

Original Article

Expression of glomerular heparan sulphate domains in murine and human lupus nephritis

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Abstract

Background. Recently, we identified specific N- and 6-O-sulphated heparan sulphate (HS) domains on activated glomerular endothelial cells. In this study, we evaluated in lupus nephritis the expression of different HS domains on glomerular endothelium and in the glomerular basement membrane (GBM).

Methods. The expression of specific glomerular HS domains and the presence of immunoglobulins (Ig) were determined by immunofluorescence staining of kidney sections of patients with nephritis due to systemic lupus erythematosus (SLE) and MRL/*lpr* lupus mice. The expression/presence of glomerular HS domains and Ig was also evaluated after eluting Ig from renal sections of lupus mice using two elution methods, and in renal sections of lupus mice treated with heparinoids.

Results. Both MRL/*lpr* mice and patients with lupus nephritis showed a decreased expression of HS in the GBM. The expression of N- and 6-O-sulphated HS domains on glomerular endothelium was decreased in MRL/*lpr* mice, but increased in SLE patients. MRL/*lpr* mice had more extensive glomerular Ig deposits than SLE patients. After elution of Ig, the glomerular endothelial expression of N- and 6-O-sulphated HS domains in MRL/*lpr* mice was recovered and even increased above normal levels, while the expression of HS in the GBM was restored to normal levels. Treatment with heparinoids prevented Ig deposition and preserved the expression of glomerular HS domains at normal levels in lupus mice.

Conclusion. The expression of specific HS domains on glomerular endothelium and in the GBM is changed

during lupus nephritis due to masking by Ig deposits and induction of inflammatory N- and 6-O-sulphated HS domains.

Keywords: albuminuria; glomerular basement membrane; glomerular endothelial cells; glomerulonephritis; heparan sulphate; heparinoids; systemic lupus erythematosus

Introduction

A hallmark of systemic lupus erythematosus (SLE) is the development of anti-nuclear autoantibodies, in particular anti-chromatin autoantibodies, that include anti-dsDNA, anti-histone and nucleosome-specific antibodies [1,2]. Furthermore, nucleosomes can be detected in the circulation of SLE patients and lupus mice, which may be the result of an aberrant apoptosis and/or an insufficient clearance of apoptotic cells/debris [2]. About 50% of patients with SLE will develop glomerulonephritis, which is characterized by granular deposits of immunoglobulins (Ig) in the mesangium and along the glomerular capillary wall, and the glomerular influx of leucocytes, leading to albuminuria, haematuria and impairment of renal function [3].

Heparan sulphate proteoglycans (HSPGs) are composed of a core protein with covalently linked linear heparan sulphate (HS) carbohydrate side chains. These HS side chains can consist of up to 150 disaccharide units of glucosamine–uronic acid residues that can be sulphated at the N-position, 2-, 3- and/or 6-O-position, and epimerized at the C-5-position [4]. The possible combinations of modifications within one HS chain gives rise to an enormous structural diversity, which is important for the specific binding of a myriad of factors. HSPGs are intrinsic constituents of the extracellular matrix, like the glomerular basement

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membrane (GBM), and responsible for the majority of anionic sites in the GBM [5]. It is assumed that the negatively charged HS in the GBM is important for the charge-selective filtration [5,6], although recently its contribution has been questioned [7]. Furthermore, HSPGs are expressed at the cell surface of virtually all cells and are involved in many biological processes, which are often mediated by the binding of relevant factors via their HS side chain. HS expressed at cell surfaces plays an important role during inflammatory processes by binding to chemokines, cytokines, selectins and integrins [8,9]. Several studies demonstrated that in particular N- and 6-O-sulphated HS domains on activated endothelial cells may serve as ligands for L-selectin and Mac-1, which are expressed on leucocytes [4,8–10].

Based on previous work, we hypothesized that in SLE, binding of anti-dsDNA and nucleosome-specific antibodies to nucleosomes in the circulation leads to neutralization of negative charges on the nucleosome, thus making it overall more positive [11]. This masking of negative charge in the nucleosome leads to an enhanced binding/deposition of the anti-nucleosome/nucleosome immune complexes to the negatively charged HS in the GBM mediated by the positively charged N-terminal histone tails within the nucleosome [11–13]. Recently, it was demonstrated with EM co-localization studies that deposited Ig is bound to extracellular chromatin bound to the GBM [14]. The amount of Ig deposits along the capillary loops and the degree of albuminuria correlated significantly with a decreased expression of HS in the GBM, while the expression of the HSPG core protein agrin remained unaltered [15]. Previously, we showed that treatment of MRL/*lpr* lupus mice with heparin or low molecular weight (LMW) heparinoids prevented albuminuria, and specifically, the deposition of nephritogenic immune complexes in the GBM. Most likely, the administered heparinoids bound to the anti-nucleosome/nucleosome immune complexes, thus preventing the binding to HS, and thereby, the deposition of these immune complexes in the glomerular capillary wall [16].

In the current study, we evaluated the expression of different HS domains either in the GBM or on

glomerular endothelial cells in murine and human lupus nephritis. The glomerular expression/presence of specific HS domains and Ig was also evaluated after elution of Ig from kidney sections of lupus mice following two methods, and in kidney sections of lupus mice treated with heparinoids.

Methods

Animals

Kidney sections were obtained as previously described from 18-week-old MRL/*lpr* mice, which were divided into three groups depending on their degree of albuminuria: 'no albuminuria' (NA, albustix <300 µg/ml), 'short albuminuria' (SA, albustix >1000 µg/ml, albuminuria for a period shorter than 7 days) and 'long albuminuria' (LA, albustix >1000 µg/ml, albuminuria for a period of 14–21 days) [15]. Kidney sections of age-matched MRL *+/+* mice were included as a control. At this stage these mice had no renal abnormalities and no albuminuria. Kidney sections of 21-week-old MRL/*lpr* mice, which were treated daily with LMW heparin or phosphate buffered saline (PBS) from week 8 until week 21, were obtained as described previously [16]. The local Animal Ethics Committee of the Radboud University Nijmegen Medical Centre approved all animal experiments.

SLE patients

Kidney biopsies from SLE patients with proliferative lupus nephritis (ISN/RPS class III or IV) (Table 1) were collected at the Division of Nephrology, Radboud University Nijmegen Medical Centre, which was approved by the local Human Ethics Committee of the Radboud University Nijmegen Medical Centre.

Immunofluorescence staining

Indirect immunofluorescence stainings were performed on 2 µm cryostat renal sections. Sections were fixed in ice-cold acetone for 10 min and incubated with primary antibodies diluted in PBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 0.05% sodium azide (IF-buffer) for 45 min at room temperature. The expression of HS in the GBM was evaluated by staining with the anti-HS mouse monoclonal

Table 1. Clinical characteristics of SLE patients

Patient no	Sex	Age	Duration of disease (months)	Serum creatinine (µmol/l)	Urinary protein (g/24 h)	Classification lupus nephritis (ISN/RPS 2003)	C3 (g/l)	C4 (g/l)	Anti-dsDNA (U/ml)
1	F	37	18	100	2.7	IV-S (A/C)	1.10	0.17	350
2	F	42	6	120	2.1	IV-S (A/C)	0.95	0.20	250
3	F	36	120	142	6.4	IV-G (A)	0.35	0.09	59
4	F	18	24	71	2.2	III (A/C) + V	0.62	0.12	600
5	F	21	2	172	15	IV-G (A/C)	0.46	0.13	17
6	F	20	2	65	2.8	III (A/C) + V	0.80	0.15	79
7	M	22	1	54	2.0	III (A/C)	0.27	0.02	847
8	F	43	0	66	2.4	III (A) + V	0.37	0.02	577
9	F	18	0	64	0.4	III (A)	0.49	0.09	706
10	M	26	51	90	0.7	III (A/C)	0.37	0.04	212

antibody JM403 (0.1 µg/ml), which recognizes HS in the GBM [17]. The glomerular endothelial expression of N- and 6-O-sulphated HS domains was evaluated by double staining with the VSV-tagged single chain anti-HS antibodies AO4B08, EW3D10 and EW4G2, recognizing N- and 6-O-sulphated HS domains (10–50 µg/ml) [18,19] and a rat anti-mouse CD31 (PECAM-1) monoclonal antibody (0.1 µg/ml, BD Biosciences, Alphen aan den Rijn, The Netherlands). To prevent non-specific mouse Ig staining on mouse renal sections, the mouse-on-mouse kit (Brunschwig Chemie, Amsterdam, The Netherlands) was used according to the manufacturer's protocol. Sections were washed in PBS and for detection incubated with the appropriate Alexa 488-conjugated secondary antibodies (5 µg/ml, Molecular Probes, Leiden, The Netherlands) or anti-VSV-Cy3 (2 µg/ml, Sigma-Aldrich Chemie) in IF-buffer for 45 min at room temperature. The presence of glomerular Ig was evaluated by incubation with the appropriate Alexa 488-conjugated anti-Ig antibodies (5 µg/ml, Molecular Probes). Subsequently, sections were washed in PBS, post-fixed with 1% paraformaldehyde in PBS, washed in PBS, and embedded in VectaShield mounting medium H-1000 (Brunschwig Chemie). The slides were evaluated by fluorescence microscopy (Leica DM4000B fluorescence microscope, Leica Microsystems B.V., Rijswijk, The Netherlands) and photographed with the Leica DFC300 FX digital camera (Leica Microsystems). The staining intensities of 25 glomeruli were scored on a scale between 0 and 10 on blinded sections by two independent observers. The *r* values between the two observers ranged from 0.8–0.95. Six mice were analysed in each group.

Elution of Ig deposits

To study whether Ig could be eluted and HS staining could be subsequently restored, we tested two elution procedures on renal sections of MRL/*lpr* mice. Method I: Non-fixed 2 µm cryostat sections were submerged in 220 ml solution of 0.15 M glycine with 5 M urea, pH 2.8, of 4°C for exactly 2 h, and subsequently, extensively washed in PBS at 20°C. Method II: Non-fixed 2 µm cryostat sections were submerged in 220 ml solution of 0.15 M glycine with 5 M urea, pH 2.8, of 20°C with a subsequent cooling-down at 4°C for exactly 2 h and followed by extensive washing in PBS at 20°C. Kidney sections of MRL +/+ mice were also subjected to the same elution procedures and used as controls to correct for Ig-independent effects of the elution procedures on the integrity of the HS domains as probed by the anti-HS antibodies. For each anti-HS antibody, the ratio was calculated by dividing the expression of untreated sections of MRL +/+ mice by the expression of MRL +/+ sections that were eluted. To evaluate the effect of Ig on the elution procedure, renal sections of normal C57/BL6 mice loaded with excessive amounts of purified rabbit anti-mouse GBM IgG were subjected to elution procedure II, as described above. Staining of anti-HS antibodies was compared with sections that were not subjected to elution procedure II.

Statistical analysis

Values are expressed as means ± SEM and significance was determined by Mann-Whitney U testing using GraphPad Prism, version 4.0 software (GraphPad Software, Inc.,

San Diego, CA). *P* values <0.05 were considered as significant.

Results

*The expression of HS domains in the GBM and on glomerular endothelial cells is decreased in MRL/*lpr* mice with albuminuria*

We evaluated the expression of HS in the GBM and on glomerular endothelial cells of 18-week-old MRL/*lpr* lupus mice with no albuminuria (NA), short albuminuria (SA) or long albuminuria (LA), and in age-matched MRL +/+ controls. Probing with the anti-HS antibody JM403, which recognizes N-unsubstituted HS domains especially in the GBM, revealed a significant decrease of HS in the GBM of the LA group compared with the MRL +/+ control group, while HS expression in the GBM of the NA and SA groups was similar to the MRL +/+ group, although there was a trend to a lower expression in the SA group (Figure 1A–E) [15]. Double staining with an anti-CD31 antibody and the anti-HS antibodies AO4B08, EW3D10 and EW4G2 [18,19], which recognize N- and 6-O-sulphated HS domains, revealed a decreased glomerular endothelial expression of the N- and 6-O-sulphated HS domains in the LA and SA groups compared with the MRL +/+ group, while the expression in the NA group was unaffected (Figure 2A–C). As will be discussed, we have evidence that these N- and 6-O-sulphated HS domains are associated with activated glomerular endothelial cells, and thus can be considered as inflammatory HS domains. We also evaluated glomerular Ig deposits, which revealed an increased presence of Ig along the capillary wall in both the SA and LA group compared with the MRL +/+ group, while the Ig deposition in the NA group was similar to the MRL +/+ group (Figure 2D). Our data suggest that Ig may mask specific HS domains in both the GBM and on glomerular endothelial cells.

*Masking of HS domains in the GBM and on glomerular endothelial cells by Ig in MRL/*lpr* mice with albuminuria*

Previously, we obtained indirect evidence that in SLE, HS in the GBM was masked by Ig deposits [11–13,15]. However, at that time we could not unmask the HS domain in the GBM that is recognized by the antibody JM403 by eluting Ig from renal sections. Most likely, the elution procedure led to destruction of the HS domain [15]. To remove Ig, we originally incubated renal sections of LA MRL/*lpr* mice in 0.15 M glycine with 5 M urea, pH 2.8, with a starting temperature of 4°C for exactly 2 h at 4°C ('Ig elution method I'), which indeed resulted in removal of Ig, as previously found [15], but not in the full reappearance of HS along the capillary wall, i.e. in the GBM as measured by JM403 and on glomerular endothelial cells as measured

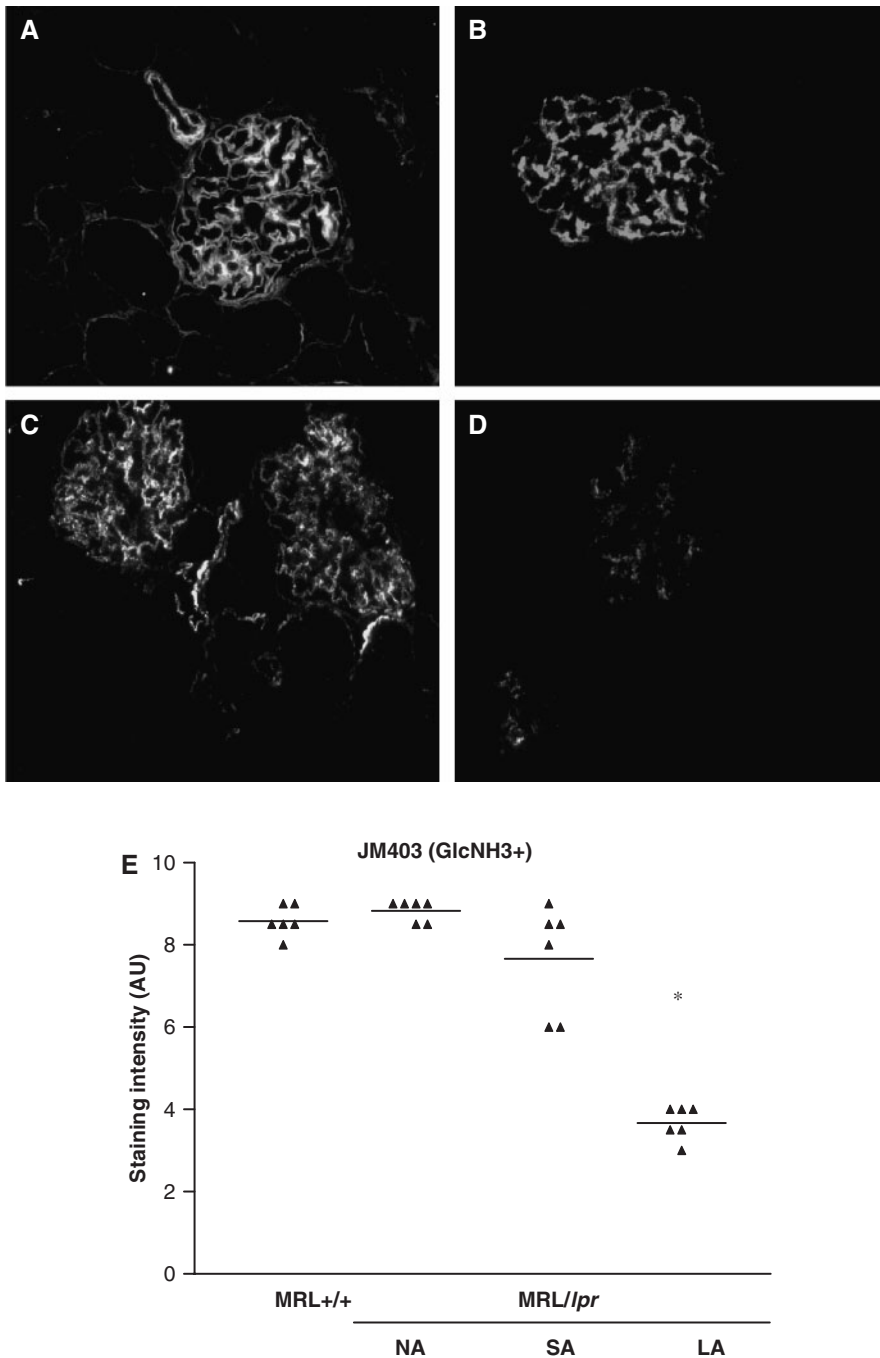


Fig. 1. Expression of heparan sulphate in the glomerular basement membrane of lupus and control mice. The expression of HS in the GBM of MRL +/+ control mice (A), MRL/*lpr* lupus mice with no albuminuria (B), short (C) and long albuminuria (D) was determined by probing with the monoclonal anti-HS antibody JM403. Staining intensity along the capillary wall was scored semi-quantitatively. Magnification 400 \times . The expression of HS in the GBM was significantly decreased in MRL/*lpr* mice with long albuminuria (E). * $P < 0.05$ compared with MRL +/+ control mice. GlcNH₃⁺, N-unsubstituted glucosamine; NA, no albuminuria; SA, short albuminuria; LA, long albuminuria.

by EW3D10 (Figure 3A and B). In the current study, we developed a slightly modified Ig elution procedure, in which we incubated renal sections of MRL +/+ mice and MRL/*lpr* mice in 0.15 M glycine with 5 M urea, pH 2.8, with a starting temperature of 20 $^{\circ}$ C followed by a cooling-down period of exactly 2 h at 4 $^{\circ}$ C ('Ig elution method II'). The use of Ig elution method II, revealed also the removal of Ig along the

capillary wall from sections of SA and LA MRL/*lpr* mice down to the levels present in MRL +/+ and NA MRL/*lpr* mice. Moreover, by using elution method II, we were now able to recover (partially) the staining for HS in the GBM as measured by JM403 and on glomerular endothelial cells as measured by EW3D10, while elution method I hardly recovered glomerular HS expression (Figure 3A and B). Next, we estimated

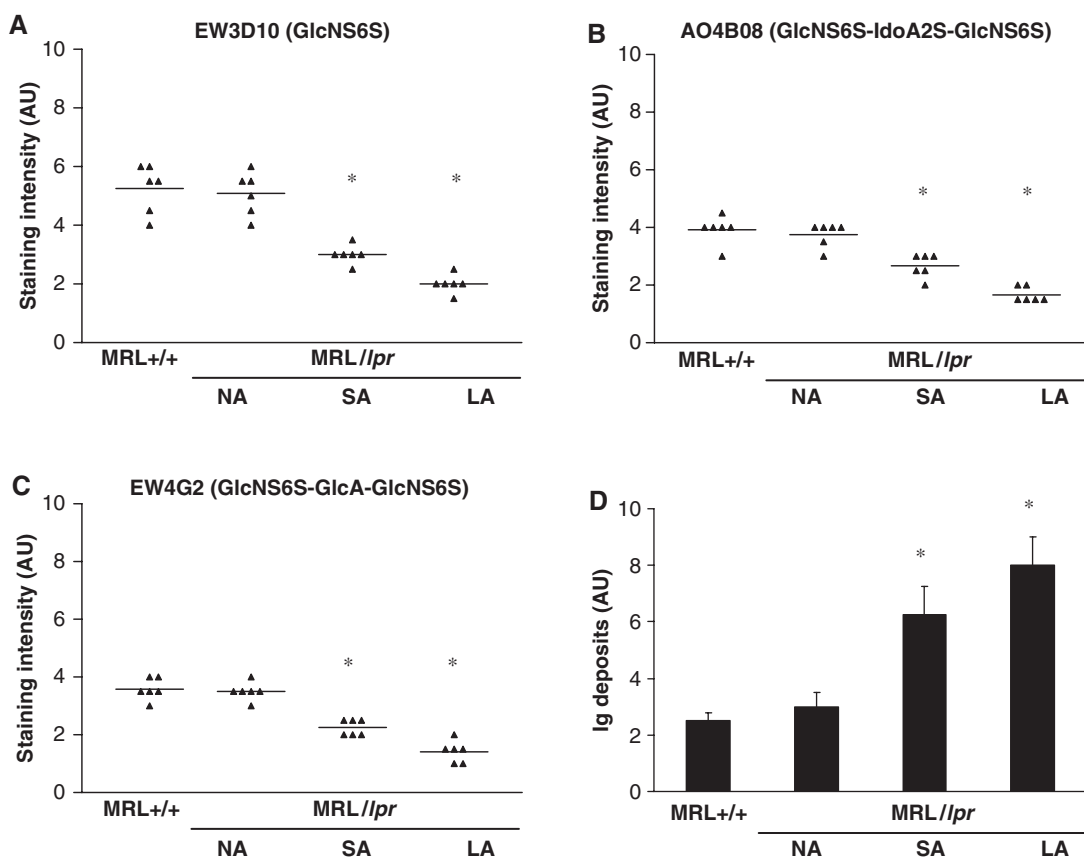


Fig. 2. Expression of N- and 6-O-sulphated heparan sulphate domains on glomerular endothelial cells and Ig deposits along the capillary wall in lupus and control mice. The glomerular endothelial expression of N- and 6-O-sulphated HS domains in MRL +/+ mice and MRL/lpr lupus mice with no albuminuria, short and long albuminuria was determined by double staining for CD31 and N- and 6-O-sulphated HS domains with the respective anti-HS single chain antibodies EW3D10 (A), AO4B08 (B) and EW4G2 (C). The epitopes of the single chain antibodies are given within parentheses. Co-staining intensities were scored semi-quantitatively and revealed a significantly decreased expression of N- and 6-O sulphated HS domains in MRL/lpr lupus mice with short and long albuminuria (A-C). Ig deposition along the capillary wall was significantly increased in MRL/lpr mice with short and long albuminuria (D). * $P < 0.05$ compared with MRL +/+ control mice. GlcNS, N-sulphated-glucosamine; S, sulphate; IdoA, iduronate; GlcA, glucuronate; NA, no albuminuria; SA, short albuminuria; LA, long albuminuria.

the direct effect of the Ig elution method I and II on the integrity of HS domains by subjecting sections of control MRL +/+ mice to elution method I and II followed by staining with the various anti-HS antibodies. This revealed that the staining by the anti-HS antibody JM403 was rather insensitive to the newly developed Ig elution method II, while the staining by the anti-HS antibodies EW3D10, AO4B08 and EW4G2 decreased about 1.5-fold after elution method II (Figure 4A and B). Knowing this, we subjected sections of LA MRL/lpr mice to the Ig elution method II, followed by staining with the anti-HS antibodies JM403 (Figure 5A), EW3D10, AO4B08 and EW4G2 (Figure 5B–D), which revealed a nearly complete recovery of expression of HS domains in LA MRL/lpr mice compared with MRL +/+ mice. However, when we corrected the staining intensities of the anti-HS antibodies for the direct effect of the elution method on the binding of the anti-HS antibodies (Figure 4B), we noticed a complete recovery of HS expression in the GBM as measured by JM403, while the expression of inflammatory N- and

6-O-sulphated HS domains on glomerular endothelial cells as measured by EW3D10, AO4B08 and EW4G2, respectively, was about 1.5-fold higher than in MRL +/+ mice (Figure 5A–D).

One could argue that Ig plays a possible protective role on the effects of elution procedure II on the integrity of the HS domains on renal sections with lupus nephritis. This could raise doubts whether the ‘correction factor’ for the effect of the elution method on the integrity of HS domains as determined on MRL +/+ kidney sections is applicable for lupus sections. Therefore, we have performed immunofluorescence stainings with the anti-HS antibodies after Ig elution method II on mouse renal sections loaded with excessive amounts of rabbit anti-GBM IgG, which mimics the glomerular Ig deposition in lupus nephritis. It appeared that the effect of the elution procedure on the integrity of the HS domains on these sections loaded with excessive amounts of rabbit anti-GBM IgG was similar, i.e. the correction factor was ~1.5-fold, to the effect of the elution procedure on renal sections of MRL +/+ mice, in the presence of

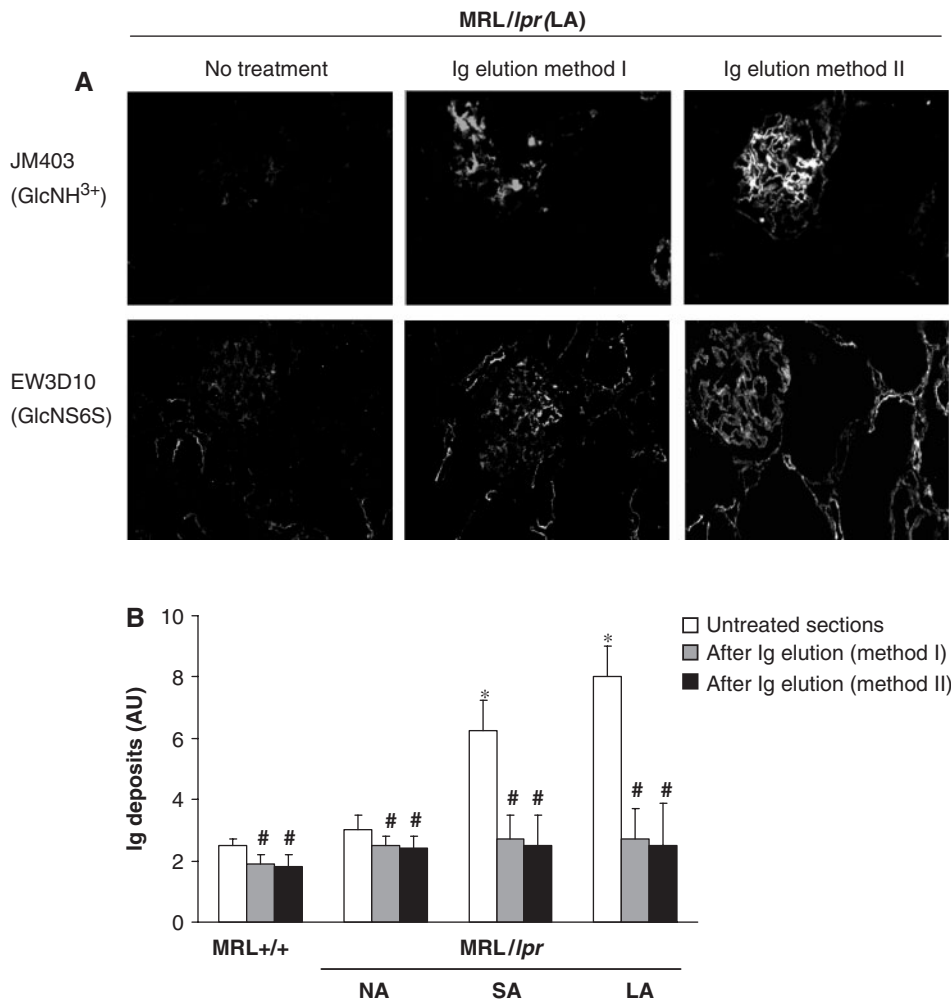


Fig. 3. Elution of Ig from renal sections of lupus mice unmasks the expression of glomerular heparan sulphate domains. Ig was eluted from renal sections of MRL/lpr mice with long albuminuria by two different methods as detailed in the Methods section and in the text (A). Both Ig elution method I and II lead to the removal of Ig (B), but only Ig elution method II leads to reappearance of HS in the GBM, as measured by staining with JM403, and on glomerular endothelial cells, as measured by staining with the anti-HS antibody EW3D10 (A). * $P < 0.05$ compared with MRL +/+ control mice. # $P < 0.05$ compared with untreated sections. Magnification 400 \times . GlcNH³⁺, N-unsubstituted glucosamine; GlcNS, N-sulphated-glucosamine; S, sulphate; NA, no albuminuria; SA, short albuminuria; LA, long albuminuria.

low amounts of Ig (data not shown). In summary, Ig masks specific HS domains in the GBM and on glomerular endothelial cells in MRL/lpr mice with albuminuria. Moreover, elution of Ig revealed an increased expression of the inflammatory N- and 6-O sulphated HS domains on glomerular endothelial cells.

Decreased expression of HS in the GBM and increased expression of inflammatory N- and 6-O-sulphated HS domains on glomerular endothelial cells in SLE patients

Analogous to the staining performed in murine lupus nephritis, we stained renal sections of SLE patients with proliferative lupus nephritis ($n = 10$; Table 1) and healthy controls ($n = 10$) with JM403, EW3D10, AO4B08 and EW4G2. As previously demonstrated [11], the expression of HS in the GBM, as measured with JM403, was decreased in SLE patients compared

with healthy controls (Figures 6A and 7A). However, and in contrast to mice with lupus nephritis, the expression of inflammatory N- and 6-O-sulphated HS domains on glomerular endothelial cells as measured with EW3D10, AO4B08 and EW4G2, and co-stained with anti-CD31, was significantly increased in SLE patients compared with healthy controls (Figures 6B and 7B–D). Evaluation of Ig deposits along the capillary wall revealed less abundant Ig deposition in SLE patients compared with mice with lupus nephritis (compare Figure 7E and Figure 2D). Four human renal biopsies were subjected to Ig elution method II, followed by staining with the anti-HS antibody JM03, which revealed a nearly complete recovery of HS expression in the GBM (data not shown). This suggests that the masking of HS in the GBM by Ig in SLE patients is comparable to that in lupus nephritis mice, while the increased expression of inflammatory N- and 6-O-sulphated HS domains

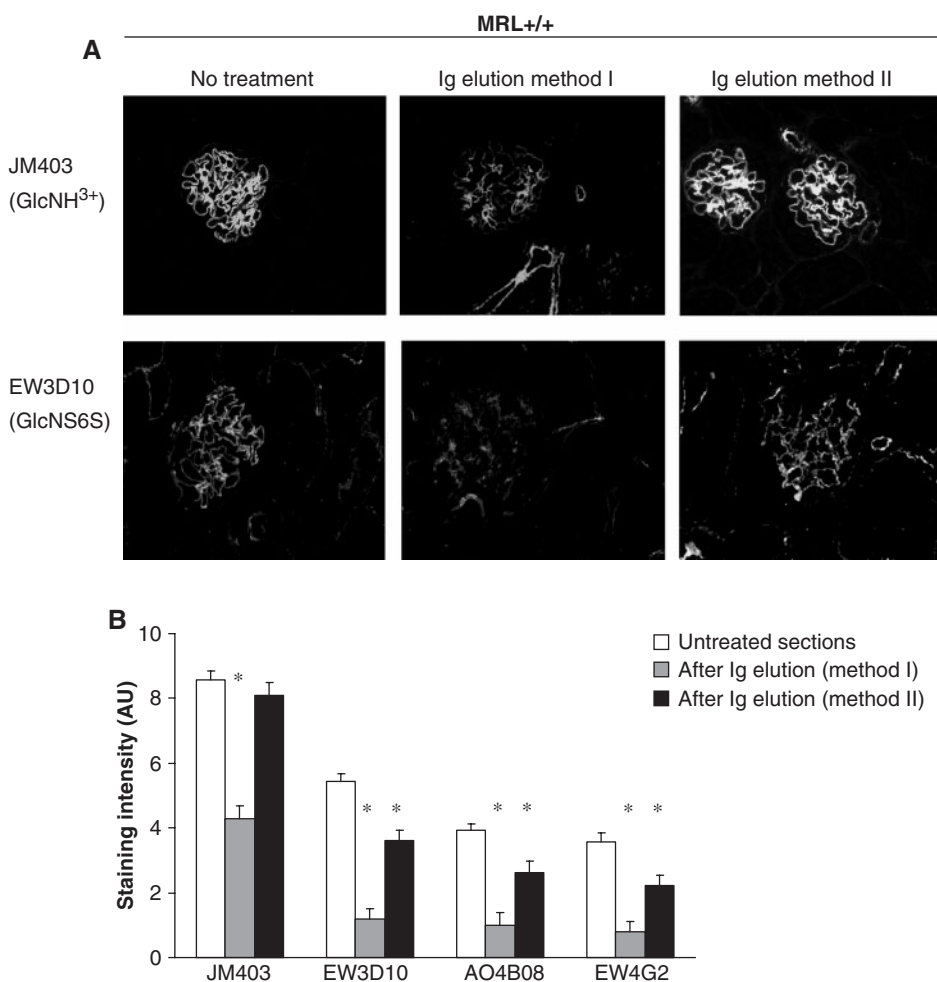


Fig. 4. Effect of the Ig elution procedure on the integrity of heparan sulphate domains in the GBM and on glomerular endothelial cells. This effect was determined by subjecting renal sections of MRL $+/+$ control mice to the Ig elution procedures (methods I and II) followed by staining with the indicated anti-HS antibodies. Representative photographs of renal sections before and after Ig elution probed with the anti-HS antibodies JM403 and EW3D10, respectively, are shown. Magnification $400\times$ (A). Semi-quantitative scoring of the staining intensities of the anti-HS antibodies JM403, EW3D10, AO4B08 and EW4G2 revealed that the epitope of JM403 is slightly affected, while the epitopes of EW3D10, AO4B08 and EW4G2 are significantly affected by the Ig elution method II (B). * $P < 0.05$ compared with untreated sections. GlcNH³⁺, N-unsubstituted glucosamine; GlcNS, N-sulphated-glucosamine.

on glomerular endothelial cells is not masked by Ig in SLE patients to such a degree as in lupus mice.

Treatment of MRL/lpr mice with LMW heparin prevents changes in the expression of HS domains in the GBM and on glomerular endothelial cells

Previously, we showed that treatment of MRL/lpr mice with LMW heparin prevents the deposition of nucleosome/anti-nucleosome immune complexes along the capillary wall and the development of albuminuria [16]. Indeed, MRL/lpr mice treated with LMW heparin showed an almost normal expression of HS in the GBM (Figure 8A), whereas the expression of the inflammatory N- and 6-O-sulphated HS domains on glomerular endothelial cells was preserved (Figure 8B–D). This preserved HS expression in the GBM and on glomerular endothelial cells was associated with a significant decrease in Ig deposits along the capillary wall compared with PBS-treated MRL/lpr

mice (Figure 8E). Notably, treatment with LMW heparin not only prevented Ig deposition and masking of HS domains, but also prevented the increased expression of the inflammatory N- and 6-O-sulphated HS domains on glomerular endothelial cells as observed in untreated MRL/lpr mice (Figure 5B–D) and SLE patients (Figure 7B–D). In summary, treatment of murine lupus nephritis with LMW heparin prevents Ig deposition along the capillary wall, is associated with a normal expression of HS in the GBM and prevents inflammation-induced upregulation of the N- and 6-O-sulphated HS domains on glomerular endothelium.

Discussion

Previously, we found indirect evidence that in lupus nephritis, HS in the GBM was masked by Ig, which leads to the reduction of functional anionic sites

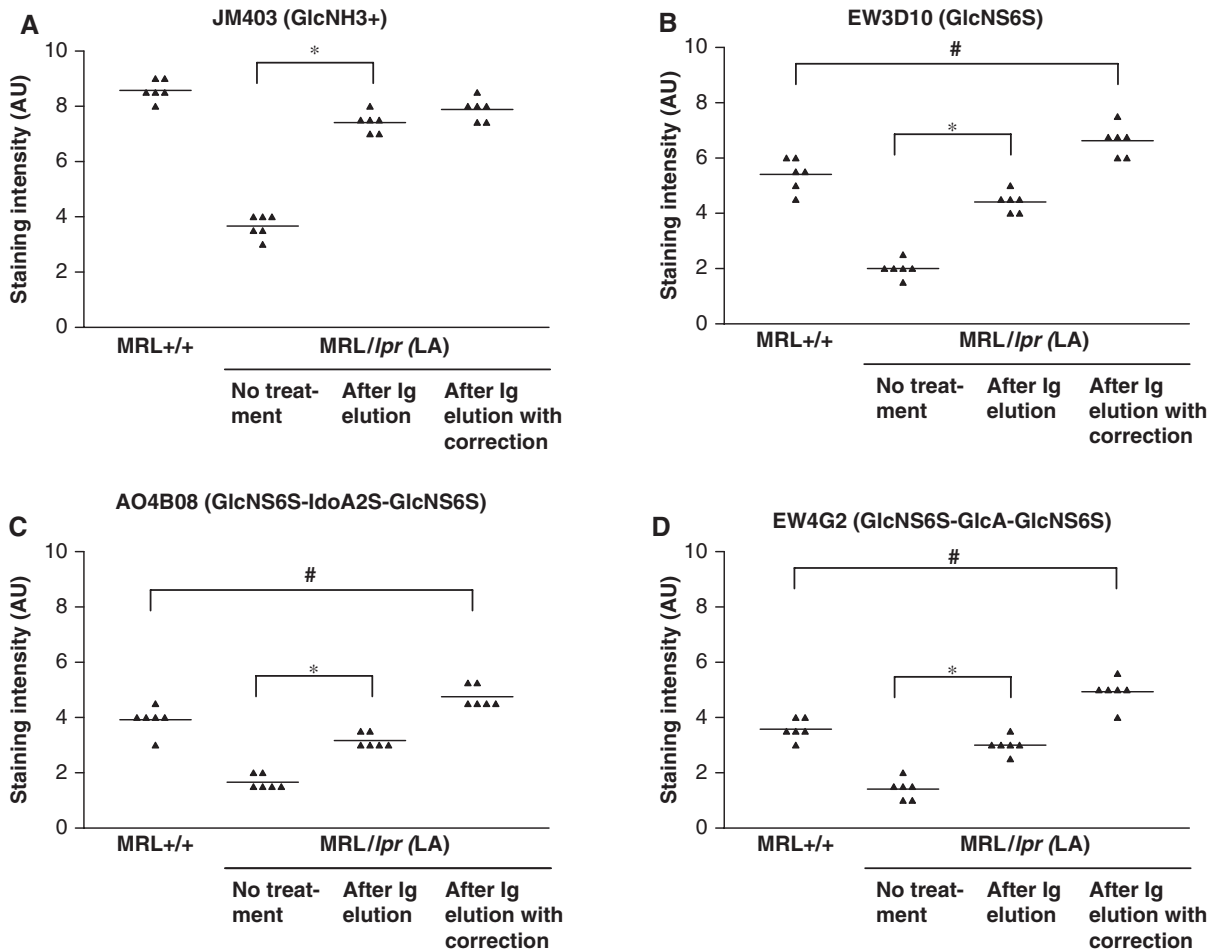


Fig. 5. Elution of Ig from renal sections of MRL/lpr mice unmasks heparan sulphate domains in the GBM and on glomerular endothelial cells. Elution of Ig (method II) from renal sections of MRL/lpr mice with long albuminuria unmasked the expression of HS in the GBM, as measured by staining with the anti-HS antibody JM403 (A), and the expression of N- and 6-O-sulphated HS domains on glomerular endothelial cells, as measured by staining with the respective anti-HS antibodies EW3D10 (B), AO4B08 (C) and EW4G2 (D). Correction of staining intensities for the effect of the Ig elution procedure on the epitopes of the anti-HS antibodies (Figure 4), revealed that the expression of the N- and 6-O-sulphated HS domains on glomerular endothelial cells was higher than in MRL +/+ control mice, while the HS expression in the GBM was similar to MRL +/+ control mice. * $P < 0.05$ compared with untreated sections. # $P < 0.05$ compared with MRL +/+ control mice. GlcNH³⁺, N-unsubstituted glucosamine; GlcNS, N-sulphated-glucosamine; S, sulphate; IdoA, iduronate; GlcA, glucuronate; LA, long albuminuria.

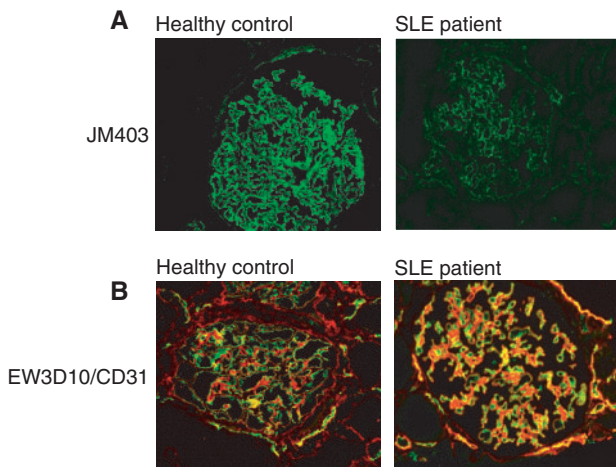


Fig. 6. Expression of heparan sulphate domains in the GBM and on glomerular endothelial cells in a patient with SLE nephritis

in the GBM and is associated with the development of albuminuria [15]. We also showed that the amount of Ig deposited along the capillary wall was significantly correlated with the degree of albuminuria. However, we never could prove unequivocally that Ig masked HS in the GBM, since elution of Ig from renal sections did remove Ig, but also affected the integrity of the HS domain in the GBM as evaluated by the monoclonal antibody JM403 [15]. In the current study, we developed a modified Ig elution method that had a less detrimental effect on the integrity of HS domains

and in a healthy control. Representative photographs showing the decreased expression of HS in the GBM (A) and the slightly increased expression of N- and 6-O-sulphated HS domains on glomerular endothelial cells (B) of a SLE patient and a healthy control, as measured by staining with the anti-HS antibody JM403 (A) or co-staining with anti-CD31 (green) and the anti-HS antibody EW3D10 (red) (B). Magnification 400 \times .

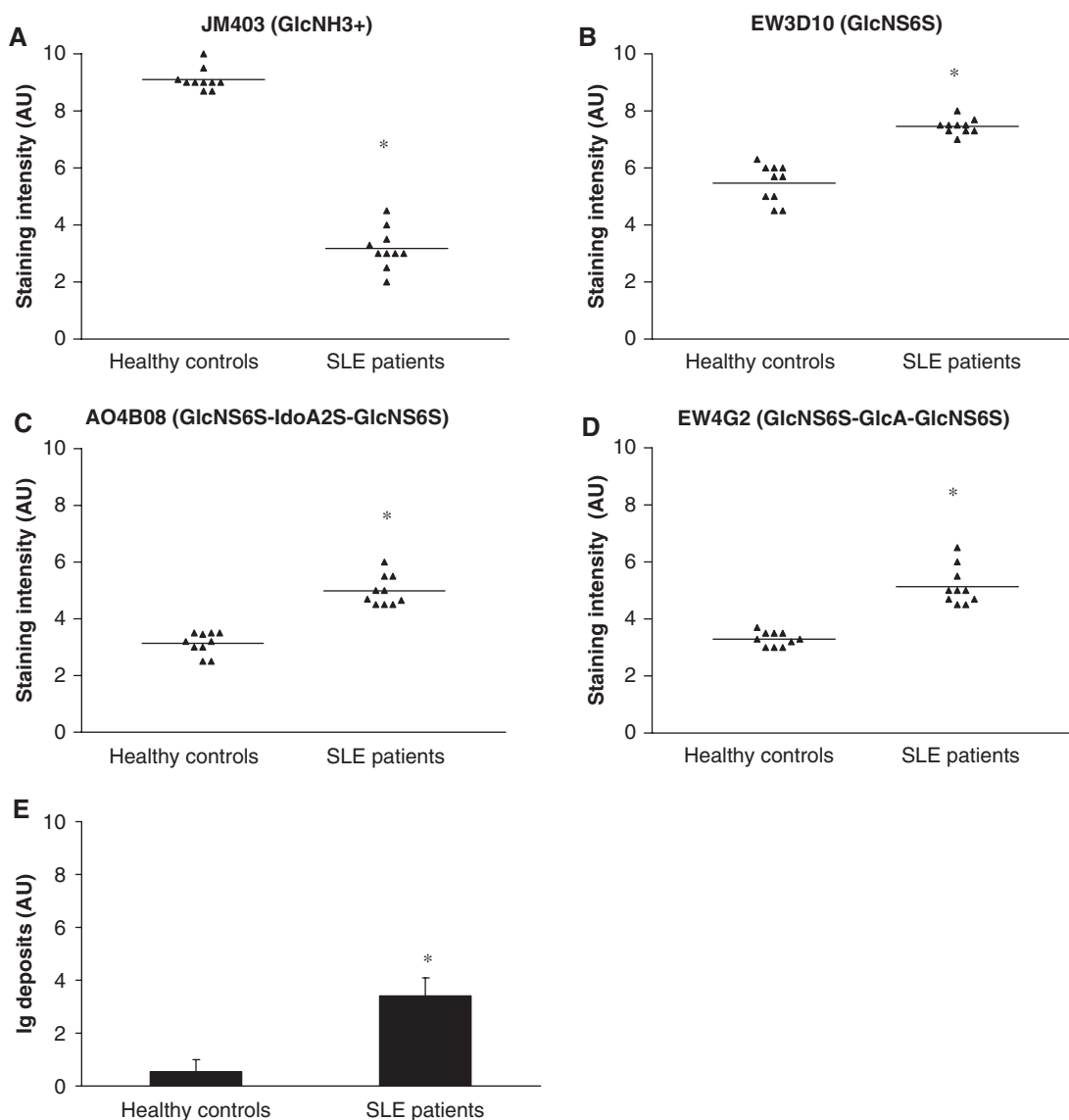


Fig. 7. Analysis of the expression of heparan sulphate domains in the GBM and on glomerular endothelial cells in SLE patients and healthy controls. The expression of HS in the GBM, as measured by staining with the anti-HS antibody JM403, was significantly decreased in SLE patients (A). The expression of N- and 6-O-sulphated HS domains on glomerular endothelial cells, as measured by co-staining with anti-CD31 and the respective anti-HS antibodies EW3D10 (B), AO4B08 (C) and EW4G2 (D), was significantly increased. Ig deposits along the capillary wall were also significantly increased in SLE patients (E). * $P < 0.01$ compared with healthy controls. GlcNH₃⁺, N-unsubstituted glucosamine; GlcNS, N-sulphated-glucosamine; S, sulphate; IdoA, iduronate; GlcA, glucuronate.

as measured by anti-HS antibodies. Using this Ig elution method, we now clearly could demonstrate that in murine lupus nephritis, HS domains in the GBM and on glomerular endothelial cells are indeed masked by deposited Ig. This is also the case for expression of HS in the GBM in SLE patients (data not shown), which indicates that in lupus nephritis HS in the GBM is masked by Ig. Elution of Ig from renal sections of mice with lupus nephritis also revealed the unmasking of specific N- and 6-O-sulphated HS domains on glomerular endothelial cells as measured by specific anti-HS antibodies. Actually, when we corrected for the effect of the Ig elution procedure on the integrity of these HS domains, the glomerular endothelial

expression of these N- and 6-O sulphated HS domains was even higher than in control mice. We have ample evidence that these N- and 6-O-sulphated HS domains can be considered as inflammatory HS domains on glomerular endothelial cells [20]. First, the expression of these N- and 6-O-sulphated HS domains by glomerular endothelial cells *in vitro* is increased after activation with proinflammatory cytokines like tumour necrosis factor (TNF)- α and interleukin (IL)-1 β . Second, these N- and 6-O-sulphated HS domains play a crucial role in the adhesion of granulocytes to glomerular endothelial cells as measured in a static *in vitro* adhesion system. Third, the glomerular endothelial expression of these N- and 6-O-sulphated

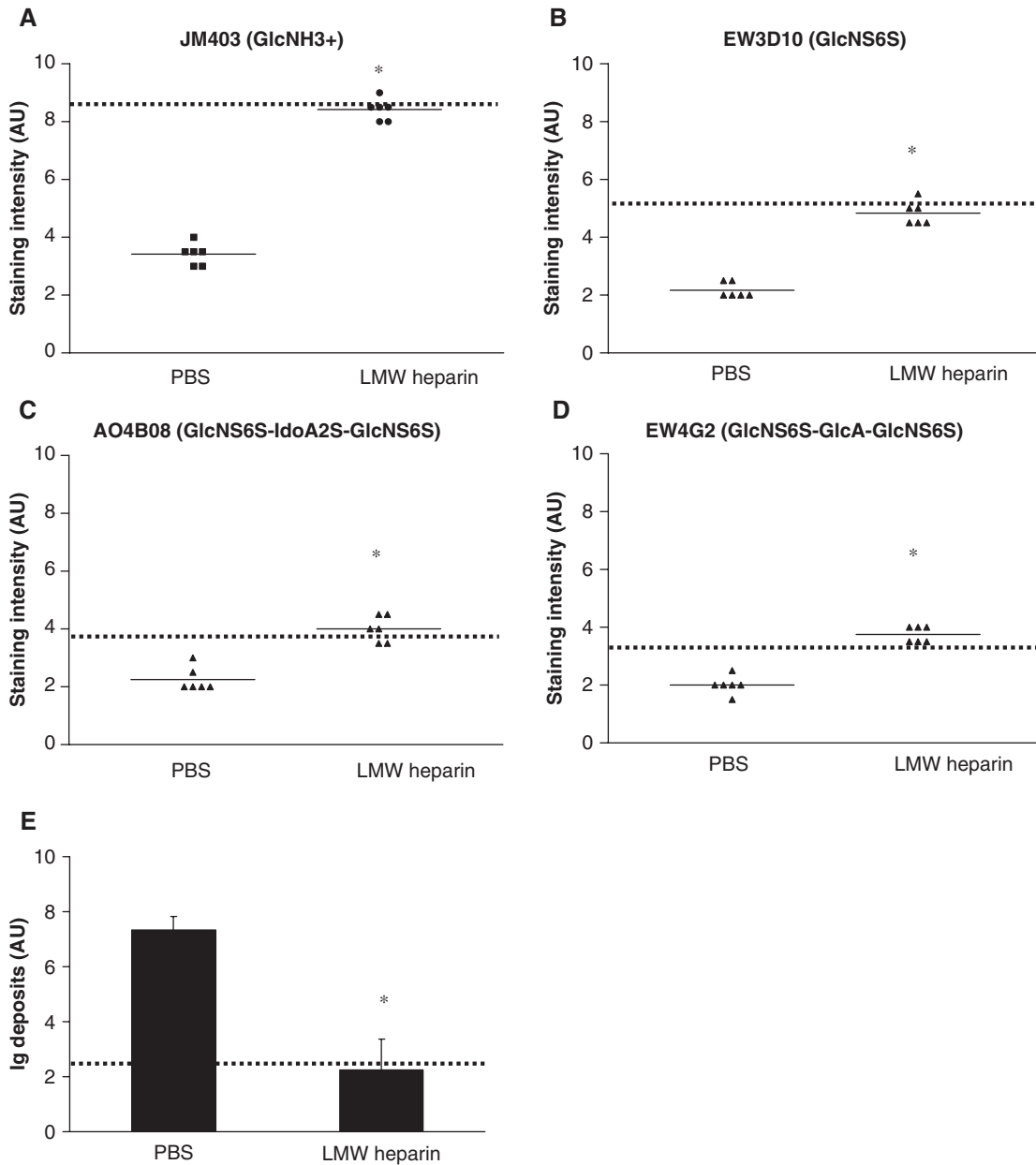


Fig. 8. The expression of glomerular heparan sulphate domains is preserved in lupus mice treated with low molecular weight (LMW) heparin. The expression of HS in the GBM, as measured by staining with JM403 (A), and the expression of N- and 6-O-sulphated HS domains on glomerular endothelial cells, as measured by co-staining with anti-CD31 and the respective anti-HS antibodies EW3D10 (B), AO4B08 (C) and EW4G2 (D), was preserved in MRL/*lpr* mice treated with LMW heparin and similar to that in MRL *+/+* control mice (dotted line). The amount of Ig along the capillary wall in MRL/*lpr* mice treated with LMW heparin was the same as in MRL *+/+* control mice (dotted line). (E), * $P < 0.05$ compared with MRL/*lpr* mice treated with PBS. GlcNH₃⁺, N-unsubstituted glucosamine; GlcNS, N-sulphated-glucosamine; S, sulphate; IdoA, iduronate; GlcA, glucuronate.

HS domains is also increased during the acute phase of experimental anti-GBM glomerulonephritis [21]. In the current study, we also could show activation of glomerular endothelial cells in lupus mice with albuminuria by measuring an increased glomerular endothelial expression of intercellular adhesion molecule (ICAM)-1 (data not shown), which also has been described by others [22]. Interestingly, without Ig elution we already could observe an increased

glomerular endothelial expression of the inflammatory N- and 6-O-sulphated HS domains in SLE patients. Apparently, there was less deposited Ig in the renal sections of the SLE patients when compared with lupus mice, which could explain that there was a lower degree or even absence of masking of glomerular endothelial HS domains in SLE patients. On the other hand, although the amount of Ig deposited along the capillary wall in SLE patients was lower compared

with lupus mice, the reduction in expression of HS in the GBM was similar to that in lupus mice. This may be explained by the preferential deposition in the GBM of the nucleosome/anti-nucleosome immune complexes [14]. In summary, in both murine lupus nephritis and in patients with proliferative SLE nephritis we could show an increased glomerular endothelial expression of inflammatory N- and 6-O-sulphated HS domains.

Recently, we have shown that the HS-specific $\beta(1-4)$ -endoglucuronidase enzyme heparanase (HPSE) that cleaves HS at specific sites [23,24] may be involved in the pathogenesis of proteinuria in overt diabetic nephropathy by degradation of HS in the GBM [25]. In the current study, we also evaluated the glomerular HPSE expression, which appeared to be slightly, but not significantly, increased in SLE patients with proliferative lupus nephritis compared with healthy controls (data not shown). Since we previously showed a normal content of glomerular HS in MRL/lpr mice, irrespective of the degree of proteinuria [15], we suggest that the increased N- and 6-O-sulphated HS expression by glomerular endothelium is due to an altered modification process of HS during lupus nephritis.

Previously, we have shown that treatment of pre-diseased lupus mice with LMW heparin prevented the binding of immune complexes to the GBM and delayed the development of lupus nephritis [16]. In addition, we now could show that this treatment prevents the increase in expression of N- and 6-O-sulphated HS domains. The anti-inflammatory effect of treatment with LMW heparin, i.e. the prevention of the increased glomerular endothelial expression of the inflammatory N- and 6-O-sulphated HS domains, may be the result of both a reduced deposition of immune complexes and a reduced glomerular influx of leucocytes.

In conclusion, the expression of specific HS domains in the GBM and on glomerular endothelial cells is changed during lupus nephritis. These changes are due to both masking by Ig and by inflammatory stimuli, while treatment with heparinoids preserves the expression of HS domains in the glomerulus by preventing both processes.

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Conflict of interest statement. None declared.

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