Heparan Sulfate Analysis from Diabetic Rat Glomeruli*†

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One of the major complicating factors in insulin-dependent diabetes mellitus is nephropathy. Several investigators have linked heparan sulfate (HS) alterations in the glomerular basement membrane (GBM) with albuminuria as a marker of abnormal blood filtration and the subsequent progression to renal failure. In this study, we examined the fine structure of HS in the glomerulus and the GBM isolated from the kidneys of rats injected with streptozotocin. Using fluorophore-assisted carbohydrate electrophoresis, we obtained disaccharide composition analyses for HS. In a time course study, we observed that normal rat HS isolated from the GBM becomes more N-sulfated as the glomeruli mature over a period of 8 weeks. Diabetic rats injected with streptozotocin at the beginning of this period showed a reversal of this trend. Using a graded sieve technique, we found that two different sizes of glomeruli could be isolated from the rat kidneys and that there was a significant difference in the HS disaccharide content between these two pools of glomeruli. Only the larger sized glomeruli had less N-sulfation of HS as a result of insulin-dependent diabetes mellitus. This change in the fine structure of HS was localized to the GBM and was not associated with cell surface HS. We also generated oligosaccharides of HS that portray fine structural alterations in the diabetic rats indicative of a loss of the sulfation of N-acetylg glucosamine.

Insulin-dependent diabetes mellitus (IDDM) is a multifaceted disease displaying a wide range of complicating factors. One of these is nephropathy, representing the single most important cause of renal failure for adults in the Western world (1). At the onset of IDDM, patients exhibit an elevated glomerular filtration rate (GFR), nephromegaly, and low levels of albuminuria (2). Within the first few years after onset, morphological changes in the kidney include the thickening of the glomerular and tubular basement membranes and the accumulation of periodic acid-Schiff-positive material in the mesangium (3–5). Although glyceremic control results in the decline of the GFR and the abatement of albuminuria, the symptoms may return in 10–20 years. As albuminuria slowly increases, the GFR begins to decrease, and an increase in blood pressure is often concomitant. Despite the common morphological changes associated with nephropathy, only 20% will result in end-stage renal disease.

The glomerulus is a tuft of anastomosing capillaries supported by a mesangial network and enclosed in Bowman capsule (6). This tuft consists of three cell populations as follows: (a) the visceral epithelial cells (podocytes), (b) the capillary endothelium, and (c) the mesangial cells. The podocytes include the external aspect of the capillary array and possess “foot” processes, called pedicels, that are connected by structures known as slit diaphragms. The endothelial cells line the inner aspect of the capillaries and possess fenestrations that lack the typical diaphragms observed in other fenestrated endothelium. The mesangial cells are axial within the glomerular tuft, resembling fibroblasts situated within their own interstitial matrix and provide a structural framework for the support of the capillaries.

The glomerulus is composed of two extracellular matrices, namely the glomerular basement membrane and the mesangial matrix. Despite their close juxtaposition and interaction at specific sites, they are distinct from one another in both their spatial orientation as well as their structural constituents. The GBM is a continuous matrix sheet underlying the inner perimeter of the capillaries and the outer perimeter of the mesangium. It is a trilaminar structure consisting of the lamina rara externa, a lamina densa, and a lamina rara interna, although it lacks a reticular lamina typical of most basement membranes (7). The structural components of these matrices that have received wide attention regarding their function in glomerular filtration are the proteoglycans (PGs). Because of their high charge to mass density, the PGs are thought to regulate blood filtration by the effect of charge and size selection. The dominant features of PGs that contribute to these biophysical properties are the nature of their glycosaminoglycans. Heparan sulfate (HS), chondroitin sulfate (CS), and hyaluronan are the only glycosaminoglycans localized to the GBM and mesangium. They are linear polymers, consisting of repeating units of GlcNAc or GalNAc, alternating with GlcUA. HS and CS are attached to the parent protein cores via a tetrasaccharide linked to serine-glycine residues. After the initial synthesis of the carbohydrate backbone, an epimerase and several sulfotransferases modify the fine structure of glycosaminoglycans. These modifications vary between cell type, localization, and environmental status.

Although little is known about the function of CS in the glomerulus, there is evidence to suggest that HS is essential for the
Heparan Sulfate in Diabetic Glomeruli

maintenance of proper glomerular filtration. For example, it has been shown that removal of HSs from the GBM in vivo in rats by digestion with heparitinase leads to increased urinary secretion of both ferritin and albumin (8, 9). Furthermore, by injecting antibodies into the bloodstream that have immunoreactivity against HS, an acute case of proteinuria developed (10). These observations are further substantiated by a study of renal biopsies from patients with diabetic nephropathy, revealing a decrease in HS staining in the GBM without corresponding changes in the core proteins of HSPGs (11).

In this paper, we examine the fine structural features of HS in glomeruli and the GBM in the STZ rat model (IDDM) (12, 13). Using fluorophore-assisted carbohydrate electrophoresis (FACE), we present disaccharide composition and oligosaccharide analyses for HS. We put these results in the context of previously reported data and highlight differences in important experimental parameters that can aid data comparison. Furthermore, we discuss the biological implications of our findings and offer potential cellular mechanisms that could be responsible for the diabetic related changes in HS noted in this study.

EXPERIMENTAL PROCEDURES

 Animals and Animal Care—Male Sprague-Dawley rats (150–174 g) were purchased from Harlan (Indianapolis, IN) and housed under conditions of constant temperature with a 12-h light/dark cycle. Food and water were available ad libitum. The rats were anesthetized with isoflurane (Abbot Laboratories, North Chicago, IL), weighed, and injected with 55 mg/kg STZ by adding the appropriate volume of the drug at 25 mg/ml in 10 mM sodium citrate, pH 4.5, via tail vein injections. The rats were euthanized by CO₂ asphyxiation at 3 days, 1, 2, 4, and 8 weeks post-injection for a time course study.

 Blood-Glucose Analysis—Three days after STZ injection, the rats were anesthetized as before, and 5 μl of blood was taken from the tail vein and placed in 95 μl of 100 mM ammonium acetate, pH 7.0. This solution was heated at 100 °C for 10 min, placed on ice for 10 min, and centrifuged at 14,000 × g for 5 min to pellet the insoluble material. 2 μl of the supernatant was transferred to a new tube, lyophilized, and directly labeled with [3H]-thymidine. The kidney was transferred to a Cellector to together with a razor blade for 10 min. Afterward, the kidney were removed, and the kidneys from each rat were minced and minced kidneys of each rat were removed and weighed. The capsules were transferred to 10 ml of phosphate-buffered saline. A standard aliquot of each was transferred to a slide, and the glomeruli were manually counted using a dissection microscope. This was repeated one time for each sample. At least 50 glomeruli were counted each time. Afterward, the glomeruli were allowed to air-dry and were reconstituted in water for area measurement. Phase contrast images were taken at 10× magnification. For each group, 40 glomeruli were traced using Image-Pro® software (Media Cybernetics, Silver Spring, MD), and the areas within the traced glomeruli were quantified. Afterward, the glomeruli were appropriately divided for further analyses as follows. One-third was transferred to a separate vial. This portion was directly prepared for HS analysis and is labeled as “glomeruli” in the figures. From the other two-thirds we isolated the “GBM.” For both the glomerular and GBM samples, one-half of the total was applied to the gel. For the time course, each data point was a compilation of 3 rats (n = 3), except for week 8, which used 12 rats per data point.

 For the preparation of HS oligosaccharides isolated from week 8 glomeruli, we took two-thirds of week 8 glomeruli and pooled the samples from each group (i.e. diabetic and control). Of this fraction, one-third of the glomeruli was used to prepare glomerular HS oligosaccharides, and the remaining two-thirds was used to prepare GBM HS oligosaccharides. Thus, each oligosaccharide sample was an average of the glomeruli from 12 rats. One-third of the total sample was applied to the FACE gel for the large glomeruli. One-half of the total sample was used for the small glomeruli. This amount of sample was required to obtain a sufficient signal for FACE. HS disaccharide analysis (Fig. 7 and the week 8 portion of the time course) was also done on a portion (one-third) of the starting material and required the equivalent of two rats to get a sufficient signal per lane of a FACE gel. In other words, these week 8 disaccharide analyses were n = 6, although the sample was derived from 12 rats. The oligosaccharide data presented in Figs. 10 and 11 represent portions of the glomeruli that were used to generate the data in Fig. 7 and the week 8 portion of the time course.

 Isolation of Glomerular Basement Membrane—Our GBM isolation procedure is based upon previous work (14). For GBM preparation, two-thirds of the glomeruli from two kidneys were transferred to 2 ml screw-cap microcentrifuge tubes. Each glomerular sample was suspended in 1 ml of room temperature distilled water containing 0.05% sodium azide, 5 mM benzamidine-HCl, 5 mM N-ethylmaleimide, 10 mM disodium EDTA, and 1 mM phenylmethanesulfonyl fluoride. The glomeruli were constantly vortexed at medium speed for 1 h and pelleted by centrifugation at 50 × g. This step was repeated one time. The pellet was further extracted for 1 h using 1 ml of 3% Triton X-100 in the protease inhibitor mixture described above, vortexed, and pelleted as before. The pellet was rinsed four times in 1 ml of distilled water and digested for 1 h with 50 units of DNase I (Ambion, Austin, TX) in 1 ml of 1 mM NaCl at 37 °C. The remaining pellet was extracted twice for 1 h each time with 1 ml of 4% sodium deoxycholate at room temperature in the protease inhibitor mixture. The resulting GBM preparation was rinsed five times with distilled water and resuspended in 900 μl of distilled water for subsequent proteinase K digestion.

 Preparation of Heparan Sulfate Samples—All samples were prepared for fluorescent derivatization in the following man-
ner. Each extract was suspended in 900 μl of distilled water, to which 50 μl of proteinase K (2.5 mg/ml) in 1 M ammonium acetate, pH 7.0, was added, and the sample was incubated at 60 °C for 2 h with vortexing at the 1-h mark. Another 50-μl aliquot of proteinase K was added, and the incubation was repeated as before. Proteinase K was inactivated by incubation at 100 °C for 10 min. The samples were then cooled on ice for 10 min, and brought to 250 μl by vacuum concentration. 1 ml of ice-cold absolute EtOH was added to each sample and maintained at −20 °C overnight. Glycosaminoglycans were pelleted by centrifugation at 14,000 × g for 20 min. Each pellet was suspended and washed with 1.25 ml of 75% EtOH, pelleted as before, and air-dried for 20 min at room temperature. Each dried pellet was resuspended in 20 μl of 100 mM ammonium acetate, pH 7.0, and digested with 4 μl each of 1 milliunit/μl heparinase, heparitinase I, and heparitinase II for complete digestion or with heparitinase I only for the generation of oligosaccharides, giving a total reaction volume of 32 μl. The digests were incubated at 37 °C for 3 h with vortexing on the hour. The endoelaminases were inactivated by incubation at 100 °C for 10 min, and the samples were subsequently cooled on ice for 10 min. For disaccharide analysis, the samples containing HS digests were lyophilized and directly derivatized with 2-aminoacridone (AMAC) (Invitrogen). For oligosaccharide analysis, the digests were precipitated in 82.5% EtOH, and the pellet was derivatized with AMAC.

Fluorescent Derivatization of Heparan Sulfate Samples with 2-Aminoacridone (15)—Each sample, representing a portion of the glomeruli or GBM from two kidneys, was labeled in an 8-μl reaction volume using PCR tubes and low retention pipette tips. Briefly, 4 μl of 12.5 mM AMAC (85% Me2SO and 15% glacial acetic acid) was added to each lyophilized sample, and the solution was incubated at 25 °C for 20 min. Then 4 μl of 1.25 mM sodium cyanoborohydride in distilled water was added, followed by an overnight (16–18 h) incubation at 37 °C. The next day, 2.9 μl of 50% glycerol containing 0.43 mM Trizma (Tris base) and 4% NaOH was added to 4 μl of each reaction mixture, and the samples were incubated at 25 °C for 20 min. 5 μl of each glycerol/reaction mixture solution was loaded onto the gel.

PAGE—HS disaccharides and oligosaccharides were analyzed using OLIGO® profiling gels and companion buffers (Prozyme, San Leandro, CA). The glass plates of the pre-cast gels were washed with distilled water and inserted into the gel box, and the wells were thoroughly washed with running buffer.

![Fluorescent Derivatization of Heparan Sulfate Samples with 2-Aminoacridone](image)

The gel box was placed in a large container filled with ice and allowed to equilibrate to 4 °C for 1–2 h. All samples were loaded simultaneously in 5-μl aliquots using a Hamilton 8-channel glass syringe (Reno, NV). The OLIGO® gels were subjected to 15 mA/gel (constant) for about 1 h and 45 min. The gels were washed with distilled water and imaged while in their glass plates.

**RESULTS**

We have used two parameters to substantiate the effect of STZ injection upon the development of experimental diabetes in our rat model. The first parameter is the fold increase in diabetic glucose concentration in the blood in relation to the blood-glucose of the control animals. Fig. 1A shows a representative FACE gel of the blood-glucose for diabetic animals compared with control. The quantification of this measure- ment is shown in Fig. 1B. Any animals with a blood-glucose level <2.0-fold of the control animals were not considered to be
diabetic, and all data derived from them were discarded. Blood-glucose levels were also confirmed at the time of sacrifice (data not shown).

The second parameter used to substantiate a diabetic state was the ratios of the average kidney weight from an animal in relation to the body weight of that animal. Fig. 1C shows the average kidney weight in relation to the body weight of the same animals and compared with the average for controls. In the STZ model, diabetic animals have lower body weights because of the inability to properly utilize blood-glucose. In contrast, their kidney weights increase as a result of renal disease.

Glomeruli were isolated from minced kidneys by graded sieving. Although unexpected, we noticed that glomeruli could be isolated from both 94–140-μm ("large") and 74–94-μm ("small") range sieves. Large and small glomeruli were readily isolated from both diabetic and control rat kidneys. The purity of the glomeruli in each fraction was noted visually and was >90% with little tubular contamination. The average size of week 8 large and small glomeruli can be seen in Fig. 2, A and B. The quantified areas of the glomeruli are shown in Fig. 2C. There was a statistical difference in these two sizes of glomeruli for both the diabetic and control rats (p < 0.0001). Although there was no difference in the large glomeruli when the diabetic rat was compared with the control rat, the small glomeruli were statistically different (0.00046). This suggests that the small diabetic glomeruli had enlarged by week 8.

To examine the changes in HS disaccharide composition in the glomeruli and GBM during the onset and development of diabetes, a time course study was implemented. As the control rats aged from day 3 to week 8, the large glomeruli population increased although the small glomeruli did not significantly change (Fig. 3A). In contrast, the small glomeruli of the diabetic rats decreased at weeks 4 and 8, whereas the large glomeruli increased proportionally (Fig. 3B). There was a statistically significant (p = 0.004) greater number (38%) of large rather than small glomeruli in the diabetic population at week 8, although they were equivalent in the control.

Fig. 4 shows a representative FACE gel of HS disaccharides 8 weeks after the onset of diabetes. Under optimum conditions, this technique resolves six major bands. The identity of these disaccharides is listed in Fig. 4 legend and includes two N-acetylated disaccharides (bands 1 and 3) and four N-sulfated disaccharides (bands 2, 4, 5, and 6). The dominant disaccharide observed in rat glomeruli and GBM is Δdi-N-acetylgalactosamine (band 1), followed in intensity by its 2–4 times less abundant N-sulfated form (band 2). The remaining disaccharides (bands 3–6) are 4–6 times less abundant than the acetylated form. As indicated in Figs. 5 and 7, we multiplied the data values of the less abundant disaccharides by factors of 2 or 4 so that their trends could be better compared with Δdi-N-acetylgalactosamine.

Depending upon the gel lot, disaccharides 5 and 6 merged, resolving five total bands instead of six. Such was the case for all time points except week 8. The resolution of disaccharides 5 and 6 did not affect our study because both of these disaccharides are N-sulfated, and our primary conclusions involved comparing the N-acetylated and the N-sulfated disaccharides. For the sake of simplicity, the 5th band in the quantified disaccharide data in Figs. 5 and 7 represents the sum of N-sulfated disaccharides 5 and 6.

We used FACE to examine HS disaccharides during a time course study (Fig. 5). N-Sulfated disaccharides are presented in
Fig. 5 as gray bars and N-acetylated disaccharides are white bars. The disaccharides derived from the GBM of the large glomeruli showed significant changes over the time course. Specifically, as the control rats aged from day 3 to 8 weeks (Fig. 5A), the primary N-acetylated disaccharide decreased (band 1), and the primary N-sulfated form significantly increased (band 2). In contrast, the diabetic N-acetylated disaccharides did not decrease over time (Fig 5B, bands 1 and 3), whereas the N-sulfated disaccharides either decreased (bands 4 and 5) or did not change at all (band 2). The GBM derived from the small glomeruli of control rats showed a reversal of the trend observed in the large glomeruli. In this extract, the primary N-sulfated disaccharide (Fig. 5C, band 2) significantly decreased over time, whereas the N-acetylated disaccharides either increased (band 3) or did not change (band 1). In contrast, the GBM derived from the small glomeruli of diabetic rats showed no changes over the time course (Fig. 5D), except for a slight increase in the minor N-acetylated disaccharide (band 3).

We compiled all of the N-sulfated disaccharides (gray bars) and compared them with the N-acetylated disaccharides (white bars) for the GBM of the large and small glomeruli (Fig. 6). The N-sulfated disaccharides in the GBM of the large glomeruli from the controls steadily increased 9.7% from day 3 to week 8 (Fig. 6A). In contrast, the N-sulfated disaccharides in the GBM
of the small glomeruli from the controls decreased $-11.0\%$ from day 3 to week 8 and $3.2\%$ for the diabetics (Fig. 6B). However, the disaccharide profile of the GBM of the large glomeruli from the diabetics was distinctly different from the controls. In this case, the $N$-sulfated disaccharides steadily decreased $-8.2\%$ from day 3 to week 8 (Fig. 6A). In this respect, the changes in the GBM of the large glomeruli from the diabetics were very similar to the changes in the GBM of the small glomeruli from both the controls and the diabetics.

Fig. 7 shows the week 8 disaccharides in greater detail. The significance of these data is more clearly observed when pooling the $N$-acetylated and $N$-sulfated disaccharides for direct comparison (Fig. 8). We observed that the $N$-acetylated disaccharides in the GBM of the large glomeruli were greater for the diabetic rats (19%, $p = 0.012$) than the control rats (Fig. 8A). Conversely, the $N$-sulfated disaccharides were less for the diabetic rats (30%, $p = 0.012$) than the control rats. There was no difference between the diabetic and control samples derived from the GBM of the small glomeruli (Fig. 8B). There was a 25% greater amount of $N$-acetylated disaccharides in the smaller control glomeruli compared with the larger control glomeruli ($p = 0.0039$) (Fig. 8C). Also, there was a 43% decrease in the amount of $N$-sulfated disaccharides in the smaller control GBM compared with the larger control GBM ($p = 0.0039$). There was no statistical difference when comparing the percentage of these two disaccharide populations between the GBM of the large and small diabetic glomeruli (Fig. 8D). In other words, the GBM of the larger diabetic glomeruli has acquired a similar HS disaccharide structure compared with the GBM of the smaller control glomeruli.

When comparing the absolute mass of HS, we observed no statistical difference among the HS fractions when comparing the diabetic rats with the control rats (Fig. 9A). The percentage of HS found in the GBM compared with total glomerular HS was not statistically different (Fig. 9B), although there was a greater percentage (39%) of HS in the GBM of large diabetic glomeruli compared with control glomeruli.

In summary of the week 8 disaccharide data, the decrease of $N$-sulfation in the diabetic rat is exclusively observed in the GBM of the large glomeruli, which resembles the fine structure found in the GBM of the small control and diabetic glomeruli. Because the mass of HS was not significantly altered by diabetes, this decrease in $N$-sulfation would result in lowering the charge density of the GBM in the large glomeruli.
Heparitinase I requires an unsulfated or monosulfated glucosamine (either N-sulfated or 6-sulfated with an N-acetyl group) in glycosidic linkage to an unsulfated glucuronic acid at the site of cleavage. In other words, this enzyme digests primarily in regions between the highly sulfated S-domain block structures in HS. Because digestion with this enzyme would yield HS oligosaccharides that are N-sulfated, we wanted to see if they were altered by diabetes.

Fig. 10, A and B, shows the FACE gels of these oligosaccharides derived from large and small glomeruli, respectively. Digestion of HS with heparitinase I yielded six major oligosaccharides (r1–r6) for samples derived from glomeruli (Fig. 10, lanes 2 and 3) and from the GBM (lanes 4 and 5). Oligosaccharide r6 was found primarily in the small glomeruli (Fig. 10B, lanes 2 and 3). The migration of the sample oligosaccharides bisect the standard tetramers in Fig. 10, lane 1 (s1, s2, s3, and s4), and migrate faster than the standard hexamers in lane 6, except for s7, which is probably a tetramer. Thus, the major sample HS oligosaccharides were primarily derived from small glomeruli.
Heparan Sulfate in Diabetic Glomeruli

FIGURE 11. Quantification of heparan sulfate oligosaccharides 8 weeks after the onset of diabetes. A and B show the quantification of the six major oligosaccharides normalized by DNA content for the large glomeruli and the GBM of the large glomeruli, respectively. C and D show the same for the small glomeruli. Each sample represents a compilation of 12 rats.

The disaccharide data did not show any difference between control and diabetic rats for the GBM derived from the small glomeruli (Fig. 8B), but the oligosaccharide data suggest a decrease in N-sulfation even in the GBM of the small glomeruli (Fig. 11D). N-Sulfated disaccharides represented only 25% of the total disaccharides in the GBM of the small control glomeruli, compared with 44% for the GBM of the large glomeruli (Fig. 8, B and A, respectively). Thus, changes in the N-sulfation of the disaccharides derived from the GBM of small glomeruli would be a lesser percentage of the total than the equivalent change in the GBM of the large glomeruli. The isolation of N-sulfated oligosaccharides from these populations could prevent the masking effect caused by the greater percentage of N-acetylated disaccharides in the GBM of the smaller glomeruli. Thus, it is possible that N-sulfation is decreased in the GBM of the smaller glomeruli, but its decrease is a smaller percentage of the total HS than when compared with the GBM of the large glomeruli. These data suggest that the N-sulfated domains of HS are significantly reduced in diabetic rats, particularly in the GBM of the large glomeruli.

DISCUSSION

Three important experimental parameters significantly influenced the results and conclusions of this study as follows: (a) the time of sacrifice, (b) the removal of lipid-soluble material from the glomerular preparations, and (c) glomerular size.

The time of sacrifice after STZ injection in the rat has varied in reported data as follows: 3 weeks (16–21), 4 weeks (19, 22, 23), 5–6 weeks (24), and 10 weeks (16). As our control rats aged from day 3 to 8 weeks, we observed a decrease in the primary N-acetylated disaccharides and an increase in the primary N-sulfated residues from the GBM of large glomeruli. This trend has not been reported by other investigators, and it demonstrates a particularly interesting process in normal glomerular maturation. It also emphasizes the importance that the time of sacrifice makes when comparing the different studies listed above. Although the physiological consequence of increasing N-sulfation in the large glomeruli during normal glomerular maturation is not clear, the fact that this trend is inhibited in the diabetic rats emphasizes its importance. Furthermore, the observation that the HS in the small glomeruli becomes less N-sulfated with time clearly shows that the two size populations are distinctly different.

Enrichment of glomeruli for insoluble matrix components was also an important factor in observing HS disaccharide changes in the diabetic rats. This matrix-enriched fraction of glomeruli is commonly referred to as GBM in the literature, although it clearly contains mesangial matrix in addition to matrix derived from the GBM. As in our study, most methods utilizing the STZ rat model have isolated this matrix fraction by osmotic lysis and detergent extraction of lipid and cell-associated products in the presence of protease inhibitors (18–20, 22, 23), whereas others have used chaotropes (16, 25) and sonication (24, 25) to achieve a similar result. At least one study only used isolated glomeruli and did not enrich for matrix components (21). We observed that only the GBM fraction of isolated glomeruli gave statistically significant changes in HS disaccharide composition when comparing the diabetic and control
Heparan Sulfate in Diabetic Glomeruli

rats. For example, it was only in the GBM fractions that we observed an increase in the N-acetylated disaccharides and a decrease in N-sulfated disaccharides for diabetic samples. In other words, diabetic related changes in the fine structure of HS were only observed when lipid- and water-soluble components were removed from the large glomeruli, and only the insoluble extracellular matrix products were prepared for analysis. This is particularly important considering that the matrix fraction of HS (Fig. 9A). We conclude that diabetic related disaccharide N-sulfation abnormalities in the glomeruli themselves were not observed because lipid-soluble HS was sufficiently to mask the 20–30% decrease present in the HS matrix. These data also imply that cell surface and water-soluble HS (60% of total) may not be altered in this model of IDDM.

The stainless steel, graded sieve isolation protocol has been the most widely used method for isolating glomeruli in our model. In the literature, there have been variations in the choice of sieve pore size for this purpose. The following sieve ranges (μm) have been utilized previously: 140–280 (25), 63–149 (18–20, 24), 105–210 (26), and 75–150 (17). There are at least two reports in which glomeruli were collected on both the 94–140- and 74–94-μm ranges that we have incorporated into our study (21, 22). It appears that these two studies pooled the glomeruli from both populations instead of keeping them separate as we were able to, because of the sensitivity of the FACE technique. One study isolated glomeruli on a 105-μm sieve but notes that smaller glomeruli could be recovered on smaller pore sizes (26). Another study made the observation that glomerular size is related to body weight in the rat body weight range of 75–300 g (24). The body weight (g) of the rat at the time of STZ injection has varied in the reported data as follows: 175 (21, 22), 80–120 (18, 24, 27), 150 (20, 25), unreported (17, 19, 23), and 350 g (16). Our rats were 150–174 g when injected. By week 8, the average weight of the control and diabetic rats was 401 (± 9.08) and 245 g (± 19.2), respectively. All of these body weights fall into the range in which glomerular size is affected by body weight. Thus, care should be taken when comparing the glomerular yield on a specific sieve from a rat on the low end of this range compared with a rat on the high end.

The isolation of two different sizes of glomeruli (i.e. large and small) was not anticipated or planned, but the importance of separating these two sizes of glomeruli is demonstrated by the observation that only the HS disaccharides from the larger diabetic glomeruli showed a decrease in N-sulfation. The HS composition of the small glomeruli was significantly less N-sulfated than the large glomeruli. This adds credibility to the glomerular area data (Fig. 2), confirming that these two populations of glomeruli possess biochemical differences and implying that there may be a physiological rationale related to glomerular size. The greater number of larger rather than smaller glomeruli in the diabetic population (Fig. 3, week 8) is consistent with data that describe an increased mean glomerular diameter for diabetic kidneys compared with control kidneys (24). All glomerular development stops 21 days after birth in the rat (6). In other words, no new glomeruli are formed after this time. Because our rats are older than 41 days, the changes we observed in the number and area of small and large glomeruli are the result of glomerular maturation and not glomerular development. Because our rats had completed glomerular development before STZ injection, the increase in the number of large glomeruli in the control population over time could not have been caused by the development of more large glomeruli, but it was likely caused by maturation of the smaller glomeruli into larger ones that were selected on the larger sieve (Fig. 3). Because the number of small control glomeruli did not consequently decrease with time, this implies that yet smaller glomeruli enlarged over time to be selected on the small sieve. These data are consistent with a kidney that gains more mass as it ages (Fig. 1). But these glomerular trends were different for the diabetic rat. The number of large diabetic glomeruli increased significantly more than for the control glomeruli by the 8-week time point. Also, the number of small diabetic glomeruli decreased in proportion to the increase in the number of large diabetic glomeruli. One interpretation of this phenomenon is abnormal hypertrophy of the smaller diabetic glomeruli into larger glomeruli. This is consistent with our observation that there was a statistically larger glomerular area (p = 0.00046) for the small glomeruli from diabetic kidneys compared with controls (Fig. 2C).

Hypertrophy is the first step to nephropathy, and this beginning stage is often called early sclerotic. Our primary biochemical observation was that HS N-sulfation decreased in the large diabetic glomeruli population when compared with the large control glomeruli. The decreased state of N-sulfation for the diabetic was nearly identical to the N-sulfation of both control and diabetic small glomeruli. Thus, the observations from the biochemical data (Figs. 5–9) and the data representing the yield of glomeruli (Fig. 3) imply that the “mechanism” for decreased N-sulfation in the diabetic rat could be influenced by hypertrophy of the diabetic smaller glomeruli. As the smaller diabetic glomeruli hypertrophied, they could have been isolated on the sieve that selected the larger glomeruli. Because the smaller glomeruli are less N-sulfated, this would dilute the extent of N-sulfation of the large glomeruli. But this mechanism is insufficient to explain the trend observed in the disaccharide time course (Fig. 5). Here we observed that N-sulfated disaccharides 4 and 5 derived from large glomeruli decreased over time, whereas those derived from the small glomeruli did not change. This observation implies that the depletion of N-sulfation in the diabetic state must also be influenced by a literal suppression of N-sulfation on the HS backbone and not exclusively caused by dilution of the large glomeruli population with hypertrophied small glomeruli.

The glomerular area (Fig. 2), glomerular yield (Fig. 3), and the biochemical data (Figs. 5–11) make it clear that the large and small glomeruli from the controls represent distinctly different populations. The data also suggest that a major difference of the diabetic large glomeruli is their inability, unlike the controls, to modulate their large glomerular HS sulfation pattern toward higher sulfation from where it was at the time (day 3) shortly after the onset of diabetes.

The suppression of N-sulfation on the HS backbone in the diabetic glomeruli could be caused by several mechanisms. As HS is polymerized, it undergoes a series of modification reactions catalyzed by at least four families of sulfotransferases and one epimerase (28). N-Sulfation of N-acetylgalcosamine occurs
Heparan Sulfate in Diabetic Glomeruli

via N-deacetylase/N-sulfotransferase in clusters along the chain. Subsequently, other sulfotransferases modify hydroxyl groups on both glucuronic acid and N-acetylglucosamine. Our data are consistent with the conclusion that N-deacetylase/N-sulfotransferase activity or expression could be impaired in IDDM, but it does not exclude the possibility that other sulfotransferases could also be impaired. One study has reported a 10% decrease in N-deacetylase/N-sulfotransferase activity in the STZ rat model (27). Another study examined serum sulfate concentrations in the STZ rat model and found no differences between the diabetic and control throughout the 12 weeks of disease (29). Although they did observe a significant ($p = 0.002$) 1.7-fold increase in the diabetic kidney, they concluded that it could not compromise biological sulfation reactions. Another means in which sulfation of HS could be altered is by suppression of the synthesis or transport of the biological sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (30).

Previously, we have shown that endogenous HS oligosaccharides purified from confluent cultures of rat mesangial cells are potent inhibitors of rat mesangial cell proliferation and that they are the metabolites of cellular HS PGs (31, 32). Intact HS chains, from which these oligosaccharides were derived, are only slightly anti-mitogenic. This suggests that fine structures within the HS oligosaccharides are critical and could be dependent upon N-sulfated regions (31). Previous studies have reported that glomeruli produce HS oligosaccharides that are similar to those produced by rat mesangial cells (32). Thus, it is possible that the diabetic-dependent structural changes of HS observed in this study could alter the mitogenic effects of these oligosaccharides derived by metabolic turnover from glomeruli.

Acknowledgments—We thank Dr. Douglas Templeton for the review and helpful suggestions and John Gallagher for kindly providing the heparan sulfate oligosaccharide standards.

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