerular basement membrane charge is imparted by the sulphated GAG side chains of proteoglycans (HS proteoglycans [HSPGs] and, less, hyaluronic acid) and to a lesser extent by carboxyl and sialyl groups of glycoproteins. These negatively charged molecules play an important role in establishing a charge-selective barrier that restricts the passage of negatively charged molecules, such as albumin and other proteins [14].

Role of glycosaminoglycans in renal pathologies: experimental data

Nowadays, the charge selectivity phenomenon has received renewed attention with the identification of mechanisms of synthesis of barrier-related molecules [15, 16]. In particular, GBM HS proteoglycans (and more specifically on perlecan, collagen XVIII, and agrin) are considered primary charge barrier components. Segmented loss of GBM HS has been reported in membranous glomerulonephritis, lupus nephritis, minimal change disease and diabetic nephropathy in humans [17, 18] and rat models of Adriamycin and Heymann nephritis [19]. Van der Born *et al*. observed that streptozotocin-induced diabetic rats with diabetic nephropathy experienced a significant decrease in glomerular HS/4-hydroxyproline ratio (showing increased collagen and relatively decreased GBM HS content) compared with control rats, and that was associated with selective proteinuria and glomerular hyperfiltration [20]. In another experimental model, non-diabetic mice knock-out for the Ext1 gene encoding a subunit of HS co-polymerase develop proteinuria that is less impressive than that expected from the available knowledge on renal physiology, suggesting that the cessation of polymerisation of podocyte-secreted HS that affects glomerular ultrafiltration charge may not be a serious cause of albuminuria, and there may be other roles of these molecules, including podocyte behaviour or morphology, as indicated in this study [21].

However, whether charge selectivity is actually important for glomerular function for the extent of proteinuria is still a matter of debate [22]. Recent *in vivo* manipulations of glomerular HS proteoglycans put in perspective (but did not exclude) a role for either molecules themselves or their anionic charge, which is altered greatly by loss of HS but caused insignificant albuminuria, in glomerular filtration [16]. On the other hand, mice without HS attachment sites on perlecan revealed normal glomerular structure and no evidence of renal disease, except slightly increased susceptibility to protein-overload albuminuria [23, 24]. Collagen XVIII mutants had mild mesangial expansion with slightly elevated serum creatinine levels [25] and podocyte-specific agrin knock-out mice had a significant GBM charge defect but normal renal function [26]. Perlecan HS and perlecan-HS/agrin double mutant rats experience significant charge reduction on GBM, but no renal dysfunction or proteinuria [27]. Lastly, the removal of HS in rats did not result in acute proteinuria [28].

Moreover, in renal biopsies of different human primary proteinuric diseases, pronounced alteration in tubulointerstitial HS proteoglycans is evident and strongly related to the inflammatory processes [29]. In fact, GAGs have a role in modulation of inflammation in tissues. Heparin sulphate proteoglycans can bind the leukocyte adhesion molecule L-selectin and chemokines, suggesting their role in inflammation [29-32]. Mouse and human glomerular endothelial cells activated by tumour necrosis factor (TNF)-α or interleukin 1-β showed increased expression of inflammatory N- and 6-O-sulphated HS domains and these are important in leukocyte trafficking and inflammation [33].

Heparin sulphate is an important constituent of subendothelial extracellular matrix and basement membrane structure and vascular HS is decreased in atherosclerosis, diabetes and during inflammation. It is also affected by lipoproteins, and lipoprotein-modulated perlecan may play an

important role in vascular smooth muscle growth, and thus in atherosclerosis [34]. Celie *et al.* showed the role of microvascular BM HS in inflammatory responses, in human renal allograft biopsies [35]. Heparin sulphate and GAGs play a major role in adhesion of leukocytes to glomerular cells, and that is important for proliferative glomerulopathies, inflammation and angiogenesis. Under dynamic flow conditions addition of HS, heparin and tinzaparin and removal of HS on mouse glomerular endothelial cells the number of rolling and adhering leukocytes decreases about 2-3-fold and the rolling velocity doubles [36]. Heparin sulphate also binds to cell surface receptors and is involved in the modulation of inflammation. CD44/HS actions are well studied in inflamed synovial membrane macrophages, and it is involved in the regulation of growth factors during inflammation and wound healing [37, 38].

Experimental data supporting the potential use of glycosaminoglycans in proteinuric diseases

Besides HSPGs, heparanase may have a role in renal diseases; together with the changes in glomerular cell-GBM interactions and loss of HS, increased heparanase release might cause the release of HS-bound factors and HS fragments in the glomeruli or changes in intracellular signalling by binding of heparanase to glomerular cells [39]. In diabetic nephropathy, the HS content of the GBM is decreased and that causes protein leak into the urinary space [40]; the increased amount of heparanase enzyme in response to hyperglycaemia may be one explanation. On the other hand, heparanase upregulation by high glucose is prevented by insulin and/or heparin in endothelial cell cultures [41]. In humans, increased heparanase level is associated with reduced HS, as observed in renal biopsies of diabetic patients with nephropathy [42], whereas in renal biopsies of different human primary proteinuric diseases pronounced alterations in tubulointerstitial HSPGs were evident and strongly related to inflammatory processes [29]. This is probably related to more relevant involvement of renal glomeruli in diabetic nephropathy than in other proteinuric diseases.

Evidence from *in vitro* and diabetic animal studies reveal that the administration of heparin increases synthesis of HS [43], and other anionic glycoproteins can effectively prevent the biochemical alterations that promote albuminuria [44]. Enoxaparin, a low-molecular weight heparin, was also tested on patients with diabetic and non-diabetic glomerulopathies. The proteinuria-decreasing effect of this heparin was found not to be related to the renin-angiotensin system, and its glomerular filter-related effect was suggested [45].

Angiotensin II (AT-II) receptor blockers are renin-angiotensin system (RAS) modulators with very well known antiproteinuric activity [46]. Angiotensin II inhibits HSPG expression in human podocytes [47] and heparins modulate AT-II signalling in glomerular cells [48], inhibiting aldosterone synthesis [49] and lowering proteinuria in diabetic patients [45], but this effect is less pronounced in other forms of proteinuric renal diseases and its relation to haemodynamic changes produced by RAS is not proven in clinical trials [45]. Of course, heparins are not easily administrable for chronic treatments.

In this context, heparinoids were considered as potentially useful antiproteinuric drugs that could have synergistic effects with an RAS modulator [50]. In particular, sulodexide, a soluble, highly purified preparation of low-molecular weight GAGs composed of fast-moving heparin (80%) and dermatan sulphate (20%) derived from porcine intestine, appeared to be a promising treatment for diabetic proteinuria partially resistant to RAS blocking agents [51]. It prevents HS degradation, reconstruction of HS content of GBM, and *in vivo* inhibition of heparanase [40].

In fact, sulodexide is concentrated in renal parenchyma for a long time after administration [52] and in preliminary trials it has been supposed that it reduces albuminuria acting *in vivo* as a heparanase inhibitor that reaches the glomerular capillary wall and prevents HS degradation, thus allowing reconstruction of HS content and restoration of glomerular basement membrane ionic permselectivity [40]. Its antiproteinuric effect appears to be mainly related to the basal proteinuria and to the treatment duration [53], independently from its antithrombotic and profibrinolytic activity.

Studies in mouse articular chondrocytes after lipopolysaccharide stimulation also showed anti-inflammatory and anti-apoptotic actions of GAGs [54]. Moreover, sulodexide seems to have powerful anti-inflammatory activity in experimental models [55]. In a model of cultured human umbilical endothelial cells exposed to high glucose concentration, sulodexide suppresses cellular inflammation and prevents glucose cytotoxicity [56]; it is able to reverse the glucose-related cell release of free oxygen radicals, monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (IL-6), and the inactivation of the cell repair mechanism induced by exposure to glucose. However, these anti-inflammatory effects have not been demonstrated in humans yet. Therefore, in rats with streptozotocin-induced diabetes, sulodexide exerts direct endothelial protective effects [57] that could also be involved in kidney protection. However, the anti-inflammatory role of

GAGs and heparin was already known in humans for decades, and especially in patients with allergy and asthma [58].

Clinical evidence of antiproteinuric effects of glycosaminoglycans

The antiproteinuric effects of GAGs, and especially of sulodexide, have been known for nearly two decades [59], and many clinical studies confirm its potential usefulness in treating nephropathies and especially in diabetic nephropathy.

In particular, the largest and more methodologically correct clinical trial was the one carried out by Gambaro *et al.*: the Diabetic nephropathy and Albuminuria Study (Di.N.A.S.) involved 223 patients with type 1 and 2 diabetes with both macro- and micro-albuminuria [60]. In this trial, 200 mg/day sulodexide treatment for 4 months was associated with 46% decrease in albumin excretion rate from baseline in diabetics who were not receiving concomitant angiotensin-converting enzyme (ACE) inhibitor treatment, and urinary albumin excretion was maintained even 2 months after drug intake interruption. The reduction in albuminuria was dose-dependent in this study, i.e. 100 mg/day sulodexide caused a mean decrease in albumin excretion rate of about 17% from baseline among the patients who were not receiving concomitant ACE inhibitor medication. The difference of albumin excretion vs. placebo was 62% in all diabetics with 200 mg/day sulodexide. The antiproteinuric effect of sulodexide appeared to be independent from the baseline blood pressure level and from the use of ACE inhibitors, meaning that even in patients already receiving ACE inhibitors, sulodexide was able to decrease the albumin excretion rate to approximately the same extent as in patients without ACE inhibitors (respectively 40% and 46% from baseline after 4 months with 200 mg/day sulodexide) [60].

However, this supposed additive effect of sulodexide was not confirmed either for ACE inhibitors when used at the maximum recommended dosage or for angiotensin receptor blockers (ARBs) also when used at the maximum recommended dosage. In fact, although in a pilot study on 149 microalbuminuric type 2 diabetic patients a trend for an increased rate of therapeutic success (return to normoalbuminuria or a decrease in albumin : creatinine ratio [ACR] of at least 50% from the baseline value) was observed for sulodexide, 200 mg/day for 6 months, compared to placebo (33.3% vs. 15.4% of the patients achieving the efficacy endpoint respectively; $p = 0.075$) [75], in the two related subsequent large trials respectively on microalbuminuric and macroalbuminuric type 2 diabetic patients the favourable trend of the preliminary pilot study for the additive effect of sulodexide in patients already treated with the maximum recommended dosages of ACE inhibitors or ARBs was not confirmed, even if the detailed results of these two studies are not known yet [76].

Other clinical trials focused on the mechanism of the antiproteinuric activity of sulodexide. Sulikowska *et al.* studied whether the albuminuria-lowering effect of sulodexide comes from its renovascular or tubular effects [61]. Dopamine infusion causes efferent arteriolar dilatation and increases creatinine clearance in normal people [62]. They tested dopamine-induced glomerular filtration response testing and urinary N-acetyl-β-D-glucosaminidase measurements to test proximal tubular integrity, besides albuminuria, on type 1 diabetic patients. Patients were divided into placebo and daily 100 mg sulodexide groups for 120 days and dopamine testing was performed only on patients taking sulodexide. Sulodexide caused a decrease in albumin excretion from 126.1 ±15.41 to 96.3 ±13.7 mg/day in the treatment group compared with a decrease from 106.8 ±21.4 to 126.8 ±29.6 mg/day in controls. N-acetyl-β-D-glucosaminidase measurements also changed from 5.1 ±0.62 to 4.7 ±0.40 U/gCre in the sulodexide group and from 5.9 ±0.87 to 6.3 ±1.35 U/gCre in the control group with placebo. The response increase in creatinine clearance to dopamine infusion was from 13.2 ±2.1 to 15.44 ±1.9% (+16.9% increase) in patients taking this drug. The conclusion was that sulodexide affects intrarenal vascular reactivity and also improves N-acetyl-β-D-glucosaminidase tests, indicating amelioration of tubular damage [61].

Whatever the main mechanism may be, the role of sulodexide as an antiproteinuric agent is suggested by the fundamental Di.N.A.S. trial and a large number of smaller studies on both type 1 and 2 diabetic patients (Table I) [53, 60, 61, 63-75]. Although these smaller studies often had an open design and a short duration, and did not involve homogeneous patient categories, nevertheless they contributed to the evidence of a clinically favourable antiproteinuric effect of sulodexide.

Other effects of glycosaminoglycans on cardiorenal physiology

The potential cardiovascular effects of sulodexide and GAGs are summarized in Table II.

Interactions with HS modify and contribute to various protein actions and intercellular signalling by cytokines and growth factors, and some proteins share binding sites with HS [78]. Extracellular matrix of blood vessel walls also contain considerable amounts of proteoglycans and systemic hypertension may change the content of subendothelial matrix of vessels: this may also contribute to increased

Table I. Clinical studies evaluating the effects of sulodexide on proteinuria and albuminuria

Number of patients	Type of patients	Dose	Duration of treatment	Main results	Researchers [Reference no.]
18	Type 2 diabetes	600 lipoprotein lipase releasing units/day *i.v.*	3 weeks	Albuminuria fall in 89% of patients, proteinuria normalization in the 9 microalbuminuric patients	Shestakova 1997 [39]
15	Type 1 diabetes	600 lipoprotein lipase releasing units/day *i.v.*	3 weeks	Albuminuria fall after the first week, maintained also 6 weeks after treatment cessation	Szelachowska 1997 [57]
15	Type 2 diabetes	600 lipoprotein lipase releasing units/day *i.m.*	4 weeks	Albuminuria fall in 60% of patients, reversed after	Sorrenti 1997 [58]
20	Type 2 diabetes	100 mg/day	4 months	Significant reduction in albumin excretion rate, fibrinogen and blood pressure	Solini 1997 [59]
53	Type 2 and type 1 diabetes	600 lipoprotein lipase releasing units/day *i.m.*	3 weeks	Significant reduction of albuminuria in 72% of patients, slower in type 2 diabetics	Skrha 1997 [60]
36	Type 1 diabetics	600 lipoprotein lipase releasing units/day *i.m.* 5 days/week	3 weeks	Significant reduction of albuminuria in 90% of patients, slower in macroalbuminuric patients	Dedov 1997 [61]
14	Type 1 diabetes	60 mg vial of sulodexide/day for 10 days, and then orally with 25 mg capsules twice a day for 21 days	31 days	Significant reduction of albuminuria with normalization in 40% of microalbuminurics and 25% of macroalbuminurics	Poplawska 1997 [62]
35	Type 2 and type 1 diabetes	600 lipoprotein lipase releasing units/day *i.m.* 5 days/week	15 days	Significant reduction of albuminuria in 70% of patients, persistent 3 weeks after treatment cessation	Perusicová 1997 [63]
20	Type 2 and type 1 diabetes	600 lipoprotein lipase releasing units/day *i.m.* 5 days/week	3 weeks	Quickly reversible albuminuria in all patients	Zalevskaia 1998 [64]
20	Type 1 diabetics	600 lipoprotein lipase releasing units/day *i.m.* 5 days/week	3 weeks	Significant reduction of albuminuria in 70% of patients, and persistent in 60% 6 weeks after drug discontinuation	Rasovski 1998 [65]
20	Type 2 and type 1 diabetics	600 lipoprotein lipase releasing units/day *i.m.* 5 days/week	3 weeks	Significant reduction in albuminuria and serum N-acetyl-β-glucosaminidase (NAG) activity	Skrha 1998 [66]
20	Type 2 and type 1 diabetics	60 mg/day *i.m.* 100 mg/day *p.o.*	3 weeks 8 weeks	Albumin excretion rate reduced after both treatment phases in macroalbuminuric, but not microalbuminuric patients	Oksa 1999 [67]
223	Type 2 and type 1 diabetics	50 mg/day, 100 mg/day, or 200 mg/day *p.o.*	4 months	Dose-dependent reduction in albumin excretion rate	Gambaro 2002 [52]

Table I. Clinical studies evaluating the effects of sulodexide on proteinuria and albuminuria – cont.

Number of patients	Type of patients	Dose	Duration of treatment	Main results	Researchers [Reference no.]
60	Type 2 and type 1 diabetics	50 mg/day *p.o.*	12 months	Albuminuria strongly reduced in all patients vs. controls and vs. baseline	Achour 2005 [68]
45	Type 1 diabetics	120 mg/day *p.o.*	6 months	Reduction in albuminuria and N-acetyl-β-D-glucosaminidase (NAG) excretion, increase in renal vascular function	Sulikowska 2006 [56]
149	Obese type 2 diabetics	200-400 mg/day *p.o.* in addition to ACEI or ARBs in resistant patients	6 months	25.3% and 33.3% of the patients respectively in the two sulodexide groups combined and in the 200 mg/day group achieved a significant reduction or normalization of albuminuria vs. 15.4% of the patients in the control group ($p = 0.26$ and $p = 0.075$ respectively)	Heerspink 2008 [69]

Table II. Potential cardiovascular beneficial effects of sulodexide and GAGs in diabetic patients

- Antithrombotic action
- Decreased oxidative stress
- Antihyperlipidaemic actions
- Prevention of glucose toxicity
- Suppression of cellular inflammation
- Cytokines and growth factors modulation
- Interactions with AT-II signalling and RAS system
- Reduction of peripheral vascular resistance and improvement of vascular elasticity
- Antiproteinuric effects

peripheral vascular resistance, as shown in animal models [79].

As stated above, at least a part of the renal histological degradation observed in diabetes is related to inflammatory processes, and sulodexide showed anti-inflammatory activity in different animal models [55]. A pilot study recently conducted on 11 healthy men concluded that it may also decrease transforming growth factor β1 (TGF-β1) release [80].

The haemorheological and lipid lowering actions of GAGs have also been known for the last two decades. In fact, sulodexide decreases triglycerides [81], increases Apo-A1 and HDL-C levels [82] and blood viscosity [83], decreases D-dimer and fibrinogen levels [84, 85], and releases tissue plasminogen activator [86, 87]. Sulodexide has a dual effect on coagulation: antithrombin catalysis by fast moving heparin component and heparin cofactor II catalysis by dermatan sulphate component [88]. It causes inhibition of thrombus formation and growth with less systemic anticoagulation than comparable antithrombotic doses of heparins [89] and may also reduce oxidative stress slightly expressed by malonylaldehyde and superoxide dismutase in diabetics [72]. Therefore, in rats with streptozotocin-induced diabetes, sulodexide exerts direct endothelial protective effects, improving acetylcholine-induced relaxation of isolated aorta and mesangial arteries and reducing the number of circulating endothelial cells [57].

Conclusions

A relatively large body of literature supports the antiproteinuric and nephroprotective effects of GAGs and sulodexide, especially in diabetic nephropathy. These could derive from their effect on vascular permeability and inflammation, and on endothelium protection and haemorheology improvement. Sulodexide has the advantage of being an oral medication with few and usually mild side effects of intestinal type (i.e. diarrhoea, nausea, dyspepsia). Its antiproteinuric and nephroprotective role in therapy may be played in patients not tolerating ARBs and ACE inhibitors or in patients who are resistant to dosages of ARBs and ACE inhibitors which are not being given at the maximum recommended level, for safety or other reasons. In fact two recent clinical trials were not able to confirm in patients already receiving the maximal recommended dosages of ARBs or ACE inhibitors the previous statistically significant additional effects of sulodexide observed by Gambaro *et al.* [60] also in patients being treated

with unspecified dosages of ACE inhibitors. Certainly, more clinical research is needed to understand which factors influence the drug efficacy and, consequently, which patients could a priori obtain the best effect from this treatment. Therefore, further clinical trials are currently ongoing with sulodexide as an antiproteinuric agent.

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