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Reduction of heparan sulphate-associated anionic sites in the glomerular basement membrane of rats with streptozotocin-induced diabetic nephropathy

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Summary Heparan sulphate-associated anionic sites in the glomerular basement membrane were studied in rats 8 months after induction of diabetes by streptozotocin and in age- and sex-matched control rats, employing the cationic dye cuprolinic blue. Morphometric analysis at the ultrastructural level was performed using a computerized image processor. The heparan sulphate specificity of the cuprolinic blue staining was demonstrated by glycosaminoglycan-degrading enzymes, showing that pretreatment of the sections with heparitinase abolished all staining, whereas chondroitinase ABC had no effect. The majority of anionic sites (74 % in diabetic and 81 % in control rats) were found within the lamina rara externa of the glomerular basement membrane. A minority of anionic sites were scattered throughout the lamina densa and lamina rara interna, and were significantly smaller than those in the lamina rara externa of the glomerular basement membrane ($p < 0.001$ and $p < 0.01$ for diabetic and control rats, respectively). Diabetic rats progressively developed albuminuria reaching 40.3 (32.2–62.0) mg/24 h after 8 months in contrast to the control animals (0.8 (0.2–0.9) mg/24 h, $p < 0.002$). At the same time, the number of heparan sulphate anionic sites and the total anionic site surface (number of anionic sites × mean anionic site surface) in the lamina rara externa of the glomerular basement membrane was reduced by 19 % ($p < 0.021$) and by 26 % ($p < 0.02$), respectively. Number and total anionic site surface in the remaining part of the glomerular basement membrane (lamina densa and lamina rara interna) were not significantly changed. We conclude that in streptozotocin-diabetic rats with an increased urinary albumin excretion, a reduced heparan sulphate charge barrier/density is found at the lamina rara externa of the glomerular basement membrane. [Diabetologia (1995) 38: 1169–1175]

Key words Albuminuria, anionic sites, cuprolinic blue, glomerular basement membrane, heparan sulphate, rat, streptozotocin.

Heparan sulphate (HS) is thought to play an important role in the permselective properties of the glomerular capillary wall [1–3], since enzymatic digestion of HS in the glomerular basement membrane (GBM) causes an increased passage of native ferritin and albumin into the urinary space [4, 5]. Furthermore, intrarenal or intravenous injection of cationic molecules leads to an increased permeability of the GBM to proteins, due to the neutralization of primarily HS-associated anionic sites of the glomerular capillary wall [6–8]. We have recently demonstrated the induction of an acute selective proteinuria after intravenous injection of a monoclonal antibody against GBM HS [9].

Diabetic nephropathy ultimately develops in 30–40 % of patients suffering from insulin-dependent diabetes mellitus and is initially characterized by micro-

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Abbreviations: HS, Heparan sulphate; GBM, glomerular basement membrane; HSPG, heparan sulphate proteoglycan; STZ, streptozotocin; LRE, lamina rara externa; LD, lamina densa; LD + LRI, lamina densa + lamina rara interna; ANOVA, analysis of variance.

albuminuria [10, 11]. The selectivity index of the proteinuria in diabetic patients with microalbuminuria [12–15] suggested a decreased charge-dependent permeability of the GBM. For that reason the significance of GBM heparan sulphate proteoglycan (HSPG) for the development of albuminuria in diabetic nephropathy has been analysed in several studies by quantitation of HSPG or HS in glomeruli or isolated GBM. Studies in humans revealed a decreased content of HS and HSPG within the GBM of kidneys from IDDM patients [16–18]. Investigations have also been performed in experimental models of diabetic nephropathy, mostly in the streptozotocin (STZ)-induced diabetes model in the rat. Biochemical quantitation of HS in the GBM, in glomeruli or in the renal cortex revealed a decreased [19–22] or unaltered [23, 24] HS content in rats with STZ-induced diabetic nephropathy. However, these studies do not discriminate between HSPG present in the peripheral GBM, or in the enlarged mesangial matrix that also contains HSPG. An alternative approach is to visualize HS and/or HSPG-core protein by immunohistochemical and histochemical methods. We found, using monoclonal antibodies, a decreased staining for HS in the GBM in kidney biopsies from diabetic patients, which correlated with the fractional protein excretion, whereas the GBM staining for the core protein of HSPG was unaltered [25, 26]. Others, however, found a decreased GBM staining for the core protein of HSPG in diabetic kidneys [27, 28]. Negatively charged molecules like HS can also be visualized by cationic dyes. For staining of HS, cuprolinic blue is very useful. Under critical electrolyte concentration this cationic dye specifically stains HS and can therefore be used as a marker for GBM HS [29]. A recent study using cuprolinic blue demonstrated a reduction of HS-associated anionic sites within the GBM in patients with diabetic nephropathy, which was inversely correlated with the degree of albuminuria [30]. A decrease of the number and density of cuprolinic blue anionic sites in the GBM of diabetic BB-rats has also been demonstrated [31]. In this study we analysed the HS-associated anionic sites in the GBM of STZ-diabetic rats with a pronounced albuminuria.

Materials and methods

Animals. Diabetes was induced in overnight-fasted male Wistar-Münich rats (Jackson Laboratory, Bar Harbor, Me., USA) of 150 g body weight by intravenous injection of 55 mg STZ/kg body weight, freshly prepared in 10 mmol/l citrate buffer (pH 4.5), 25 mg/ml. Animals were treated three times a week (Monday, Wednesday, Friday at 17.00 hours) with a low dose (1.2 IU) of bovine insulin (Ultralente, Novo, Copenhagen, Denmark) to maintain blood glucose levels around 25 mmol/l. Sex- and age-matched rats that did not receive STZ served as controls. The rats were followed-up monthly for body weight, blood glucose and albuminuria as described previously [32]. Blood glucose level was determined by Haemo-Glukotest 1-44R (Boehringer Mannhein, Mannheim, Germany) and albuminuria by rocket electrophoresis according to Laurell [33] using goat anti-rat albumin and rat albumin as the standard (both from Nordic, Tilburg, The Netherlands).

Tissue collection and cuprolinic blue staining. Eight months after induction of diabetes, rats were anaesthetized by Inactin (Byk Gulden, Konstanz, Germany); control rats, 140 mg/kg body weight; diabetic rats, 110 mg/kg body weight; kidneys were removed, decapsulated and weighed. Small cubes (1 mm^3) of renal cortex tissue were fixed for 2 h by immersion fixation in lysine – periodate – 2% paraformaldehyde fixative, cryoprotected in 2.3 mol/l sucrose for 1 h, snap-frozen and stored in liquid nitrogen. To avoid penetration problems of the cationic dye, 30-µm cryostat sections were cut, rinsed for 10 min in 0.05 mol/l sodium acetate buffer (pH 5.6), and stained overnight at room temperature in 0.05 sodium acetate buffer (pH 5.6) containing 2% glutaraldehyde, 0.2 mol/l $MgCl_2$ and 0.2 % cuprolinic blue (BDH Chemicals, Poole, Dorset, UK). Sections were rinsed three times for 10 min in the same buffer without cuprolinic blue, incubated for 1 h in a 1% sodium tungstate solution followed by an additional 30 min in 1 % sodium tungstate in 50 % ethanol, dehydrated in graded ethanol and embedded in Epon 812. Ultrathin sections were prepared on a LKB Ultratome, poststained for 45 min with uranyl acetate and examined in a JEOL 1200 EX II electron microscope (Jeol, Tokyo, Japan). Specificity of the cuprolinic blue staining for HS was controlled in two diabetic and two control rats by a 1-h preincubation (before cuprolinic blue staining) at 37 °C of 30-µm cryostat sections with 1 U/ml heparitinase from *Flavobacterium heparinum* (Seikagaku, Tokyo, Japan) in 0.1 mol/l sodium acetate buffer containing 10 mmol/l Ca^{++} (pH 7.0), or with 0.1 U/ml chondroitinase ABC from *Proteus vulgaris* (Seikagaku) in 0.1 mol/l Tris/HCl (pH 8.0), or the buffers alone without the enzymes.

Morphometric analysis. Thirty-five photomicrographs of randomly-chosen cross-sectioned glomerular capillary loops with a final magnification of 68,000 × were taken from at least three randomly-chosen glomeruli per animal. The photomicrographs were analysed using a CCD RGB camera (Sony CA-325 AP) with a macro objective, attached to a VIDAS^{plus} image analysis system (Kontron Inc., München, Germany). After the image was digitized, three lines were drawn interactively: the GBM was lined between the epithelial foot processes and the outer GBM and between the endothelial cells and the inner GBM. A third line was drawn just beneath the anionic sites in the lamina rara externa (LRE) of the GBM. The image was corrected for unequal lighting and background staining. A fixed threshold was applied to determine areas of positive staining. Since we observed that the anionic sites in the LRE were larger in size than those found in the remaining part of the GBM (lamina densa and lamina rara interna (LD + LRI)), anionic site analysis was performed separately for the LRE and LD + LRI. From the resulting binary images of each photomicrograph the following parameters were calculated: a) the number of anionic sites per µm GBM; b) the mean anionic site surface (nm^2), which gives an impression of the mean surface (= area) of each individual anionic site; c) the *total* anionic site surface (nm^2/µm GBM), defined as the number of anionic sites/µm GBM multiplied with the mean anionic site surface (a × b); d) the thickness of the LRE and LD + LRI (nm); and e) the *relative* anionic site surface (%). Latter parameter was calculated as follows: [(total anionic site surface LRE or LD + LRI/total surface LRE or LD + LRI) × 100 %].

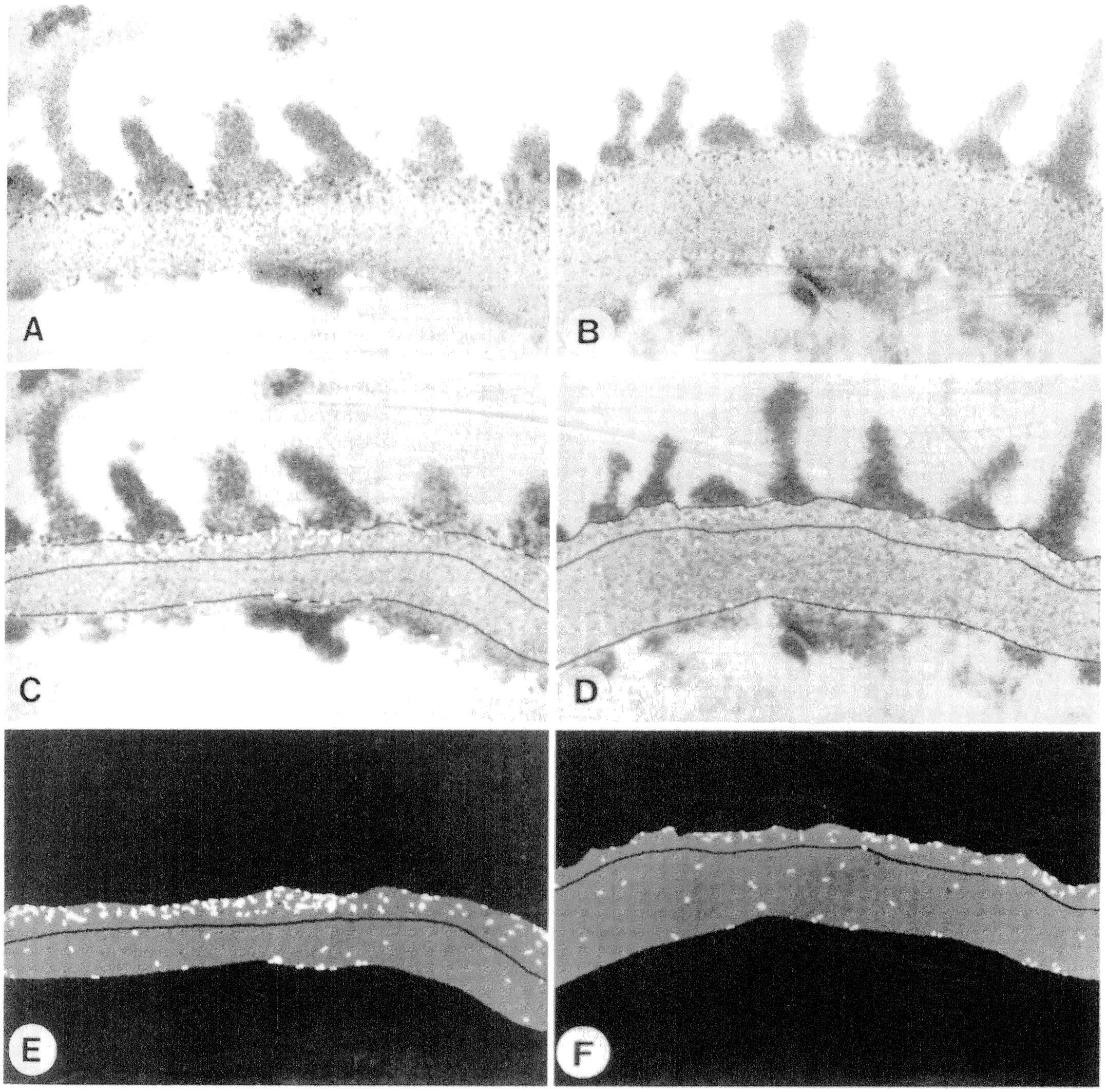

Fig. 1 (A–F). Staining of the HS-associated anionic sites in the GBM of a control (**A, C, E**) and a diabetic (**B, D, F**) rat. Note that the majority of the anionic sites are localized in the LRE of the GBM. The original photomicrographs are seen in (**A**) and (**B**). These images were digitized, three lines were drawn interactively as indicated in Methods, and the computerized anionic sites are shown in white (**C**) and (**D**). The corresponding binary images, on which the analysis was performed, are shown in (**E**) and (**F**). (Magnification: 50,000 ×)

and expresses the percentage of the LRE or LD + LRI which is occupied by anionic sites. The analysis of the GBM anionic sites after pretreatment with glycosaminoglycan-degrading enzymes was also done on 35 photomicrographs from at least three randomly-chosen glomeruli/animal.

Statistical analysis

For statistical analysis the Mann-Whitney test was used for albuminuria, blood glucose concentration, body weight and kidney weight (Table 1). To analyse the morphometric data we used a special analysis of variance (ANOVA) approach, namely a single-factor nested design with random nesting factor, the single factor being presence/absence of diabetes, and animals as the nesting factor. p-values less than 0.05 were regarded as significant. Values are expressed as median values and (range).

Results

Development of diabetic nephropathy in STZ-diabetic rats. After a diabetes duration of 8 months an increased urinary albumin excretion developed and en-

Table 1. Characteristics of the rats 8 months after induction of diabetes

Parameter	Diabetic rats ($n = 8$)	Control rats ($n = 5$)	p-value Mann-Whitney
Albuminuria (mg/24 h)	40.3 (32.2–62.0)	0.8 (0.2–0.9)	< 0.002
Blood glucose (mmol/l)	23.0 (20.9–25.2)	4.8 (4.5–5.1)	< 0.002
Body weight (g)	228 (212–256)	370 (368–391)	< 0.002
Wet kidney weight (g)	3.12 (2.96–3.26)	2.34 (2.25–2.51)	< 0.002

Values are median (range)

Table 2. Influence of glycosaminoglycan-degrading enzymes on the number of anionic sites in the LRE or LD + LRI

Rat no.	No pretreatment		Heparitinase		Chondroitinase ABC	
	LRE	LD + LRI	LRE	LD + LRI	LRE	LD + LRI
Diabetic						
1	36	12	3	3	34	9
2	32	9	3	2	37	10
Control						
1	37	8	2	1	34	6
2	40	11	4	2	35	8

Table 3. Analysis of the HS-associated anionic sites of the GBM

Parameter	Diabetic rats ($n = 8$)	Control rats ($n = 5$)	p-value ANOVA
Lamina rara externa (LRE)			
Number of anionic sites (sites/μm GBM)	34 (25–42)	42 (39–46)	< 0.02
Surface/anionic site (nm^2)	150 (119–168)	163 (139–220)	NS
Total anionic site surface (nm^2/μm GBM)	4957 (2975–7056)	6670 (5838–8580)	< 0.02
Thickness LRE (nm)	76 (63–88)	74 (72–86)	NS
Relative anionic site surface (%)	6.79 (4.64–8.82)	9.31 (7.48–12.15)	< 0.03
Lamina densa and lamina rara interna (LD + LRI)			
Number of anionic sites (sites/μm GBM)	12 (3–18)	10 (9–14)	NS
Surface/anionic site (nm^2)	66 (57–82)[a]	62 (60–79)[b]	NS
Total anionic site surface (nm^2/μm GBM)	895 (195–1148)	711 (600–936)	NS
Thickness LD + LRI (nm)	208 (166–242)	162 (144–186)	< 0.01
Relative anionic site surface (%)	0.45 (0.14–0.64)	0.44 (0.37–0.53)	NS

Values are median (range)
[a] LD + LRI vs LRE, $p < 0.001$; [b] LD + LRI vs LRE, $p < 0.01$

largement of the kidneys, which are both characteristic for diabetic nephropathy (Table 1). The diabetic animals were hyperglycaemic during the entire study period as controlled by monthly blood glucose monitoring. The blood glucose values in Table 1 correspond to the blood glucose levels measured just before the animals were killed. Table 1 also shows that the diabetic rats gained less weight compared to control rats. Light microscopy demonstrated a moderate increase of the mesangial matrix in the glomeruli of most diabetic rats as described previously [32].

Analysis of the HS-associated anionic sites in the GBM. The cuprolinic blue staining procedure yielded a homogeneously-stained thin layer of anionic sites within the LRE of the GBM. These sites represent, in the diabetic rats, 74 % and in the control rats, 81 % of the total number of anionic sites within the GBM. The other anionic sites were scattered throughout the LD + LRI (Fig. 1 A, B). Figure 1 also demonstrates the three lines which indicate the epithelial and endothelial borders of the GBM and which mark the layer of anionic sites in the LRE (Fig. 1 C, D). The corresponding binary images are shown in Figure 1 (E, F). The HS-specificity of the staining was demonstrated by pretreatment of sections originating from two diabetic and two control rats with glycosaminoglycan-degrading enzymes. As can be seen in Table 2, pretreatment of the sections with heparitinase (an enzyme that specifically cleaves HS) reduced the number of anionic sites in the LRE and LD + LRI to background levels, whereas chondroitinase ABC (an enzyme that specifically cleaves chondroitin sulphate A and C and dermatan sulphate) had hardly any effect on the number of anionic sites in the LRE and LD + LRI. Preincubation of the sections with acetate or. Tris buffer alone (control buffers for the glycosaminoglycan-degrading enzymes) had no influence on the number of anionic sites in both LRE and LD + LRI (not

shown). This indicates that in both the diabetic and control rats the GBM anionic sites are HS-associated.

The results of the analysis of the anionic sites are shown in Table 3. The number of anionic sites in the LRE was significantly reduced in the diabetic rats by 19% compared to the control animals ($p < 0.02$). Since it has been suggested that the size of the anionic sites gives information about the proteoglycan structure [29], we also measured the mean anionic site surface in every individual rat. Although the mean anionic site surface in the LRE was not significantly different between both groups, there was a tendency towards smaller sites in the diabetic rats. The total anionic site surface in the LRE was reduced by 26% ($p < 0.02$), the relative anionic site surface in the LRE was significantly decreased in the diabetic rats by 27% compared to control rats ($p < 0.03$), which was not due to a difference in the thickness of the LRE. These data suggest a reduced HS-associated charge barrier within the LRE in the diabetic rats. A minority of anionic sites were found in the LD + LRI in both diabetic and control rats. Latter sites were significantly smaller than the anionic sites in the LRE ($p < 0.001$ for diabetic rats and $p < 0.01$ for control rats). Consequently, lower values compared to the LRE were found for both the total and relative anionic site surface (Table 3). From these data it can be calculated that 15 or 10% of the total anionic site surface in the GBM is located in the LD + LRI for the diabetic or control rats, respectively. This suggests, using this technique, that the HS-associated anionic sites in the LRE are the major determinants for GBM charge.

Since all diabetic animals were only analysed 8 months after diabetes induction, we did not find a correlation between the different anionic site parameters and albuminuria. This is probably due to the fact that the analysis was done only at one time point (8 months) and not also at earlier time points.

Discussion

In this study the HS-associated anionic sites in the GBM of albuminuric STZ-diabetic rats and control rats were analysed using the cationic dye cuprolinic blue. It has been documented that under critical electrolyte conditions, this dye specifically stains HS in extracellular matrix [29], as also revealed by our control experiments with diabetic and control animals using glycosaminoglycan-degrading enzymes.

We found in this STZ-induced diabetic nephropathy model and in normal rats that the majority of the HS-associated anionic sites are localized in the LRE of the GBM. This was also found by Vernier et al. [30] in the kidneys of insulin-dependent diabetic patients and by Chakrabarti et al [31] in the kidneys of diabetic BB-rats. The results show that in the LRE of diabetic rats the number of anionic sites, the total anionic site surface and the relative anionic site surface were significantly decreased compared to control rats. These differences cannot be explained by differences in the LRE width, since these were equal in both groups. These results strongly suggest a reduced HS-associated charge barrier within the LRE in experimental diabetic nephropathy. No alterations were observed for the anionic sites in the LD + LRI of the GBM. The number of anionic sites in the LD + LRI represents 26 and 19% of the total number of anionic sites in the GBM for diabetic and control rats, respectively. Furthermore, the mean surface per anionic site in the LD + LRI is significantly smaller than in the LRE. Since it has been suggested that the size of the anionic sites gives some information about the structure of the HSPG molecule [29], this analysis suggests that two types of GBM HS are present. The major population is identified as larger anionic sites in the LRE which are subject to alterations in diabetes, and a minor population, recognized as smaller anionic sites in the LD + LRI, which remain unaltered in diabetes. In a previous study [34], we suggested the existence of at least two different types of basement membrane HSPG, based on differences in glomerular staining pattern. Using a monoclonal antibody against the core protein of the large EHS-HSPG we observed strong mesangial and weak GBM staining, whereas monoclonal antibodies against the core protein of GBM HSPG yielded weak mesangial and strong GBM staining. Whether these two different HSPG populations are related to the two different HS populations as found in the present study is not known. Furthermore, no data are available whether the expression of these two different types of HSPG in diabetes are regulated separately and differently. At the same time, one should realize that histological processing can markedly influence the localization of HS-associated anionic sites in basement membranes, as shown by Chan et al. [35]. By changing the fixative from glutaraldehyde to formaldehyde the cuprolinic blue HS-associated anionic sites and the HSPG-core protein (visualized by the immunogold technique) were found to co-localize over the LD of basement membranes. This observation later led to the suggestion that the lamina lucidae/rarae of basement membranes were artefacts, and that HSPG is localized throughout the whole GBM [36]. This might indicate that the localization of HS-associated anionic sites in the LRE is due to redistribution during histological processing. Even if this is true, the total anionic site surface of all GBM anionic sites (LRE + LD + LRI) still decreased by 21% ($p < 0.02$) and the relative anionic site surface of all GBM anionic sites decreased even by 26% as a consequence of the increased thickness of the GBM ($p < 0.002$). Therefore, irrespective of eventual redistribution of the HS-associated anionic

sites due to histological processing, our results indicate a reduced HS-associated charge barrier in the GBM of long-term diabetic rats, that might be explained by a reduced number of HSPG molecules in the GBM and/or an altered structure; e.g., reduced charge of HS.

In a previous study on isolated glomeruli of diabetic rats 8 months after STZ injection, no absolute difference in GBM HS content was found between diabetic and control rats [32]. The HS measurements of that study were done on glomerular extracts with an inhibition-ELISA, using anti-HS monoclonal antibody JM-403, specific for GBM HS [9]. Combining the results of the present study with those of the study mentioned above supports the idea of alterations in the structure of GBM HS. The most likely explanation for this is a reduction of the sulphation of GBM HS in diabetic rats. Undersulphation of HS both from the GBM [24] as well as from other tissues [37, 38] in STZ rats has been reported. Recently, it has been shown that culture of mesangial cells on non-enzymatic glycated mesangial matrix or prolonged exposure to elevated glucose levels leads to the production of undersulphated proteoglycans [39, 40]. HS sulphation occurs in the Golgi-apparatus of the producing cell and is determined by the key enzyme glucosaminyl N-deacetylase [41]. A decreased activity of this enzyme has been found in STZ-diabetic rats [42–44]. Finally, at present the influence of other diabetes-related factors such as non-enzymatic glycation on the staining properties of cuprolinic blue in diabetic GBM is not known. Non-enzymatic glycation of intrinsic GBM constituents and the cross-linking of non-enzymatic glycated serum proteins to the GBM leads to an altered three-dimensional architecture of the GBM [18, 45], that might interfere with the staining of the HS-associated anionic sites. In conclusion, this study demonstrates a reduced HS charge barrier in the LRE of the GBM of rats with STZ-induced diabetes, which might explain the increased urinary albumin excretion found in these animals.

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