Diabetes results in structural alteration of chondroitin sulfate/dermatan sulfate in the rat kidney: effects on the binding to extracellular matrix components

Darukeshwara Joladarashi, Paramahans V Salimath, and Nandini D Chilkunda

Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore 570 020, Karnataka, India

Received on October 4, 2010; revised on February 24, 2011; accepted on March 10, 2011

Chondroitin sulfate (CS)/dermatan sulfate (DS) is a group of sulfated polymers, which play an essential role in various biological phenomena. In the kidney, they are present in small but significant amounts. Studies on their structure–function relationship in the kidney and their changes during diabetic conditions have not been rigorously looked into, which is the focus of this paper. The CS/DS content decreased significantly (14%) during diabetic conditions. This was accompanied by a decrease in the CS/heparan sulfate ratio. Disaccharide composition analysis revealed fine structural changes especially with respect to the E unit [glucuronic acid β1-3 N-acetyl β-galactosamine (4,6-O-sulfate)] and the degree of sulfation. The mRNA expression levels of major enzymes involved in the synthesis of the “E”-disaccharide unit showed a decrease during diabetes. The changes in CS/DS had implications on ligand-binding properties when tested in vitro for binding to major extracellular matrix (ECM) components such as type IV collagen, laminin, and fibronectin. Thus, this study provides insights into the structure–function relationship of CS/DS in the kidney during diabetes and alterations of which could aggravate conditions such as diabetic nephropathy by virtue of them being a part of ECM components.

Keywords: chondroitin sulfate / dermatan sulfate / diabetes / E-disaccharide unit / extracellular matrix components

Introduction

Chondroitin sulfate (CS)/dermatan sulfate (DS) along with heparan sulfate (HS), keratan sulfate and hyaluronic acid form a unique class of molecules called glycosaminoglycans (GAGs; Kjellen and Lindahl 1991). They are present as components of various extracellular matrices (ECMs) as well as at the cell surface. They are synthesized as side chains of proteoglycans (PGs) and play an important role in various biological phenomena such as cell proliferation (Yamaguchi et al. 1990), migration (Lane and Solursh 1991), neurotogenesis (Nandini et al. 2004), growth factor binding (Nandini and Sugahara 2006), wound healing (Penc et al. 1998) etc. to name a few. They are characterized by the disaccharide glucuronic acid (GlcA)/iduronic acid (IdoA) 1-3 linked to N-acetyl-β-galactosamine (GalNAc), which can be differentially sulfated resulting in microheterogeneity and fine structural variations bringing about differences in their nature and functional properties (Silbert and Sugumaran 2002).

The kidney has both HS and CS/DS wherein HS constitutes 80–90% of the total sulfated GAGs. However, in the embryonic kidney, CS accounts for 75%, indicating that both HS and CS contribute to kidney organogenesis (Steer et al. 2004). There are, however, few reports available with respect to the structure of CS/DS in the kidney and their modulation during pathological conditions such as diabetes despite their important functions in various tissues (Rolls et al. 2006; Bakalash et al. 2007) and lower organisms (Gamain et al. 2002; Dinglasan et al. 2007). Various CS/DS PGs in the kidney have been demonstrated by immunohistochemistry (Williams et al. 2007). The absence of decorin, which is a DS PG, has been shown to adversely influence the tubulointerstitial fibrosis of the obstructed kidney by an enhanced apoptosis and increased inflammatory reaction (Schaefer et al. 2002). However, the structure–function relationship of CS/DS chains associated with them has not been rigorously studied. Basement membrane-specific CS/DS has been found to be abnormally associated with the glomerular capillary basement membrane of diabetic rats (McCarthy et al. 1994), and recently, an increase in the levels of NG2 PG, a large integral membrane-spanning CS PG, which interacts with molecules on both sides of the plasma membrane, has been observed (Xiong et al. 2007). Alterations in the basement membrane CS PG have been looked into in the rat model of polycystic kidney disease, wherein altered distribution of CS PGs was observed during the onset and recovery phases (Ehara et al. 1994). Furthermore, an increase in CS/DS PGs was observed in obstructed rat...
kidneys, which do not get ameliorated on treatment with heparin (Pecly et al. 2006). The mechanism underlying the overproduction, however, remains to be elucidated.

Diabetes is a metabolic disorder that is characterized by sustained hyperglycemia. In the kidney, prolonged exposure to high levels of blood glucose leads to secondary complication such as diabetic nephropathy, which ultimately leads to end-stage renal disease (Fowler 2008). A number of studies have been carried out implicating HS, a major component in the kidney for charge selectivity in the glomerular basement membrane (Parasthasarthy and Sprio 1982). There are very few reports pertaining to CS/DS during diabetic conditions in the kidney. In one such report, an increase in the CS content has been demonstrated (Cadaval et al. 2000), whereas in another a decrease has been observed in the DS content, leaving CS unaltered (Saraswathi and Vasan 1983). However, nothing is said about the structure–function relationship of the kidney CS/DS. Hence, in the present study, an attempt was made to elucidate the structural features of kidney CS/DS, changes occurring during diabetic condition and their implications toward the binding to some of the major ECM components such as type IV collagen, laminin and fibronectin.

### Results

**Effect of diabetes on basic parameters**

Rats experimentally induced with diabetes using streptozotocin (STZ) showed high levels of fasting blood glucose and urine sugar. It showed characteristic features of hyperphagia, polydipsia and polyuria without a concomitant increase in weight gain (Table I). Diabetic condition also resulted in an increase in relative weight of the kidney and the glomerular filtration rate (GFR), which was measured in terms of creatinine clearance that is in agreement with our earlier reports (Nandini et al. 2003).

**Effect of diabetes on total sulfated GAGs, HS and CS/DS of the kidney**

Harvested kidneys were dried and defatted and taken up for the isolation of GAGs. Isolated GAGs were desalted and estimated by the 1,9-dimethylmethylen blue (DMMB) method for sulfated GAGs. The amount of total sulfated GAGs, which include both HS and CS/DS, was significantly reduced in diabetic animals and was to the tune of 12% when compared with control animals. This decrease in GAGs appeared to be due to both HS and CS/DS, which was decreased to the extent of 8 and 14%, respectively (Figure 1A). Digestion of the total GAG preparation with nitrous acid and subsequent estimation of undigested GAGs by dye-binding assay from control and diabetic rats gave similar results (data not shown). The changes in individual GAGs resulted in a significant decrease in the CS/HS ratio among the diabetic groups when compared with the control groups (Figure 1B).

<table>
<thead>
<tr>
<th>Table I. Effect of diabetes on various parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters</td>
</tr>
<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Body weight (g)</td>
</tr>
<tr>
<td>Initial</td>
</tr>
<tr>
<td>Final</td>
</tr>
<tr>
<td>Gain</td>
</tr>
<tr>
<td>Fasting blood sugar (mg/dL)</td>
</tr>
<tr>
<td>Urine output (mL/day)</td>
</tr>
<tr>
<td>Urine sugar (g/day)</td>
</tr>
<tr>
<td>Kidney weight (g/100 g body weight)</td>
</tr>
<tr>
<td>GFR (mL/min)</td>
</tr>
</tbody>
</table>

Values are the mean ± SD of control (n = 6) and diabetic (n = 11) rats. Values are significant at **P < 0.01 and ***P < 0.001 when compared with control.

![Fig. 1. Sulfated GAGs in control and diabetic rat kidneys. GAGs were isolated from the kidneys of control and diabetic rats. Amount of HS and CS/DS were determined by DMMB assay after differential digestion with either chondroitinase ABC or HNO2 (see Materials and methods) (A). The ratio of CS/HS was calculated from the amounts of CS/DS and HS obtained (B). Values are represented as the mean ± SD of two independent experiments carried out in duplicates on pooled control (n = 6) and diabetic (n = 11) rat kidneys. *P < 0.05, **P < 0.01.](image-url)
and 6-**disaccharides.** Among the various disaccharides, the retention times and co-elution with standard authentic preparations. One unique feature observed is the presence of 1-3GalNAc(4S) obtained on treatment with chondroitinase ABC (Nandini et al. 2005).

Disaccharide composition analysis of CS/DS isolated from control and diabetic rat kidneys

Purified CS/DS was further taken up for disaccharide composition analysis. Depolymerization with chondroitinase ABC to remove HS and hyaluronic acid, respectively, as given in Materials and methods. The identity of purified CS/DS was confirmed by agarose gel electrophoresis (Figure 2). Digestion with chondroitinase ABC resulted in the disappearance of the band, thus confirming it to be CS/DS.

Digestibility of the CS/DS preparations from control and diabetic rat kidneys by various chondroitinases

Chondroitinases ABC, AC and B digest CS/DS chains according to their specificities. The resistant fragment remaining was measured using metachromatic dye, DMMB, which binds to sulfated GAGs and long oligosaccharides. Due to its binding, the digestibility by a differential action of lyases can be measured (Farndale et al. 1986). Digestion by chondroitinase ABC showed 95% digestibility of the CS/DS preparations from both the groups. Digestivity toward chondroitinase AC was similar (30%) for both the preparations (Figure 4). Digestibility to chondroitinase AC was also tested on different amounts of CS/DS preparations by increasing the amount of enzyme, duration of incubation etc. However, the digestibility did not exceed 30–35% (data not shown). Since the CS/DS preparation exhibited high amounts of 4-O-sulfated units, it was interesting to determine their amenability to chondroitinase B. Chondroitinase B cleaves GalNAc and IdoA linkage in an endolytic fashion but not the GalNAc–GlcA linkage in CS/DS chains. Intriguingly, the digestibility of CS/DS from the diabetic rat kidney by chondroitinase B was significantly higher than that of the control rat kidney, which was of the order 28 and 8%, respectively. Analyses of chondroitinase B digests after 2AB labeling on a Superdex peptide column revealed that although CS/DS from the control rats showed the presence of larger amounts of longer oligosaccharides, CS/DS from the diabetic rat kidney was markedly characterized by the presence of disaccharides (Figure 5). This supports the data obtained by the differential digestibility to chondroitinase B and the loss of the binding ability to DMMB dye.

Expression levels of major biosynthetic enzymes involved in the synthesis of the “**E**”-disaccharide unit of CS/DS in control and diabetic rat kidneys

In order to determine if there were alterations in the mRNA expression of major biosynthetic enzymes involved in the synthesis of the “**E**”-disaccharide unit, namely C4ST-1, D4ST-1 and GalNAc4S-6ST in diabetic rats, rat kidneys were harvested and the total RNA was isolated. It was converted to cDNA using random hexa primers and amplified with specific primers as detailed in Materials and methods. The size of the amplicons was determined by 1.5% agarose gel electrophoresis (Figure 6A). Actin was used as an internal control to normalize the expression levels. Results revealed that the expression levels of C4ST-1, D4ST-1 and GalNAc4S-6ST were markedly reduced in diabetic rats, validating the data obtained by HPLC (Figure 6B–D). Among C4ST-1 and D4ST-1, the expression levels were higher for C4ST-1.

**Fig. 2.** Agarose gel electrophoresis of CS/DS from the kidney. Purified CS/DS obtained from the control rat kidney (given as a representative) was subjected to 1% agarose gel electrophoresis and spots visualized by Alcian blue staining. Lane 1, standard CS/DS (50 µg); lane 2, kidney GAGs (50 µg); lane 3, purified CS/DS from the kidney (50 µg); lane 4, chondroitinase ABC digested CS/DS (50 µg); lane 5, kidney GAGs (100 µg).

**Agarose gel electrophoresis of purified CS/DS**

Kidney GAGs were digested with freshly prepared nitrous acid and hyaluronidase from *Streptomyces hyalurolyticus* to remove HS and hyaluronic acid, respectively, as given in Materials and methods. The identity of purified CS/DS was confirmed by agarose gel electrophoresis (Figure 2). Digestion with chondroitinase ABC resulted in the disappearance of the band, thus confirming it to be CS/DS.

**Disaccharide composition analysis of CS/DS isolated from control and diabetic rat kidneys**

In order to determine if there were alterations in the mRNA expression of major biosynthetic enzymes involved in the synthesis of the “**E**”-disaccharide unit, namely C4ST-1, D4ST-1 and GalNAc4S-6ST in diabetic rats, rat kidneys were harvested and the total RNA was isolated. It was converted to cDNA using random hexa primers and amplified with specific primers as detailed in Materials and methods. The size of the amplicons was determined by 1.5% agarose gel electrophoresis (Figure 6A). Actin was used as an internal control to normalize the expression levels. Results revealed that the expression levels of C4ST-1, D4ST-1 and GalNAc4S-6ST were markedly reduced in diabetic rats, validating the data obtained by HPLC (Figure 6B–D). Among C4ST-1 and D4ST-1, the expression levels were higher for C4ST-1.

**Binding studies by solid-phase immunoassay**

To determine if the fine structural variations in CS/DS impacted binding to various ECM components, binding was tested in vitro against type IV collagen, laminin and fibronectin. CS/DS preparations from both control and diabetic rat...
kidneys were individually immobilized onto poly-L-lysine-coated wells and tested for the binding to the ECM components by immunoassay as mentioned in Materials and methods. Although the binding to varying amounts of type IV collagen and laminin were tested using anti-type IV collagen and anti-laminin, respectively, binding to fibronectin was tested by determining a decrease in the reactivity to CS-56. All the three ECM components tested bound to the CS/DS preparations from control and diabetic rat kidneys. Results showed that the CS/DS preparation from diabetic rats bound less amounts of type IV collagen, which was in particular significantly reduced at 100 and 200 ng (Figure 7A). With laminin also CS/DS from diabetic rats exhibited lesser tendency to bind when compared with that from control rats. Significant difference was observed at 100 and 300 ng of laminin (Figure 7B). Testing for the binding with varying amounts of fibronectin revealed that the reactivity of CS/DS to CS-56 decreased with increased amounts of fibronectin. In other words, binding of CS/DS to fibronectin increased with increased amounts of fibronectin. CS/DS from diabetic rats showed decreased reactivity to CS-56 compared with control rats in the presence of fibronectin, indicating that they bind more amount of fibronectin (Figure 7C).

The binding of ECM components to the CS/DS preparations were specific since the binding was completely abrogated on the digestion of CS/DS preparations by chondroitinase ABC (data not shown).
Binding to CS-56 anti-CS antibody
To determine if CS/DS preparations from both control and diabetic rat kidneys were immobilized in equal measures on poly-L-lysine-coated plates, their reactivity to CS-56 was determined by enzyme-linked immunosorbent assay (ELISA). Antibody CS-56, a CS-specific antibody, is widely used for the recognition of CS variants (Bjornsson 2000). Results revealed that the recognition by CS-56 is similar in both the preparations (Figure 8).

Discussion
The present study deals with fine structural alterations of kidney CS/DS during diabetic condition and its implications on the binding to various major ECM components. CS/DS is an important class of molecules, which perform a wide variety of functions in different tissues. However, information on kidney CS/DS during pathological conditions, such as diabetes, is few. Such studies previously carried out by other workers have focused on quantitative changes but not on structural changes. Therefore, an attempt was made to determine changes in their fine structural features and establish their structure–function relationship.

To begin with, GAGs were isolated from the kidney of control and diabetic rats. The kidney is rich in HS along with significant amounts of CS/DS. A decrease in the amounts of CS/DS as well as HS was observed in diabetic rats, which is in accordance with our earlier report (Nandini et al. 2003). As a result, a concomitant decrease in the CS/HS ratio was observed, which points out to major changes occurring in GAGs of the kidney during diabetes. A decrease in the HS content has been implicated in the excretion of proteins such as albumin during diabetic nephropathy (Tamsma et al. 1994). CS/DS was subsequently purified and the identity was confirmed by agarose gel electrophoresis after digesting it with chondroitinase ABC. Among various disaccharides of CS/DS, a significant and specific reduction in the E unit was observed in the diabetic rat kidney. This was accompanied by a decrease in the degree of sulfation (small but significant). The role of E unit in the kidney needs to be critically looked into since it is present in appreciable amounts, contributing to as much as 20% of the total CS/DS disaccharides. The importance of E unit in the kidney, in particular, has not been elucidated yet. However, in organs such as brain, E units are known to play vital roles in its development (Purushothaman et al. 2007), presumably by binding to various growth factors (Deepa et al. 2002; Umehara et al. 2004). Moreover, the sulfation of CS/DS has been implicated in various biological activities (Silbert and Sugumaran 2002). It is thus conceivable that the decrease in the E-disaccharide unit and changes in sulfation of CS/DS might affect its interaction with various growth factors and ECM components in the kidney.

CS/DS from the kidney was determined to be a hybrid structure based on their amenability to chondroitinas ABC and B. Most of the CS/DS isolated till date have been demonstrated to be a hybrid structure (Nandini et al. 2005), which further adds to the complexity of existing structures. The hybrid nature of CS/DS has physiological importance in various organs by their ability to bind growth factors as exemplified by CS/DS hybrid chains of pig brain (Bao et al. 2005) and endocan, a PG secreted by endothelial cells (Bechard et al. 2001). An interesting feature of an increase in contiguous IdoA-containing moieties presumably from -IdoA-GalNAc- residues in CS/DS of
the diabetic rat kidney calls for increasing studies to determine changes in such moieties during pathological conditions, such as diabetes. IdoA, in particular, has the tendency to form various conformations thereby conferring it with inherent plasticity for interaction with various molecules (Ferro et al. 1986). The final structure of CS/DS is subject to different levels of regulation in vivo, which is poorly understood (Maccarana et al. 2009). The binding of growth factors to HS and DS requires IdoA residues (Hileman et al. 1998; Penc et al. 1998) with notable exception of CS-E, which exhibits high affinity binding to various growth factors despite the absence of IdoA-containing moieties (Deepa et al. 2002), thereby implying the importance of the E units, the sulfation and the conformation of sugar moieties. Regulation of the fine structural features of CS/DS has been observed by growth factors such as transforming growth factor (TGF)-β (Tiedemann et al. 2005). This is an interesting observation especially in light of the fact that there is an increase in TGF-β production in the kidney during diabetes, which promotes the accumulation of ECM components (Sharma and Ziyadeh 1995). Decorin, a DS PG, has been found to increase in the diabetic rat kidney (Schaefer et al. 2001). Core proteins of PGs have a role to play in the assembly of GAG chains. This was observed in rat glypican1 when alterations made to its globular domain resulted in changes in HS and CS moieties (Chen and Lander 2001).

Biosynthesis of CS/DS is a complex process involving multiple steps. A decrease in major biosynthetic enzymes, namely C4ST-1, D4ST-1 and GalNAc4S-6ST, involved in the

---

**Fig. 5.** Analysis of chondroitinase B digests of the purified CS/DS preparation from the kidney on a Superdex peptide column. The purified CS/DS preparation from the kidney (2 µg as sulfated GAG) was digested with 2 mIU of chondroitinase B as described in Materials and methods. All digests were individually labeled with 2AB (for details, see Materials and methods) and analyzed by gel filtration on a Superdex peptide column using 0.2 M ammonium bicarbonate containing 7% 1-propanol as eluent. The elution positions of the authentic unsaturated CS-derived disaccharides/oligosaccharides are indicated as follows; arrow 1, disaccharides/higher oligosaccharides; arrow 2, octasaccharides; arrow 3, hexasaccharides; arrow 4, tetrasaccharides; and arrow 5, disaccharides; star, unknown peak; $V_o$ and $V_t$ were around 1.6 and 4.8 mL, respectively. The profiles given are that of control (A) and diabetic (B).
synthesis of the E unit during diabetes was observed, indicating that the structural changes observed are regulated at the genetic level. C4ST-1, in particular, has been observed to regulate the expression of the E-disaccharide unit. Interestingly, overexpression of D4ST-1 and C4ST-2 could not compensate for decreased E-disaccharide as a result of decreased expression of C4ST1 in murine sog9 cells (Uyama et al. 2006). On the contrary, in a study conducted by Pacheco et al. (2009), it was observed that a decrease in D4ST-1 expression led to decreases in the E-disaccharide unit in primary human lung fibroblasts. The differences between the observations could be due to differences in the cell lines employed in conducting such a study. Our studies revealed that both C4ST-1 and D4ST-1 expression levels were decreased correlating with the decrease in the E-disaccharide unit. How much of it is due to the GlcA or IdoA-containing E unit is not known. However, it can be hypothesized that most of the E-disaccharide unit must be having GlcA since the expression levels of C4ST-1 was much higher than that of D4ST-1. GalNAc4S-6ST, on the other hand, has been shown to transfer sulfate mainly to position 6 of the internal GalNAc residues of CS-A and DS (Ito and Habuchi 2000). The decrease in expression levels of E-disaccharide synthesizing enzymes in the kidney during diabetes reflects the changes in the structure of CS/DS that could have far reaching implications.

Fig. 6. The mRNA expression of CS/DS biosynthetic enzymes involved in the synthesis of the E-disaccharide unit of the kidney. Kidney tissue was harvested and RNA was isolated, reverse transcribed and amplified employing different cycles by adding requisite primers. Amplicon size was observed by the agarose gel electrophoresis (A). Bands were quantitated by densitometry using EASY WIN-32 software and normalized against the respective actin that was used as an internal control. The expression of mRNA was evaluated for C4ST-1 (B), D4ST-1 (C) and GalNAc4S-6ST (D). Expression was carried out in duplicates for at least five animals per group. Electrophorogram of the mRNA expression profile of a control and a diabetic rat is given as a representative. Values are given as the mean ± SD. *P < 0.05, **P < 0.01.

Does the structural variations observed during diabetes result in functional variations? This question was addressed by determining the binding of isolated CS/DS chains to major ECM components such as type IV collagen, laminin and fibronectin by solid-phase immunosassay. Binding was determined by immobilizing fixed amounts of them and individually testing them with various amounts of ECM components. Here, CS/DS was immobilized on poly-L-lysine-coated plates, which has been employed by other groups for preparations of carbohydrate microarray-based approach for rapid, facile analysis of GAG–protein interactions. Poly-L-lysine, in particular, was found to be flexible and efficiently and uniformly bound various GAGs non-covalently (Shipp and Hsieh-Wilson 2007). There was a decreased binding toward type IV collagen and laminin by the CS/DS preparation from diabetic rats. It was observed earlier that interactions between heparin and type IV collagen could be competed almost to the same extent by CS chains, implicating it in the binding (Tsilibary et al. 1988). On the other hand, CS/DS from diabetic rats bound more amounts of fibronectin than that from control rats. This might be due to the sulfation degree or changes in the amount or alterations of specific sequences within the chain. It was observed by earlier workers that DS, in particular, bound to fibronectin (Stamatoglou and Keller 1982). Since our studies observed that CS/DS from diabetic rats have more contiguous IdoA-containing sequence, the binding to fibronectin might have been more than that of control rats. The binding was specific to CS/DS chains since digesting it resulted in a complete loss of the activity. The changes observed were...
presumably due to specific binding to ECM components rather than varying response due to differences in the amount of CS/DS immobilized since immobilizing similar amounts of CS/DS showed equal binding toward CS-56, an antibody specific to CS. It would be interesting to study the biological implications of these kinds of interactions especially during
pathological conditions and the domains responsible for the binding in greater detail.

The ECM components have a role in maintaining the integrity of the cell (Jane et al. 1999). Interaction between ECM components and GAGs is essential for extracellular morphology and cell adhesion (Barkalow and Schwarzbaver 1994). In the kidney, both HS and CS/DS are the components of ECMs as well as cell surface. HS, a major GAG component of the kidney, has been implicated in charge selective properties, although few reports contrary to this have also appeared. Wijnhoven et al. (2007) have demonstrated in recent days that intravenously injected heparinase-treated rats did not show increased protein excretion despite the loss of HS and disruption of GBM changed. Using the isolated perfused kidney, other workers have implicated CS and HA present in endothelial glycocalyx as determinants of glomerular permeability (Jeansson and Haraldsson 2003).

In conclusion, CS/DS chains in the kidney are complex and heterogeneous in structure, which gets altered during diabetic condition thereby affecting its functional properties. It presumably plays an important role in the kidney along with HS in controlling permselectivity by virtue of it being present in significant amounts.

Materials and methods

Materials

Chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4), standard CS unsaturated disaccharides and CS B from the porcine kidney were obtained from Associates of Cape Cod, USA. Chondroitinases AC (EC 4.2.2.5) and B (EC 4.2.2) from *Flavobacterium heparinum*, hyaluronidase (EC 4.2.2.1) from *Streptomyces hyalurolyticus* (S. hyalurolyticus). CS-56 anti-CS, type IV collagen from Engelbreth-Holm-Swarm murine sarcoma basement membrane, laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane, fibronectin from bovine plasma, STZ, 2AB, sodium cyanoborohydride (NaBH₃CN), DMBB and primers for semi-quantitative reverse transcription–polymerase chain reactions (RT–PCR) were from Aldrich Chemical Co., USA. Anti-type IV collagen was from Abcam, UK. Prepacked disposable PD-10 columns containing Sephadex G-25 (medium) and Superdex peptide™ (10/300 GL) column were purchased from GE Healthcare, Sweden. RT–PCR kit was procured from Genel Pvt. Ltd, India. Glucose oxidase/peroxidase (GOD/POD) kit was purchased from Span Diagnostic Limited, India. All other chemicals and reagents used were of analytical grade.

Animals and diet

Male Wistar rats (OUTB-Wistar IND cftri) weighing around 120–140 g were taken for the study from Institute Animal House Facility. The study had the clearance of Institutional Animal Ethical Committee. The rats were fed with AIN-76 diet (Bieri et al. 1997). They had free access to food and water.

Induction of diabetes

The rats were initially grouped into two groups of which one group served as a non-diabetic control and the other group served as diabetic and both of them were age-matched. Diabetes was induced in rats by a single intraperitoneal injection of STZ (Hatch et al. 1995) at 55 mg/kg body weight in freshly prepared citrate buffer (0.1 M, pH 4.5). Control rats were sham-injected with citrate buffer. Five days after STZ injection, the blood glucose level was monitored using GOD/POD diagnostic kit. Diabetic status and kidney functions at the end of the experimental period were assessed by measuring fasting blood glucose, urine sugar, urine volume and GFR. Creatinine clearance was used to measure the GFR levels in control and diabetic rats.

Isolation of CS/DS from the kidney

Rats were killed at the end of the experimental period of 60 days. The kidney devoid of external fat was cut into small pieces and kept in acetone at 4°C for ~15 days with fresh acetone replacement every day. Later, dried kidneys were powdered thoroughly and stored at 4°C until use. The powdered kidney was suspended in 20 mL of phosphate buffer (0.1 M, pH 6.5; Scott 1960). Papain solution [10 mg papain in 1 mL phosphate buffer containing ethylenediaminetetraacetic acid (0.005 M)] was first activated by keeping at 65°C for 30 min in a water bath. The activated papain solution (1 mL) was added to the tissue suspension and digested for 2 days at 65°C in an oven. An aliquot of fresh enzyme solution was added at the end of 24 h. After digestion, the reaction mixture was centrifuged at 3000 g for 15 min. To the supernatant, one-third volume of 40% trichloroacetic acid was added to precipitate out the proteins. The precipitate was discarded after centrifugation. To the supernatant, 4 volumes of ethanol...
containing 1.2% potassium acetate were added and left at 4°C overnight. The precipitate was collected by centrifugation and reconstituted with water.

Purification of CS/DS

Nitrous acid treatment. The GAGs obtained after alcohol precipitation were treated with freshly prepared nitrous acid, which was generated by mixing equal volumes of 0.5 mmol of sulfuric acid and 0.5 mmol of barium nitrite and left at room temperature for 40 min (Shively and Conrad 1976). An aliquot of freshly prepared nitrous acid was added again and the incubation continued for further 40 min. The treated sample was neutralized with 0.5 M Na₂CO₃ and desalted on a column of Sephadex G-50 (1 x 56 cm) using 0.2 M ammonium bicarbonate as the eluent at a flow rate of 0.6 mL/min. In all fractions, sulfated GAGs were estimated by the DMMB method (Chandrashekar et al. 1987). The putative CS/DS-rich fractions were pooled and freeze-dried repeatedly by reconstituting in water.

Hyaluronidase digestion. The CS/DS obtained after nitrous acid digestion was dissolved in 0.02 M acetate buffer containing 0.15 M NaCl, pH 6, and digested with hyaluronidase from S. hyalurolyticus at 60°C for 3 h in a water bath with intermittent shaking. After digestion, the digest was treated with trichloroacetic acid to remove proteins and adjusted to 64% ethanol to precipitate the remaining GAGs. Then, CS/DS was desalted using PD-10 columns.

Agarose gel electrophoresis. Agarose gel was casted on the boat to 0.5 cm thickness using 1% agarose. Total kidney GAGs (50 µg), purified CS/DS (50 µg) and equal amount of chondroitinase ABC-digested CS/DS were reconstituted individually in 15 µL of barium acetate buffer (0.05 M, pH 5.0) containing 20% glycerol. The samples were applied after placing the boat with the gel in the electrophoresis chamber containing barium acetate buffer and run for 3 h at 80 V. After the run, the gel was stained in 0.1% Alcian blue overnight and then destained with sodium acetate buffer (0.01 M, pH 5.5) till the gel was cleared of background color.

Disaccharide composition analysis by HPLC. Disaccharide composition analysis of purified CS/DS isolated and purified from control and diabetic rat kidneys were carried out after digesting 4 µg (as sulfated GAG) with 10 mIU of chondroitinase ABC for 1 h at 37°C (Saito et al. 1968). The digested products were derivatized with 2AB (Kinoshita and Sugahara 1999). Excess 2AB was removed by paper chromatography using a solvent system consisting of butanol: ethanol:water (4:1:1; Bigge et al. 1995). The 2AB-labeled disaccharides were diluted to 200 µL with 16 mM NaH₂PO₄ and an aliquot analyzed by anion-exchange HPLC on a PA-03 silica column (YMC-Pack PA, Kyoto, Japan) by gradient elution using a solvent system of 16 and 530 mM NaH₂PO₄ run over a period of 1 h by fluorescence detection with excitation and emission wavelengths set at 330 and 420 nm, respectively.

Determination of the digestibility of the purified CS/DS by various CS lyses. The purified CS/DS (8 µg), from control and diabetic groups, was digested with 10 mIU of chondroitinase ABC or 2 mIU of chondroitinase AC at 37°C or 2 mIU of chondroitinase B at 30°C for 1 h in a total volume of 20 µL. After enzymatic treatment, undigested CS/DS was estimated with the metachromatic dye DMMB, which complexes with sulfated GAGs and long oligosaccharides, but not with short oligosaccharides. Briefly, 30 µL of 0.05 M acetate buffer (pH 6.8) and 400 µL of DMMB solution were added to a 10 µL aliquot of the above digest, and the absorbance was measured at 525 nm. The loss of reactivity toward the dye was checked after each digestion, and the amount remaining was calculated based on the absorbance value using the calibration curve obtained with varying amounts of standard commercial CS/DS (0.4–4.0 µg). The amount of GAG before digestion was taken as 100%.

Gel filtration analysis of the chondroitinase B digests of purified CS/DS preparations on a Superdex peptide column. The purified CS/DS preparation (2 µg as sulfated GAG) was digested with 1 mIU chondroitinase B at 30°C for 1 h. The digests were individually labeled with the fluorophore 2AB and processed as mentioned earlier. Each digest was made up with 100 µL of 0.2 M ammonium bicarbonate containing 7% 1-propanol, and a 5 µL aliquot was analyzed by gel filtration on a Superdex peptide column using the above-mentioned solvent as eluent at a flow rate of 0.1 mL/min employing fluorescence detection.

Isolation of total RNA

Total RNA was isolated from 100 mg of freshly harvested rat kidneys by the Trizol method. The concentration of RNA was determined by the absorption ratio at 260 and 280 nm, and quality was checked by using formaldehyde gel electrophoresis for 28S and 18S RNA. The isolated RNA was converted into cDNA by using GeNeiT™ M-MuLV RT–PCR kit.

Reverse transcription–PCRs
cDNA obtained by reverse transcription was subjected to RT–PCR amplicons using primers corresponding to: C4ST-1, forward primer 5′-GAAGCACCTGTTGTTGATG-3′ and reverse primer 5′-GTAGTTCCGGTGACCTTTCAG-3′; D4ST-1, forward primer 5′-TAGGGCCCCCTACCTCAAGC-3′ and reverse primer 5′-AATGACATGGGGCCACAC-3′; GalNAc4S-6ST, forward primer 5′-ATCCAGTTTTTGACCT-3′ and reverse primer 5′-CCAGTCTTTGCTTGGCAT-3′; and Actin, forward primer 5′-CTATGAAATGTGACGTGGACATCG-3′ and reverse primer 5′-CTTAGAAGCATTGTGCGTACATG-3′. The reaction mixture included 2 µL cDNA, 2.5 µL 10 X PCR buffer, 1 µL dNTPs (10 mM), 1 µL (1.5 U) Taq polymerase, 16.5 µL DEPC water and 1 µL each forward and reverse primers (10 pmol). The thermal cycling program consisted of denaturation for 30 s at 94°C, annealing for 40 s, followed by 25, 30 and 35 cycles of amplification and extension for 1 min at 72°C. Annealing temperatures maintained were 62°C for C4ST-1, 67°C for D4ST-1 and 68°C for GalNAc4S-6ST. The amplicons thus obtained were analyzed
on 1.5% agarose gel. To account for the variability in total RNA input, the expressions of the enzyme obtained were normalized to corresponding actin levels from two increasing cycles in the samples. Densitometry of the amplicons was recorded and calculated using the EASY WIN-32 Image software. Reverse transcription and amplification were done independently for at least five rats per group in duplicates, and relative fold expression compared with actin levels were averaged expressed as the mean ± SD. Suitable negative controls were used to detect non-specific amplifications, if any.

Solid state binding immunoassay of CS/DS preparations to ECM components. It was carried out by immobilizing of CS/DS preparation (100 ng) on poly-L-lysine-coated wells (400 ng/well) overnight. The wells were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 37°C for 1 h. The components to be tested for binding were added individually in varying amounts for overnight binding. Excess components were washed off and amount bound was determined by adding a specific primary antibody (for type IV collagen and laminin) followed by the alkaline phosphatase (ALP)-tagged secondary antibody. Color was developed by adding a chromogenic substrate p-nitrophenyl phosphate (pNPP) and absorbance noted at 405 nm. The assay was carried out using proper controls. To test for specific binding of CS/DS to fibronectin initial steps of immobilization and binding was similar to that of type IV collagen and laminin. Binding was evaluated by determining the decrease in reactivity to CS-56. To demonstrate the specificity of binding, the CS/DS was digested by adding chondroitinase ABC into wells and incubated for 20 min and tested for binding as above.

Reactivity of CS/DS to CS-56 (anti-CS). Preparations of CS/DS from control and diabetic rat kidneys (100 ng/well) were immobilized in a 96-well ELISA plate overnight which was coated prior by poly-L-lysine (400 ng/well). It was then blocked with 1% BSA in PBS at 37°C for 1 h. Reactivity to CS-56 was tested by using it as a primary antibody, followed by an ALP-tagged secondary antibody. Absorbance was read at 405 nm after developing color using pNPP as a chromogenic substance.

Statistical analysis. All values are expressed as the mean ± SD done in duplicates or triplicates of two independent experiments. Statistical analyses between the groups were performed by the unpaired Student’s t-test. A value of \( P < 0.05 \) was considered to indicate significant difference between the groups.

Funding
This work was supported by a grant (SR/SO/HS-28/2009) from Department of Science and Technology, New Delhi, India.

Acknowledgements
The authors thank Dr V. Prakash, Director, CFTRI, Mysore, for his kind support and encouragement to carry out the investigation. Mr J. Darukeyshwara thanks Indian Council of Medical Research (ICMR), New Delhi, India, for the award of Senior Research Fellowship.

Conflict of interest
None declared.

Abbreviations
2AB, 2-aminobenzamide; ALP, alkaline phosphatase; BSA, bovine serum albumin; CS, chondroitin sulfate; DMMB, 1,9-dimethylmethylene blue; DS, dermatan sulfate; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; GAG(s), glycosaminoglycan(s); GalNAc, N-acetylgalactosamine; GFR, glomerular filtration rate; GlcA, D-glucuronic acid; GOD/POD, glucose oxidase/peroxidase; HS, heparan sulfate; ΔHexUA, 4-deoxy-a-L-threo-hex-4-enepyranosyluronic acid; HPLC, high-performance liquid chromatography; IdoA, L-iduronic acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PG(s), proteoglycan(s); pNPP, p-nitrophenyl phosphate; RT, reverse transcriptase; STZ, streptozotocin; TGF, transforming growth factor.

References


