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Decrease of Heparan Sulfate Staining in the Glomerular Basement Membrane in Murine Lupus Nephritis

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Recently we found in biopsies of human lupus nephritis a nearly complete loss of heparan sulfate (HS) staining in the glomerular basement membrane (GBM). To clarify the relationship between HS staining and albuminuria in lupus nephritis, we studied MRL/lpr mice with short (<7 days) or prolonged duration of albuminuria (14–21 days) and compared these with mice of different ages without albuminuria. Kidney sections were stained for mouse immunoglobulin (Ig), HS, heparan sulfate proteoglycan (HSPG)-core protein and laminin in immunofluorescence. In mice with prolonged albuminuria HS staining in the glomerular capillary loops had almost completely disappeared, whereas staining was unaltered in non-albuminuric mice (P = 0.001). In mice with short duration of albuminuria, there was a tendency toward a decrease of HS staining (P = 0.06). The expression of HSPG-core protein and other extracellular matrix (ECM) components was unaltered in all groups. HS staining correlated inversely with albuminuria (r = -0.55; P < 0.001) and with staining of Ig deposits in the capillary loops (r = -0.74; P < 0.001). Despite the nearly complete loss of HS staining in the GBM in mice with prolonged albuminuria, there was no change in glomerular HS content as assessed by agarose electrophoresis and HS inhibition ELISA. We conclude that the development of albuminuria in MRL/lpr mice is accompanied by a loss of HS staining in the GBM, probably due to the masking of HS by deposits of Ig. In vitro studies revealed that autoantibodies complexed to nucleosomal antigens can inhibit the binding of the anti-HS monoclonal antibody to HS. Whether this also occurs in vivo remains to be determined. (Am J Pathol 1995, 146:753–763)

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the occurrence of a multitude of autoantibodies, primarily directed against nuclear antigens. Glomerulonephritis, characterized by proteinuria and deterioration of renal function, is one of the most serious manifestations of this disease. The MRL/lpr (MRL/lpr/lpr) mouse strain is a generally accepted animal model for SLE. As in human SLE, numerous autoantibodies occur and most animals develop albuminuria and progressive loss of kidney function between 3 and 6 months of age with a 50% mortality rate at 6 months. Histologically, a diffuse proliferative glomerulonephritis is observed, and immunofluorescence studies reveal variable amounts of deposits of immunoglobulins (Ig) and complement factors in the mesangium and along the glomerular capillary walls.

Deposition of heparan sulfate (HS) reactive autoantibodies has been proposed as an early event in the development of lupus nephritis. HS is the negatively charged glycosaminoglycan side chain of heparan sulfate proteoglycan (HSPG). It is an intrinsic constituent of the glomerular basement membrane (GBM) and responsible for the majority of the anionic sites in the GBM. These anionic sites are responsible for the charge dependent permeability of the GBM, although the relative contribution of

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other negative charged molecules within the glomerular capillary wall, like the sialoprotein podocalyxin, is still a matter of debate. Consequently, loss of these anionic sites leads to albuminuria. Recently, we found that staining of HS in the GBM with the anti-HS monoclonal antibody (MAb) JM-403 had completely disappeared in 12 of 13 renal biopsies of human lupus nephritis, whereas staining of the HSPG-core protein was unaltered.

To clarify the relationship between albuminuria and HS staining in SLE, we studied MRL/I mice with different stages of renal disease and compared these with mice without albuminuria of different ages. In these groups of mice, we studied by immunofluorescence the relation of deposited Ig with the staining of HS, HSPG-core protein and laminin in the glomeruli. We measured the glomerular HS content in the glomeruli of these mice to elucidate whether a potential decreased staining of HS was due to decreased HS content of the GBM or to blocking of the staining by deposited immune complexes. Since we have found that autoantibodies complexed to nucleosomal antigens can bind to HS in vitro and to GBM-HS in vivo, we examined in ELISA whether the binding of the anti-HS MAb to HS could be inhibited by these complexes.

Materials and Methods

Animals

MRL1 (MRL, lpr/lpr) mice were bred in the animal facilities of the University of Nijmegen from stock originally obtained via the Scripps Clinic and Research Foundation (La Jolla, CA) from the Jackson Laboratory (Bar Harbor, ME). Five groups of mice were selected based on age and magnitude of albuminuria, which was screened every week with Albustix (Boehringer Mannheim, Mannheim, Germany). Group 1 consisted of 12-week-old mice (n = 8) without albuminuria (Albustix < 300 µg/ml). Group 2 consisted of 36-week-old mice (n = 8) without albuminuria (Albustix < 300 µg/ml). Group 3 consisted of 18- to 24-week-old mice (n = 8) without albuminuria (Albustix < 300 µg/ml). Group 4 consisted of 18- to 24-week-old mice (n = 8) with short duration of albuminuria (Albustix < 1000 µg/ml, albuminuria period < 7 days). Group 5 consisted of 18- to 24-week-old mice (n = 8) which had a positive result on screening (Albustix > 1000 µg/ml) on three subsequent occasions (albuminuria period at least 14 days, but not more than 21 days).

After assigning a mouse to one of the five study groups, urine was collected the next day for 18 hours in a metabolic cage with free access to water. Mice assigned to groups 1, 2, 3, and 4 were sacrificed after collection of the urine. After the kidneys were perfused with saline before removal, a part was immediately snap frozen in liquid N2 for immunofluorescence and light microscopy. From animals assigned to group 5 another urine sample was collected after 14 days, just before the kidneys were taken for immunofluorescence and light microscopy. Urinary albumin content was measured by a radial immunodiffusion technique as described. Physiological albuminuria was determined by measuring albuminuria in 50 BALB/c mice. The upper level was defined as the mean albuminuria plus twice the standard deviation.

In a separate experiment animals were divided into 5 groups in exactly the same way as described above (n = 24 per group). The kidneys of these animals were flushed with iron oxide (Fe3O4) for the isolation of glomeruli (see below).

Histology

For light microscopy tissue fragments fixed in Bouin’s solution were dehydrated and embedded in paraplast (Amstelstad B.V., Amsterdam, The Netherlands). Four-micron sections were stained with periodic acid—Schiff and silver methenamine.

Immunofluorescence was performed on 2-µ cryostat sections of all kidneys to study deposition of mouse Ig and staining of ECM components. Deposition of mouse Ig was studied in direct immunofluorescence by incubating the sections with FITC-labeled F(ab)2 sheep anti-mouse Ig (Cappel, Organon Technika NV, Turnhout, Belgium) 10 mg/ml diluted 1:750 in PBS containing 1% (w/v) BSA. The presence of HS was examined by incubating the sections with a biotinylated (1 mg/ml) mouse anti-rat HS MAb that only recognizes HS in basement membranes (JM-403). The sections were first treated with an avidin-biotin blocking kit (Vector Laboratories Inc., Burlingame, CA) before they were incubated with the biotinylated JM-403 MAb in a dilution of 1:60. FITC-labeled streptavidin (Extravidin, Sigma, St. Louis, MO) 1.5 mg/ml diluted 1:400 was used to develop the sections. The sections were stained in indirect immunofluorescence with a goat anti-human HSPG-core antiserum prepared in our laboratory, diluted 1:200. FITC-labeled rabbit anti-goat Ig (de Beer Med BV, Hilveranbeek, The Netherlands), 16 mg/ml diluted 1:500 was used as a secondary antibody. The sections were also stained with a rabbit...
anti-EHS laminin antiserum (diluted 1:400, prepared in our laboratory) followed by FITC-labeled mouse anti-rabbit Ig, which was affinity purified and preabsorbed for human, mouse, goat and sheep Ig (Jackson Laboratories Inc., West Grove, PA) 1 mg/ml diluted 1:40, as secondary antibody. The anti-rat and anti-human ECM antibodies used in these studies all cross-reacted with mouse constituents in immunofluorescence.

After the staining procedure, the sections were embedded in aquamount (BDH Ltd, Poole, UK) and examined with a Zeiss fluorescence microscope. Blinded sections were examined by three independent investigators. The intensity of the staining in the capillary loops and the mesangium was scored semi-quantitatively on a 0 to 4+ scale in at least 30 glomeruli per mouse kidney. The 0 to 4 scale was defined as follows. For staining of ECM components in capillary loops, no staining at all was scored 0, staining of 25% of all capillary loops and/or 75% decrease of staining intensity (as compared with a normal BALB/c glomerulus) was scored 1; staining of 50% of all capillary loops and/or 50% decrease of staining intensity was scored 2; staining of 75% of all capillary loops and/or 25% decrease of staining intensity was scored 3, and normal staining of all capillary loops was scored 4. For staining of ECM in the mesangium, not the intensity but the amount of mesangial staining was scored, since staining intensity of ECM components in the mesangium in all animals was equal. No staining at all was scored 0; mild staining was scored 1; moderate staining was scored 2; strong staining was scored 3; and severe staining was scored 4.

**Measurement of HS in Glomeruli**

The isolation of glomeruli was performed as described before. All steps were carried out in the presence of protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 0.05% Na<sub>3</sub>No<sub>2</sub>, 20 mmol/L EDTA, and 100 KI units Trasylol). The obtained material consisted of more than 95% glomeruli. The number of glomeruli isolated was assessed by counting in triplicate in a hemocytometer. Next, the glomeruli were spun down and treated with 1 mg papain (from papaya latex, Sigma) in 1 ml 0.05 mol/L Na-acetate, 2 mol/L NaCl, 10 mmol/L cysteine-HCl (pH 5.7) overnight at 55 C to isolate the glycosaminoglycans (GAGs). Protein was precipitated using trichloroacetic acid in a final concentration of 5% (w/v), and after centrifugation 10% (w/v) 3 mol/L Na-acetate solution was added. GAGs were precipitated by adding cold ethanol (–80 C for 30 minutes), spun down, and dried. The isolated GAGs were tested by agarose electrophoresis on a 1% (w/v) agarose gel with 50 mmol/L Ba-acetate, pH 5.0, as electrophoresis buffer. A mixture of HS (20 ng/ml), dermatan sulfate (10 ng/ml), and chondroitin sulfate (10 ng/ml) was used as marker. The GAGs on the gel were stained with AgNO<sub>3</sub>.

Furthermore, HS isolated from glomeruli was measured in an inhibition ELISA (submitted for publication). In brief, Nunc-immuno plates (Maxisorp F96, GIBCO BRL, Paisley, UK) were coated with HS (Seikagaku Kogyo Ltd., Tokyo, Japan) 50 µg/ml in PBS overnight at room temperature. Plates were blocked with PBS containing 1% (w/v) gelatin, 150 µl/well for 2 hours at 37 C. A constant amount of MAb JM-403, giving 50% of the maximal ELISA signal, was added to a serially diluted sample of the glomerular digest in PBS/1% gelatin and incubated for 1 hour at 37 C. Thereafter, this mixture was added to the HS-coated plates (100 µl/well) and incubated for 1 hour at 37 C. After washing the plates, a peroxidase-labeled goat anti-mouse IgM antiserum (Southern, Birmingham, AL) was added, diluted 1:1000 in PBS containing 0.05% Tween 20, 100 µl/well. The plates were washed again and 3,5,3′,5′-tetramethylbenzidine (Merck, Darmstadt, Germany) 100 µg/ml in 0.1 mol/L sodium acetate (pH 5.5) containing 0.003% H<sub>2</sub>O<sub>2</sub> was added, 100 µl/well. The color development was stopped by adding 100 µl 2 mol/L H<sub>2</sub>SO<sub>4</sub> per well after 15 minutes.
and the OD at 450 nm was measured in a Titertek multiscan. Inhibition was calculated according to the following formula: \[1 - \left(\frac{A_{450} + \text{inhibitor}}{A_{450} - \text{inhibitor}}\right)\times 100\%\]. Then, the 50% inhibition point was assessed and compared with a standard curve.

Inhibition of Binding of the Anti-HS MAb JM-403 to HS in ELISA by Nucleosomes and Immune Complexes

Whether nucleosomes or immune complexes containing nucleosomal antigens were able to inhibit the binding of the anti-HS MAb to HS was evaluated in ELISA. After coating and blocking Nunc plates as described above, plates were incubated for 1 hour at room temperature with a serial dilution of nucleosomes, purified non-complexed anti-nucleosome MAbs and anti-nucleosome MAbs complexed to nucleosomal particles as described. After washing the plates, a constant amount of MAb JM-403 giving 50% of the maximal absorption signal was added for 1 hour at room temperature. Next, plates were developed as described above. To control for nonspecific inhibition, the same preparations were tested for their ability to inhibit an anti-HSPG core MAb (Chemicon International Inc, Veenendaal, The Netherlands) to HSPG. EHS-HSPG (Collaborative Biomedical Products, Bedford, MA) was coated 50 pg/ml, 100 µl/well in 0.05 mol/L carbonate buffer pH 9.6 to Nunc plates overnight at room temperature, and the ELISA was further carried out as described for the anti-HS ELISA.

Statistical Analysis

Statistical analysis was performed using the Mann-Whitney U test. \(P < 0.05\) (two sided) was considered to be statistically significant. Spearman's correlation coefficient was used in linear regression analyses.

Results

Albuminuria and Renal Histology

The selection of animals for the different groups was based on a screening by Albustix. This screening proved to be a good selection procedure for the albumin excretion during 18 hours. Non-albuminuric mice in groups 1 (12 weeks old), 2 (36 weeks old), and 3 (18–24 weeks old) did show a somewhat variable urinary albumin excretion that did not, however, exceed the upper level of the normal physiological albuminuria in mice (120 µg/18 hours) (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (Median in Weeks)</th>
<th>Albuminuria (Median in µg/18 Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>15 (3–56)*</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>53 (27–120)</td>
</tr>
<tr>
<td>3</td>
<td>21 (18–24)†</td>
<td>52 (20–100)</td>
</tr>
<tr>
<td>4</td>
<td>23 (18–24)†</td>
<td>1,700 (1,200–21,400)</td>
</tr>
<tr>
<td>5</td>
<td>19 (18–24)†</td>
<td>3,400 (1,000–18,200)</td>
</tr>
</tbody>
</table>

*Range of albuminuria in parentheses.
†Age range in parentheses.
‡Second measurement of albuminuria, which took place 2 weeks after the first.

of group 4 (short duration of albuminuria) and group 5 (albuminuria for 2 to 3 weeks) did show a marked albuminuria that increased over time in group 5 animals.

Glomerular deposition of mouse Ig in all mice that did not show albuminuria (groups 1, 2, and 3) was predominantly located in the mesangial areas and increased with age. In addition to increased mesangial Ig deposits, mice with albuminuria showed also a significant increase in Ig deposition along the glomerular capillary loops, which was most pronounced in mice with prolonged albuminuria (Figures 1 and 2).

Expansion of mesangial matrix, as assessed by light microscopy, was seen in all groups of mice when compared with young (12-week-old) mice. This expansion was most marked in mice with prolonged albuminuria, although it was also seen in mice with recent onset of albuminuria and, to a lesser extent, in age-matched non-albuminuric mice. Together with this mesangial expansion we found an increase in mesangial staining for the ECM components HSPG-core protein (Figure 4) and laminin (not shown). The anti-HS MAb JM-403 did not reveal HS in the mesangium but only recognized HS in the GBM. When staining for ECM components was scored in the capillary loops, it was found that HSPG-core protein and laminin (Figures 3 and 4) were equally detected in all groups. In contrast, HS staining was markedly decreased in the capillary loops of glomeruli of mice with prolonged albuminuria (group 5), compared with young 12-week-old mice (group 1) and non-albuminuric age-matched controls (group 3). In mice with short duration of albuminuria (group 4) there was a tendency toward a decrease in HS staining that, however, did not reach statistical significance \((P = 0.06)\) (Figures 3 and 4).

There was an inverse correlation between HS staining and albuminuria \((r_a = -0.55, P < 0.001)\) (Figure 5) and between HS staining and deposits of Ig located along the capillary loops \((r_a = -0.74, P <\)
Together, no elution procedure led to total removal of Ig deposits. However, they induced a total reduction of HS staining. All other elution procedures using heparin or 2 mol/L NaCl, there was no loss of HS staining on normal kidney sections. Taken together, no elution procedure led to total removal of Ig, while leaving HS intact.

**Elution Studies**

Because the above-mentioned data suggested that HS was masked by Ig, we tried to elute Ig from the sections to restore the HS staining in the GBM. After elution using heparin or 2 mol/L NaCl, there was no effect or only a slight effect on Ig deposits. After elution using glycine–HCl, pH 2.8, Ig depositions were partly removed. However, on a normal mouse kidney section, HS was partly degraded, leading to reduction of HS staining. All other elution procedures mentioned in Materials and Methods led to complete removal of Ig deposits. However, they induced a total loss of HS staining on normal kidney sections. Taken together, no elution procedure led to total removal of Ig, while leaving HS intact.

**Measurement of Glomerular HS Content**

Glomeruli were isolated and pooled for each group. From an equal amount of glomeruli the GAGs were extracted and analyzed on agarose gel electrophoresis. Despite the almost complete disappearance of HS staining in mice with prolonged albuminuria, we found comparable amounts of HS and, although to lesser extent, equal amounts of dermatan and chondroitin sulfate in glomeruli of mice from all groups (Figure 7). When HS from the GBM was measured more quantitatively in an inhibition ELISA using the anti-HS MAb JM-403, the HS content did also not markedly differ in any of the groups examined (Table 2).

**In Vitro ELISA Studies**

To elucidate in more detail potential mechanisms for the decrease in HS staining in the GBM, we analyzed whether nucleosomes, pure non-complexed mono-
clonal anti-nuclear antibodies or monoclonal anti-nuclear antibodies complexed to nucleosomal particles (described in ref. 22) could inhibit the binding of JM-403 to HS in ELISA. As shown in Figure 8, immune complexes and nucleosomes were able to inhibit dose-dependently this binding, whereas the pure, non-complexed monoclonal anti-nuclear antibody did not inhibit. In contrast nucleosomes or immune complexes were not able to inhibit the binding of an anti-HSPG MAb to HSPG.

Discussion

Alterations of GBM-HS have been observed in various glomerular diseases with albuminuria such as diabetic nephropathy, membranous glomerulonephritis, and SLE. In SLE it has been suggested that the binding to the GBM of HS-reactive antibodies or immune complexes initiates renal disease, and it has been found that anti-HS reactivity is associated with renal disease in human SLE. Recently, we reported that in the majority of biopsies of patients with lupus nephritis, HS staining in the glomerular capillary loops was completely negative, whereas staining for the HSPG-core protein was still intact. On the other hand, the relationship between albuminuria and HS staining is not unequivocal, since in patients with IgA nephropathy and Alport's syndrome and a marked albuminuria, we found a normal staining for GBM-HS.

We studied the relationship between HS staining and albuminuria in lupus-prone MRL/l mice with different stages of renal disease. When we examined deposition of Ig in these groups of mice we observed that a significant increase of mesangial Ig deposition in mice with increasing age, compared with young mice 12 weeks of age, was not related to increased glomerular permeability for proteins. When the Ig deposits were located in glomerular capillary loops a correlation was found with albuminuria. These findings suggest that deposition of Ig in the glomerular capillary loops is causally related to the development of albuminuria. Linear regression analysis revealed a strong correlation between these two parameters.

Both the extent of Ig deposits in the capillary loops and the albuminuria were correlated with a decreased staining of GBM-HS, while the staining for HSPG-core protein and laminin was not altered in all groups studied. This means that in mice with prolonged albuminuria, the expression or the accessibility of the HS side chain is diminished, while this is unaltered for its carrier protein (HSPG-core protein). These findings are in line with a recent report in which administration of polyclonal rabbit anti-HSPG antibodies (reactive with the core protein, but not with the side chain) in NZB/W mice did not competitively inhibit the binding of autoantibodies to HS in the GBM. The negative correlation between GBM-HS and albuminuria in our study suggests that the neutralization of HS related anionic sites is related to albuminuria in lupus nephritis. The negative correlation between GBM-HS staining and capillary loop Ig deposition suggests that the blocking of the negative charge in the GBM was caused by the deposition of immune complexes in the capillary loops. Although a correlation does not prove a cause and effect, this hypothesis is in line with an earlier report which stated that treatment of lupus mice with corticosteroids decreased the localization of immunoreactants in the GBM. This treatment preserved the presence of anionic sites within the GBM, linking, as we found, the status of anionic sites to the magnitude of GBM deposition. The assumption that
HS associated anionic sites are covered by immunoreactants was confirmed by the finding that the glomerular content of HS was unchanged in all experimental groups as assessed by agarose electrophoresis and quantitative HS inhibition ELISA. Experiments which attempted to elute the Ig and uncover HS failed because no elution procedure was capable of eluting all deposited Ig while leaving HS intact. Removal of Ig is only possible by procedures which also destroy HS. However, we were able to “uncover” HS in vitro, since papain digestion of isolated glomeruli lead to HS which could be shown in the inhibition ELISA and in agarose electrophoresis.

These experiments indicate that HS is still present in the GBM but that its accessibility for the anti-HS MAb is masked by immune complexes occurring during this disease. For lupus nephritis, a decrease of the net negative charge of the GBM due to the binding of cationic proteins or immune complexes has been suggested before. In this respect, deposition of neutrophilic or platelet-derived cationic proteins has been mentioned. Also the disappearance of anionic sites, as a consequence of immune complex deposition, has been described in lupus nephritis. In NZB/W mice it was shown that a decrease in anionic sites preceded the deposition of immune complexes in the glomerular capillary wall. In another study in NZB/W mice, it has been shown that deposition of immune complexes in the GBM is associated with loss and/or redistribution of fixed anionic sites in areas of increased permeability to anionic protein.

We also studied albuminuric and non-albuminuric NZBW and GVH mice and found also in these strains disappearance of HS staining in albuminuric mice (data not shown), showing that this phenomenon occurs also in these models of lupus nephritis. Theoretically, two mechanisms could explain the relationship between decrease of HS associated anionic sites and albuminuria. First, the decrease itself could be instrumental in the development of albuminuria. Arguments in favor of this explanation are that binding of cationic molecules or of the anti-HS MAb JM-403 to HS can induce albuminuria. Also our observation that reduction of HS staining was correlated to albuminuria in nonimmunological glomerular diseases like diabetic nephropathy and adriamycin
nephropathy supports this assumption. An alternative explanation could be that the reduction of HS associated negative charges in the GBM facilitates further deposition of immune complexes, which is then responsible for the ensuing albuminuria, as suggested by the correlation between GBM Ig deposits and albuminuria. One should realize, however, that these mechanisms are not mutually exclusive and could act in concert.

Previously, we have shown that anti-nuclear antibodies complexed to nucleosomal antigens are able to bind to HS in vitro and to the GBM in vivo. Nucleosomes are built up by histones and DNA and are present in nuclei of cells. In these HS or GBM reactive immune complexes, it is assumed that the positively charged histones interact with high affinity with the negatively charged HS in the GBM. The relevance of histones in lupus nephritis with albuminuria is further substantiated by the recent detection of histones in immune deposits of lupus mice with albuminuria, whereas in lupus mice of the same age without albuminuria, histones were seldom found. Also, in biopsies of patients with lupus nephritis, histones have been identified in the deposits along the capillary wall. In the study described in this paper, we show that nucleosomes and MAbs complexed to nucleosomal particles are able to inhibit the binding of the anti-HS MAb JM-403 to HS in ELISA whereas...
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3.5 - 2.5  2  ow  u.

rs = 0.55

p<0.001

mm  m  m

m  m

\[ \text{Figure 5. Correlation between GBM-HS staining and albuminuria.} \]

Figure 6. Correlation between GBM-HS staining and Ig deposits in the glomerular capillary loops.

Figure 7. Agarose gel electrophoresis of glycosaminoglycans extracted from glomeruli of the five groups of mice studied. Lane 1: markers, HS (20 mg), dermatan sulfate (DS, 10 mg), and chondroitin sulfate (CS, 10 mg); GAGs isolated from young 12-week-old mice (lane 2); from 3-week-old non-albuminuric mice (lane 3); from age-matched non-albuminuric controls (lane 4); from mice with short duration of albuminuria (lane 5); from mice with prolonged albuminuria (lane 6).

in vitro studies suggest that HS may be masked by histone-containing immune deposits.

Acknowledgment

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