Protection of Endothelial Cells by Dextran Sulfate in Rats with Thrombotic Microangiopathy

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The characteristic features of thrombotic microangiopathy (TMA) include glomerular and peritubular capillary endothelial cell injury in association with loss of heparan sulfate proteoglycans on the cell surface and thrombus formation, followed by subsequent ischemic tubulointerstitial damage. It therefore was hypothesized that dextran sulfate (DXS) may protect the kidney against endothelial damage in a model of TMA. TMA was induced in rats by renal artery perfusion of an antiglomerular endothelial antibody, followed by the administration of DXS or vehicle. Renal damage was assessed by histologic analysis and measurements of blood urea nitrogen and creatinine. Whereas control rats developed severe renal failure with extensive glomerular and tubular injury, administration of DXS significantly protected renal function and preserved the glomerular endothelium and peritubular capillaries. The beneficial effect of DXS could be attributed to the ability of DXS to protect endothelial cells from coagulation and complement activation, as demonstrated by the histologic analysis. In addition, binding of the administered DXS to the surface of the glomerular endothelium was confirmed in TMA rats, suggesting that DXS acts as a “repair coat” of injured glomerular endothelium. In conclusion, DXS protects the kidney from experimental TMA. This protection may be mediated by DXS’s binding directly to the surface of glomerular endothelium and amelioration of coagulation, complement activation, and cellular matrix loss.


Injury to the vascular endothelium is critical in inflammation and promotion of a procoagulant state and is likely to be of major importance in the pathogenesis of various kidney disorders, such as glomerulonephritis, vasculitis, allograft rejection, ischemia-reperfusion injury, and thrombotic microangiopathy (TMA) (1–4). Endothelial injury is also involved in the initiation and propagation of glomerulosclerosis and ESRD (5–11).

The hemolytic uremic syndrome (HUS) and related TMA are clinical syndromes characterized by thrombocytopenia, nonimmune hemolytic anemia, and variable degrees of renal insufficiency (10–13). Most cases of HUS are secondary to enteric infection with certain verotoxin-producing strains of Escherichia coli (particularly O157:H7) (14), but some cases are associated with genetic defects in complement inhibition on endothelium (15). Regardless, the hallmark of HUS is injury to the glomerular and other renal microvascular endothelium, characterized by arteriolar and capillary swelling, apoptosis, and/or detachment of endothelial cells (16,17). Although most patients have complete recovery of renal function, a significant number of patients develop eventual end-stage renal failure. To understand better the pathophysiology and potential therapies, we recently developed a model of TMA in rats by selective renal artery perfusion with anti-endothelial cell antibody (1). This model is characterized by severe glomerular and peritubular capillary endothelial injury, showing the characteristic features of clinical TMA. Similar to severe forms of HUS, the renal injury does not resolve, and progressive glomerular and tubulointerstitial damage ensues (1,4). To date, therapy for the renal involvement in TMA is limited.

Physiologically, endothelial cells (EC) are covered with a layer of heparan sulfate proteoglycans (HSPG), which are crucial for the anticoagulant and anti-inflammatory properties of the endothelium. HSPG is shed from the cell membrane on activation of EC, exposing a procoagulant and proinflammatory cell surface (18). Moreover, HSPG regulate multiple functions such as leukocyte–endothelial interactions and extravasation (19) and are released rapidly under conditions of inflammation and tissue damage (20).

Dextran sulfate (DXS) is a semisynthetic analog of the glycosaminoglycan family, which includes heparin, heparan sulfate, dermatan sulfate, and chondroitin sulfate. Several studies reported that certain glycosaminoglycans prevent the complement cascade by activating C1 inhibitor-mediated inactivation of C1s (21–23). Wuillemin et al. (24) indicated that DXS has the
most potent C1s inactivation ability among various glycosaminoglycans. Taken together, we hypothesized that the DXS treatment in experimental TMA could reduce microvascular injury and protect renal functions.

**Materials and Methods**

**Animal Model and Experimental Study Design**

Male Wistar rats were purchased from Nippon Seibutsu Zairyo Center Co. (Saitama, Japan). All rats were housed in cages in a temperature- and light-controlled environment in an accredited animal care facility, with free access to water. All studies conformed to the principles of the Guide for Animal Experimentation at the University of Tokyo.

**Chemical Reagent**

DXS was purchased from Kowa Pharmaceutical Company (Osaka, Japan). Injection was performed via superior mesenteric artery at a final concentration of 50 mg/kg in saline. Biotinylated DXS was prepared by linking hydrazide biotin to the DXS, by a commercial method, which is available from Dojin-Chemical (Kumamoto, Japan).

**Purification of Goat Antiglomerular EC IgG**

Goat anti-EC IgG for renal artery perfusion was purified by using a caprylic acid precipitation method, as described previously (1). The purity of antiglomerular EC IgG was checked by SDS-PAGE.

**Induction of the Renal TMA Model**

The left kidney was removed 1 d before the induction of disease. The renal TMA model was induced by selective renal artery perfusion of the right kidney, through the superior mesenteric artery, with the purified IgG fraction of goat anti-glomerular endothelium (GEN) antibody, as reported previously (1). Kidneys were perfused with 0.2 ml of PBS (pH 7.2), followed by 50 mg/kg body wt anti-GEN EC IgG or PBS (control). Ischemia time was <6 min.

**Experimental Protocol**

For investigating the effect of DXS in this model, experimental TMA was induced as described above in 41 male rats that weighed 200 to 230 g. Rats were divided into two groups: An experimental group received 50 mg/kg DXS (n = 22), and a control group received PBS (n = 19) administered via the right renal artery 10 min after the anti-GEN antibody perfusion. Twenty-four hours after renal perfusion, a blood sample was obtained via the tail vein for the measurement of serum blood urea nitrogen (BUN) and creatinine levels. Two days after induction of the disease, a blood sample was obtained by cardiac puncture. The rats were killed, and a biopsy was taken for histologic analysis. For demonstrating functioning endothelium, 250 μg of biotinylated lectin (Lycopersicum esculentum lectin; Vector Laboratories, Burlingame, CA) was injected via the tail vein exactly 4 min before the rats were killed.

**Serum BUN and Creatinine Measurements**

Serum BUN levels were determined colorimetrically with a commercial kit that used the urease-indophenol method to measure urea nitrogen (Wako Pure Chemical Industries, Tokyo, Japan). Serum creatinine levels were determined colorimetrically with a commercial kit that used the Jaffé’s method (Wako Pure Chemical Industries).

**Assessing Renal Pathology**

Tissue fixed in methyl Carnoy’s solution was processed and paraffin-embedded. Three-micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. An indirect immunoperoxidase method was used to identify the following antigens: Proliferating cell nuclear antigen (PCNA) with anti-PCNA (PC10, mouse IgG2a; DAKO, Glostrup, Denmark), vascular endothelial growth factor (VEGF) with anti-VEGF (rabbit IgG; Santa Cruz Biotechnology, Santa Cruz, CA). To identify the renal microvascular EC, we chose two methods. One is an indirect immunoperoxidase method, which used mAb JC-12 (Bender MedSystems, Vienna, Austria) (6). The other is the lectin perfusion method, which used biotinylated lectin (25). Single-labeling studies used biotinylated species-specific secondary antibodies, followed by streptavidin–horseradish peroxidase (HRP) complex (DAKO). Chromogenic color was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Double-labeling studies were performed as described previously (25), using biotinylated species-specific secondary antibodies, followed by streptavidin–HRP complex (DAKO), with labeling being revealed using DAB as a chromogen (brown). After staining the first antigen, the remaining peroxidase activity was extinguished with 3% H2O2 in methanol for 10 min. The remaining biotin was blocked by incubation with avidin solution (Vector Laboratories), after which the first antibodies for the second antigen were applied, followed by suitable secondary biotinylated antibodies. Color was developed with DAB plus 0.08% nickel (black). Paraffin sections were also applied to the terminal dUTP nick-end labeling (TUNEL) staining method using a commercially available kit (Treivgen, Gaithersburg, MD).

Tissues for immunofluorescent staining were embedded in OCT compound (Miles, Inc., Elkhart, IN) and snap-frozen in liquid N2. Four-micrometer frozen sections were cut on a rotary microtome and mounted onto silanated microscope slides. Fibrin was detected by staining with FITC-conjugated goat anti-fibrinogen IgG (Cappel, Durham, NC). Deposition of the pathogenic goat anti-EC antibody was detected with a biotinylated anti-goat IgG (secondary antibody; DAKO), followed by incubation with Oregon green/Neutralite avidin (Molecular Probes, Eugene, OR). C3 deposition was detected by staining with FITC-conjugated goat anti-rat C3 (Cappel). Frozen sections were also used to an indirect immunoperoxidase staining method. To identify heparan sulfate, anti–heparan sulfate mouse IgM (JM403, Seikagaku Kogyo, Tokyo, Japan) was used, followed by anti-mouse IgM-HRP complex (Cappel).

**Quantification of Renal Histology**

Quantification was performed in a blinded manner on 25 randomly selected glomeruli/perfused kidney on all experimental and control animals, for each of the following variables. For glomerular thrombi, a semiquantitative scoring system was used as follows: 0, normal; 1, 0 to 25% of glomerular area involved; 2, 25 to 50% of glomerular tuft area involved; 3, 50 to 75% of tuft area involved; 4, 75 to 100% of tuft area involved. For glomerular EC analysis, a semiquantitative scoring system was also used as follows: 0, no positive glomerular tuft staining for endothelium; 1, 1 to 25% of glomerular tuft positive for endothelium; 2, 25 to 50% positive; 3, 50 to 75% positive; 4, 75 to 100% positive. PAS stain quantification was performed separately in two different parts. For glomerular scoring, 25 randomly selected glomeruli were quantified as follows: 0, normal; 1, 0 to 25% of glomerular area involved; 2, 25 to 50% of glomerular tuft area involved; 3, 50 to 75% of tuft area involved; 4, >75% of tuft area involved. For tubular scoring, 25 randomly selected ×400 pictures were quantified as follows: 0, normal; 1, 0 to 25% of tubule area involved; 2, 25 to 50% of tubule area involved; 3, 50 to 75% of tubule involved; 4, >75% of tubule are involved.

For analysis of C3 and anti-GEN antibody deposition, image analysis was used. In brief, the photographs of 20 randomly selected glomeruli were saved in JPEG files, and the electric densities were measured.
using an image processing and analysis program Image-J software (NIH, MD). The percentage of positive area in glomeruli was calculated under the threshold setting by 130 to 255.

**Binding of Biotin-Labeled DXS to Vasculature in Rats with TMA**

Left kidney was removed 1 d before the experiment. Rats with TMA were induced by selective renal artery perfusion of the right kidney with the goat anti-glomerular EC antibody as described above. Rats without TMA were induced by selective renal artery perfusion of the right kidney with PBS. After 10 min, both groups’ kidneys were perfused with 50 mg/kg biotin-labeled DXS. One day after the injection, rats were killed and tissues were embedded in OCT compound and snap-frozen in liquid N₂. Deposition of the DXS in the kidney was detected with biotinylated DXS, followed by incubation with streptavidin Alex488 (Molecular Probes, Eugene, OR).

**Electron Microscopy**

Kidney specimens for electron microscopy (EM) were fixed in ice-cold 1.4% glutaraldehyde (pH 7.4), postfixed in osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-100CX II (Nihon Denshi, Tokyo, Japan) electron microscope.

**Statistical Analyses**

Differences between the DXS and vehicle-treated groups were evaluated with the unpaired t test. Nonparametric data, including the histologic scores, were analyzed by Mann-Whitney test. Values were considered significant at P < 0.05.

**Results**

**Renal Function Preserved in Animals Treated with DXS**

Serum BUN and creatinine levels of normal rats at the same ages of experimental TMA rats were 14.88 ± 1.01 and 0.58 ± 0.03 mg/dl, respectively. There was a significant increase in serum BUN levels in control TMA rats that were given DXS treatment on day 1 and day 2 with measures of 57.10 ± 6.11 and 63.53 ± 9.99 mg/dl, respectively (P < 0.001 versus normal). In contrast, serum BUN levels in TMA rats that were given DXS treatment on day 1 and day 2 were 41.65 ± 5.14 and 40.98 ± 6.39 mg/dl, respectively (P < 0.05 versus vehicle treatment at each time point).

Similar to BUN, DXS treatment significantly improved serum creatinine level compared with control TMA rats that were given vehicle treatment on day 1 (0.84 ± 0.08 and 1.07 ± 0.10 mg/dl, respectively; P < 0.05) and on day 2 (0.86 ± 0.11 and 1.30 ± 0.21 mg/dl, respectively; P < 0.05). Taken together, these results indicated that DXS treatment improved renal function in rats with experimental TMA.

**DXS Administration Ameliorated Histologic Injury in Rats with TMA**

Light microscopic analysis with PAS staining revealed characteristic changes of TMA in the control vehicle-treated group (Figure 1). Glomeruli demonstrated generalized hypocellularity, with indistinct capillary walls, often accompanied by intraluminal thrombi. Many tubules were dilated, often with sloughing of epithelial cells into the tubule lumen, cast formation, or patchy areas of necrosis. In contrast, both the glomerular and tubular abnormalities were significantly improved in the DXS-treated rats (Figure 1, Table 1).

**Thrombus Formation**

For evaluating thrombus formation in TMA, glomerular fibrin deposition was assessed by immunofluorescent staining with antifibrinogen antibody. Glomerular thrombi were common and more severe in control TMA vehicle-treated rats, compared with DXS-treated rats with TMA, although the difference did not reach statistical significance (0.80 ± 0.14; DXS-treated group, 1.48 ± 0.25; vehicle-treated group; P = 0.063; Figure 2, A and B, Table 1).

**Complement Activation Inhibited by DXS in TMA Animal**

Complement activation in glomeruli, a previously reported feature of this model, was assessed by immunofluorescent staining with anti-C3 antibody. C3 deposition was observed along the capillary wall in both experimental and control animals. However, the intensity of C3 staining was significantly decreased by DXS treatment (0.96 ± 0.24%; DXS-treated group, 26.78 ± 5.76%; vehicle-treated group, P < 0.0001; Figure 2, C and D, Table 2).

**DXS Administration Protected Glomerular Endothelium and Vasculature**

Changes in EC morphology and density were studied by staining tissue sections with the EC-specific mouse mAb JG-12. The majority of glomeruli and peritubular capillaries demonstrated a decrease in JG-12 staining in control TMA rats (Figure 3A). However, the reduction in JG-12 staining was significantly milder in experimental TMA rats given DXS (vehicle-treated group 1.85 ± 0.12; DXS-treated group, 2.97 ± 0.14; P < 0.0001; Figure 3B, Table 1). In addition, lectin staining showed that glomerular endothelium and peritubular capillaries were severely damaged in vehicle-treated rats (Figure 3C). These data showing preservation of glomeruli endothelium suggested that DXS might be acting as a protectant factor for EC.

**Cellular Apoptosis and Proliferation**

TUNEL-positive signal had not been seen in the glomeruli of both vehicle- and DXS-treated groups (data not shown). However, a diffuse and significant increase in the number of PCNA-positive cells was noted in vehicle-treated glomeruli (Figure 3C), although few PCNA-positive cells were observed in the glomeruli in DXS-treated rats (Figure 3D). Nuclear positive signals for PCNA were observed in the glomeruli without lectin staining. It is interesting that staining of VEGF showed a similar pattern; positive signals were observed in vehicle-treated glomeruli but not in DXS treatment (Figure 3, E and F).

**EM Analysis**

In control TMA vehicle-treated rats, glomeruli showed severe stasis of red blood cells within dilated capillary lumen (Figure 4A). Fragmented red blood cells and fibrin strands were occasionally noticed. Glomerular capillaries demonstrated diffuse subendothelial expansion and swelling or degenerative
changes of EC (Figure 4B). In contrast, glomeruli in TMA DXS-treated rats maintained normal structure, and endothelial fenestrations remained intact (A and C). Dextran sulfate (DXS)-treated rats had less injury and showed preservation of glomerular and tubular architecture (B and D). Magnification, ×200 in A and B; ×400 in C and D.

Binding of Biotin-Labeled DXS to Vasculature in Rats with TMA

To confirm the binding of DXS to the glomerular endothelium, rats with TMA or without TMA received biotin-labeled DXS injection 10 min after injection of anti-GEN antibody or PBS, respectively. One day after injection, kidney sections were analyzed by fluorescence microscopy. Biotin-DXS staining was detected on the surface of the glomerular endothelium in the kidney with TMA, whereas no staining was detected in the kidney without TMA (Figure 5). These results indicated that DXS could bind only to the surface of injured endothelium.

DXS Does Not Affect Anti-GEN Antibody Binding

Finally, to ensure that the results of this study were not due simply to differences in the binding of the disease-inducing antibody, we examined anti-GEN antibody deposition by staining tissue sections with the anti-goat IgG antibody. The density of goat anti-GEN was observed without any differences between vehicle-treated and DXS-treated rats (Table 2).
Discussion

TMA is a significant cause of acute renal failure, and in some instances, patients progress to ESRD. The experimental model of TMA has provided a unique opportunity to study disease mechanisms and potential therapies. For example, VEGF protects the kidney in this model (26). The beneficial effect of VEGF may comprise not only a survival factor but also an angiogenic factor for glomerular capillary endothelium. Neutralization studies of a specific complement regulatory protein (27) and studies with C6-deficient animals have also shown a crucial role of complement activation in glomerular endothelium injury in this model (28). In this study, we show that DXS injection after the injection of antiglomerular EC antibody protected the glomerular endothelium and preserved renal functions. Although TMA can be caused by several diseases, they all share common histologic features within the kidney. These include significant renal microvascular EC injury, complement activation, platelet infiltration, fibrin deposition, and renal failure (1,4). These changes are due to injury primarily to the glomerular EC. This provides the rationale for our study to test the hypothesis that DXS, a member of the glycosaminoglycan family, can protect animals with experimental TMA. The major findings in our study were that DXS treatment significantly improved renal function, and this was accompanied by marked preservation of tissue injury. Staining with JG-12 antibody and estimation of glomerular endothelium by perfusion of biotinylated lectin demonstrated that DXS treatment protected the glomerular endothelium from the damage in rats with experimental TMA. We observed more PCNA-positive cells as well as upregulation of VEGF in vehicle-treated glomeruli. It is likely that these results reflect compensatory responses against glomerular endothelial damage, as we reported previously (1).

DXS can protect the endothelium via several potential mechanisms. DXS has an anticoagulation effect, although the affinity

Table 2. Image analysis of histologic and immunochemical studies

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<th>Characteristic</th>
<th>Vehicle-Treated</th>
<th>DXS-Treated</th>
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<tr>
<td>Glomerular goat antiglomerular endothelial cell IgG–positive area (%)</td>
<td>58.16 ± 6.57</td>
<td>51.97 ± 7.19</td>
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<tr>
<td>Glomerular C3 deposition–positive area (%)</td>
<td>26.78 ± 5.76a</td>
<td>0.96 ± 0.24</td>
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P < 0.0001 vehicle-treated versus DXS-treated group.

Figure 2. Thrombus formation and complement activation in the glomeruli of TMA rats. Marked deposition of fibrin was observed in the glomeruli of TMA rats (A). DXS treatment demonstrated less fibrin deposition (B). Similarly, marked deposition of C3 was observed in the glomeruli of TMA rats (C). DXS treatment resulted in less C3 deposition in glomeruli (D). Magnification, ×400.
of DXS to antithrombin is three orders smaller than the affinity of heparin (29). It has also been known for a decade that low molecular weight DXS is an efficient inhibitor of complement activation (24). Indeed, our results show that glomerular C3 staining was reduced in the DXS group compared with control. Studies have shown that DXS inhibits all three pathways of complement activation and dose-dependently protected pig cells from deposition of human complement, and the EC-protective effect of DXS correlated with binding of the substance to the cells (30).

Another important mechanism of endothelium protection was proposed by Laumonier et al. (30), who suggested that DXS acted locally and might functionally replace HSPG that are known to be shed from the EC surface upon activation. HSPG modulate the actions of a large number of extracellular ligands (34,35) and are involved in the preservation of the critical anticoagulant surface of vascular EC (36). They called these effects of DXS a “repair coat” by re-establishing an anticoagulant and anti-inflammatory surface (30). In this study, by using a specific antibody for glomerular heparan sulfate, we showed that most parts of glomerular loops lost HSPG in TMA-induced rats, whereas HSPG covered all parts of glomerular loops in normal rats (Figure 6). In addition, by using biotin-labeled DXS, we showed that DXS binds to the glomerular endothelium in TMA-induced rats. The binding of DXS to the endothelium requires EC damage, because DXS did not bind to the endothelium of uninjured normal rats.

Recent analysis has revealed that HSPG exists on the surface of the glomerular basement membrane (GBM) and endothelium (37). HSPG on the GBM is considered to play the key role for charge selective moiety of glomerular protein filtration, because quantitative changes in HSPG have been observed in a number of proteinuric nephropathies (38). HSPG on the glomerular endothelium is considered to play a role in leukocyte infiltration, because lack of HSPG promotes the increase in vascular permeability and leukocyte extravasation under inflammatory conditions (19). However, proteinuria or leukocyte infiltration into the glomeruli could not be observed in this model of TMA (1). This result also suggests that replaced DXS bears functions, such as “repair coat,” other than having been previously reported. Another possible mechanism of renoprotection by DXS is prevention of the deposition of anti-GEN IgG on the surface of glomerular endothelium, but this is unlikely because our immunofluorescence studies demonstrated that the deposition of anti-GEN IgG was not different between the two groups.

Although DXS injection via right renal artery was effective in this study, intravenous injection of DXS at the same dosage had no beneficial effects on renal functions, EC injury, or complement activation (data not shown). A possible explanation for this difference by injection route might be dose-dependence of DXS, i.e., a much higher dose of DXS might be needed. It should be noted that no signs of acute toxicity of DXS administration were observed by either way of injection.

In conclusion, DXS has an important protective effect in experimental TMA. This is likely mediated by complement inhibition, anticoagulation, and endothelial protection by re-establishing the intact surface of injured endothelium. Supple-

**Figure 3.** Preservation of the glomerular and peritubular capillary network by DXS treatment. JG-12 staining demonstrated preservation of the glomerular and peritubular capillary endothelium in TMA rats with DXS treatment (B) than in TMA rats without DXS treatment (A). Sections were double-stained with biotinylated lectin and anti–proliferating cell nuclear antigen (PCNA). Lectin staining (brown) demonstrated preservation of the glomerular and peritubular capillary endothelium in TMA rats by DXS treatment (D) than in TMA rats without DXS treatment (C). In contrast, PCNA-positive cells (black) in the glomeruli without DXS treatment (D) were markedly increased than those in the glomeruli without DXS treatment (C). Expressions of vascular endothelial growth factor in the glomeruli without DXS treatment (E) were markedly increased than those in the glomeruli without DXS treatment (F). Magnification, ×400.
Figure 4. Electron microscopic analysis of the glomeruli in control TMA vehicle-treated rats (A and B) and TMA DXS-treated rats (C and D). (A) Marked reduction or occlusion of the capillary lumina by red blood cells, fibrin strands (F), expanded subendothelial space, and swelling of endothelial cells (EC). Electron lucent fluffy materials (arrow), fragmented red blood cells (*), and fibrin strands occupied the subendothelial spaces. Endothelial cells (E) had enlarged cytoplasm with an increased number of organelle. (B) In the most severely affected area, capillary lumina revealed irregular dilation with marked fibrinous exudate (F), amorphous material, cellular debris (arrowhead), and fragmented blood cells. Denudation and fragmentation of EC (arrow) was noticed. (C) Almost normal configuration of a glomerulus. A few red blood cells could be detected in the loops. (D) Glomerular EC showed no remarkable change and preserved fenestrations (arrow). Magnification, ×2000 in A, B, and C; ×12,000 in D.

Figure 5. Binding of biotin-labeled DXS to rat glomerular endothelium. Marked binding of DXS was observed in the glomeruli of TMA-induced rats (A). In contrast, no binding of DXS was observed in the glomeruli of non-TMA rats (B). Magnification, ×200.
mentation of glycosaminoglycans may present a new approach for treatment of glomerular endothelial injury.

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References

Figure 6. Immunodetection of heparan sulfate proteoglycan (HSPG) molecules in the glomeruli. HSPG covered the glomerular capillary loops in normal rats (A). However, marked decrease of HSPG was observed in the glomerular capillary loops of TMA-induced rats (B). Magnification, ×400.


