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C.L.R. Belmiro¹*, R.G. Gonçalves²*, E.O. Kozlowski¹, A.F. Werneck¹, C.M. Takyia³, M. Leite-Jr.² and M.S.G. Pavão¹

¹Laboratório de Bioquímica e Biologia Celular de Glicoconjugados, Programa de Glicobiologia, Instituto de Bioquímica Médica and Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil
²Serviço de Nefrologia, Departamento de Clínica Médica, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil
³Departamento de Histologia e Embriologia, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

Abstract

Selectins play an essential role in most inflammatory reactions, mediating the initial leukocyte-rolling event on activated endothelium. Heparin and dermatan sulfate (DS) bind and block P- and L-selectin function in vitro. Recently, we reported that subcutaneous administration of DS inhibits colon inflammation in rats by reducing macrophage and T-cell recruitment, and macrophage activation. In the present study, we examined the effect of porcine intestinal mucosa DS on renal inflammation and fibrosis in mice after unilateral ureteral obstruction (UUO). Twenty-four adult male Swiss mice weighing 20-25 g were divided into 4 groups: group C (N = 6) was not subjected to any surgical manipulation; group SH (N = 6) was subjected to surgical manipulation but without ureter ligation; group UUO (N = 6) was subjected to unilateral ureteral obstruction and received no treatment; group UUO plus DS (N = 6) was subjected to UUO and received DS (4 mg/kg) subcutaneously, daily, for 14 days. An immunoblot study was also performed for TGF-β. Collagen (stained area ~3700 µm²), MCP-1 (stained area ~1700 µm²), TGF-β (stained area ~13% of total area), macrophage (number of cells ~40), and myofibroblast (stained area ~1900 µm²) levels were significantly (P < 0.05) higher in the UUO group compared to control. DS treatment significantly (P < 0.05) reduced the content of collagen (stained area ~700 µm²), MCP-1 (stained area ~160 µm²) and TGF-β (stained area ~5% of total area), in addition to myofibroblast (stained area ~190 µm²) and macrophage (number of cells ~32) accumulation in the obstructed kidney. Overall, these results indicate that DS attenuates kidney inflammation by reducing macrophage recruitment, myofibroblast population and fibrosis in mice submitted to UUO.

Key words: Glycosaminoglycans; Dermatan sulfate; Kidney; Inflammation

Introduction

Unilateral ureteral obstruction (UUO) is an experimental model used widely to study the pathogenesis of tubulointerstitial fibrosis because it is highly reproducible and induces pathogenic events similar to those observed in human renal fibrosis (1,2).

Renal interstitial fibrosis resulting from ureteral obstruction is associated with the development of an inflammatory process, consisting of chemokine-mediated macrophage recruitment, transforming growth factor β (TGF-β) production and myofibroblast accumulation. These events lead to collagen deposition and fibrosis (3,4) and so far no specific treatment is available that can efficiently block or even reverse the progression of renal disease.

Cellular adhesion molecules such as selectins play an essential role in most inflammatory reactions, mediating...
the initial leukocyte rolling events on activated endotheli-
um. During inflammation, P-selectin-glycoprotein-ligand 1 (PSGL-1) on the surface of leukocytes binds to P-
selectin on the activated endothelial cells, reducing the
velocity of the leukocytes and allowing their contact with
endothelial adhesion molecules such as intercellular
adhesion molecule 1 (I-CAM-1), and transmigration (5,6).
Therefore, it is possible that selectins on the surface of
peritubular capillary endothelial cells facilitate leukocyte
recruitment to the inflamed renal interstitium.

Heparin is an anticoagulant glycosaminoglycan that
acts by activating antithrombin in order to inhibit thrombin
and factor Xa (7,8). It has been recently shown that, in
addition to having an anticoagulant effect, heparin inhibits
inflammation. The molecular mechanism of this effect
involves P- and L-selectin blockage, which reduces leu-
cocyte recruitment (9,10). Unfractionated heparin has a
higher binding affinity for P-selectin when compared to low
molecular weight heparin (LMW-Hep) (11). This difference
may be associated with the higher effect of unfractionated
heparin in suppressing P-selectin function in vivo (12). In
fact, we recently reported that LMW-Hep attenuates fibrosis
in obstructed kidneys by down-regulating the synthesis of
collagen, fibronectin and TGF-β, but it is not able to inhibit
macrophage recruitment (13).

Dermatan sulfate (DS) is a sulfated glycosaminoglycan
consisting of alternating units of N-acetyl-D-
galactosamine (GalNAc) and iduronic acid (IdoA), which
can be sulfated mainly at the carbon 4 (C4) of GalNAc and
the carbon 2 (C2) of the IdoA residues (14). DS has been
successfully used in humans to treat and prevent
heparin-induced thrombocytopenia (15). It has also been
shown to bind and block P- and L-selectin function in vitro
(16,17). Recently, we reported that subcutaneous
administration of DS attenuates colon inflammation in
rats by reducing macrophage and T-cell recruitment and
macrophage activation (18), suggesting its potential use as
an anti-inflammatory drug.

In the present investigation, we extended the study
of the anti-inflammatory effect of DS by examining its
effect in mice after UUO by measuring the content of
monocyte chemoattractant protein-1 (MCP-1) and
TGF-β, as well as macrophage, myofibroblast and col-
lagen accumulation in the kidney. The results indicate
that DS has a protective effect against the progression
of kidney inflammation by reducing macrophage and
myofibroblast accumulation and fibrosis.

Material and Methods

Animals and experimental protocol

All animal work was carried out in accordance with
the Brazilian Animal Protection Law and the Ethics
Committee for Animal Use of Universidade Federal do
Rio de Janeiro. The study was performed on 24 adult
male Swiss mice weighing 20-25 g. The mice were kept
on a 12-h light/dark cycle at 25°C and fed a standard
mice chow and water ad libitum. Ureteral obstruction
was performed as follows: mice were anesthetized sub-
cutaneously with ketamine (35 mg/kg) and xylazine (5
mg/kg). Under sterile conditions, an abdominal midline
incision was made, the left ureter was exposed and
ligated at two points using 6-0 silk, and the ureter was
sectioned between the ligatures. The skin was sutured
for approximation and the mice were kept in regular
cages. The mice were divided into 4 groups. A group of
6 mice served as control and was not subjected to any
surgical manipulation (group C). The SH group (sham-
operated animals, N = 6) was subjected to the surgical
procedures, except that the left ureter was manipulated
without ligation and sectioning. The UUO group (N = 6)
was subjected to ureteral obstruction and given no
treatment. Another group of 6 mice was subjected to
ureteral obstruction and was given porcine intestinal
mucosa DS (4 mg·kg⁻¹·day⁻¹, dissolved in 0.30 mL sa-
line; Sigma, USA) subcutaneously, once daily (group
UUO+DS) for 14 days.

Tissue preparation

At the end of 14 days, the mice were sacrificed under
anesthesia and the kidneys were removed. Animals were
perfused with citrate buffer (3.5%), pH 7.4, via the left
cardiac ventricle for 20 min. Kidneys were removed and
sectioned mid-frontally into two pieces. One fragment
was immersed in 10% buffered formaldehyde solution
and embedded in paraffin for histological examination
and the other was immersed in acetone for hydroxy-
proline analysis. Kidney paraffin sections of 5 µm were
stained with hematoxylin-eosin (HE), and a modified
Sirius red technique (19,20) for collagen staining. Tis-
tue samples were immediately embedded in Tissue-Tek
O.C.T. compound (Miles Scientific Laboratories Ltd.,
USA) and snap-frozen in isopentane in a liquid nitrogen
bath. Samples were then stored at -80°C until processing,
and cut into 6-µm sections with a cryostat maintained at
-20°C. Tissue sections were air-dried and fixed for
10 min in 1:1 chloroform-acetone. For the detection of
macrophages and TGF-β, a rat anti-mouse F4/80 anti-
body (1:1000; Serotec, USA) and a pan-specific rabbit
anti-TGF-β antibody (1:100; R&D Systems, USA) were
used, respectively (18). Antibodies were detected with
the Rat IgG kit (Vector Labs, USA) for F4/80, and the
Histar 40000 mouse on Mouse kit (Serotec) for TGF-β
and developed with the Dako LSAB® 2 kit/horseradish
peroxidase (HRP; Dako Corp., USA) using diaminoben-
zidine (DAB) as the chromogen substrate (Liquid DAB; 
Dako).

Interstitial myofibroblasts were quantified using an
α-smooth muscle actin (α-SMA) monoclonal antibody
(1:100; Dako Corp.). An anti-MCP-1 polyclonal antibody
Histomorphometry

Histomorphometry was performed using an imaging analysis system consisting of a digital camera (Coolpix 990, Nikon, Japan) coupled to a light microscope (Eclipse 400, Nikon). Fifteen fields of renal cortex and of medulla from Sirius red-stained sections, as well as sections stained with anti-F4/80 and anti-TGF-β, anti-MCP-1 and anti-α-SMA antibodies were obtained for each animal, using a 40X magnification objective lens.

The immunostaining was quantified as stained area or number of cells (µm²) or as cells associated with a brownish color around the nucleus. There is a clear distinction between nuclei associated with immunostaining and those devoid of the brownish color. Quantification was estimated on captured high-quality images (2048 x 1536 pixels buffer) by considering the percentage of stained areas in the total histological fields using the Image Pro Plus 4.5.1 software (Media Cybernetics, USA).

Determination of hydroxyproline

The amount of hydroxyproline (the collagen-specific hydroxylated form of the amino acid proline) in the renal tissue was estimated by a modification of the method of Stegemann and Stalder (21). Briefly, the samples were immersed in acetone for 24 h at 4°C and dried in an oven at 60°C. About 30 mg of the dried material was subjected to acid hydrolysis with 6 N hydrochloric acid (HCl) at 107°C for 18 h. HCl was then removed by evaporation and the hydrolyzed material was mixed with 200 µL buffer (5% citric acid, 1H₂O, 1.2% acetic acid, 12% sodium acetate, 3H₂O, and 3.4% sodium hydroxide, pH 6.0) diluted 1:10. The mixture was incubated with 1 mL chloramine-T solution for 20 min at room temperature and 1 mL aldehyde/perchloric acid solution was added and incubated for another 15 min at 60°C. Absorbance at 570 nm was recorded after 20 min. The concentration was estimated with a standard curve using a solution of analytical grade hydroxyproline.

Immunoblotting for TGF-β

Kidney slices were quickly washed in ice-cold phosphate-buffered saline (PBS), minced, and 50 mL buffer (10% sodium dodecyl sulfate (SDS), 20% glycerol, 0.2 M dithiothreitol, 20 mM Tris-HCl, pH 6.8) was added to the extracts. Kidney extracts were recovered in a tube, centrifuged, and boiled for 10 min. Samples were submitted to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Brazil). The proteins immobilized on the membranes were immediately blocked for 1 h at room temperature with a solution of 5% nonfat milk in Tris-buffered saline-Tween 20 (0.001% TBS-T). The membranes were then incubated with a pan-specific rabbit anti-TGF-β antibody (1.5 µg/mL; R&D Systems). After 5 washes in TBS-T (3 min each), the membranes were incubated with an anti-rabbit peroxidase-conjugated antibody (1:1000 in TBS-T; Promega, USA), washed again as described above, and the bands were developed using the West Pico Pierce kit (Pierce, USA).

To check sample loading, membranes were treated with stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7) for 30 min at 50°C, washed five times in TBS-T, and blocked again as described above. The membranes were then incubated with a rabbit polyclonal anti-actin antibody (1:3000 in TBS-T; Sigma). After 5 washes in TBS-T (3 min each), the membranes were incubated with an anti-rabbit peroxidase-conjugated antibody (1:1000 in TBS-T-milk) and developed as described above.

Statistical analysis

Data are reported as means ± SD or median and range. Comparisons between groups were done by one-way ANOVA, or by the Kruskal-Wallis test followed by the Tukey post-test, with the level of significance set at P < 0.05.

Results

Morphological changes in obstructed kidneys

The effect of subcutaneous administration of DS on the progression of renal disease was examined in mice. Histological analysis of HE-stained sections revealed an evident increase in cellular infiltration in obstructed kidneys (Figure 1). DS administration to obstructed mice clearly attenuated cellular infiltration.

Collagen

Collagen content was estimated by histomorphometry of Sirius red-stained sections and by measuring hydroxyproline in renal tissues (Figure 2), as described in Material and Methods. The histomorphometry of Sirius red-stained sections, reported as µm² (Figure 2A and B), revealed a ~7.7-fold increase in collagen deposition in the interstitium of obstructed kidneys (group UUO; median value = 3717.1) compared to group C (median value = 494.9; P < 0.05). No significant difference was observed between groups C, SH, and UUO+DS (median value = 1153.5). Collagen deposition was lower in the UUO+DS group than in the UUO group (median value = 3717.1; P < 0.05), representing a ~3.2-fold reduction.

Determination of the hydroxyproline content (µg/
mg tissue dry weight; Figure 2C) revealed a ~3.2-fold increase in obstructed kidneys (group UUO; median value = 3.21) compared to non-obstructed kidneys (group C) (median value = 1.0; P < 0.05). Daily subcutaneous administration of DS for 14 days to mice after UUO significantly reduced hydroxyproline content in the interstitial space of obstructed kidneys (group UUO+DS) to a median value of 1.7 compared to group UUO (P < 0.05; Figure 2C).

**Myofibroblast accumulation**

The increased synthesis of extracellular matrix at the site of inflammation is associated with the accumulation of myofibroblast in the renal interstitium. Thus, we questioned whether the effect of DS on collagen deposition was related to a decrease in myofibroblast accumulation in the kidney. The myofibroblast content was estimated by histomorphometry of α-SMA-stained renal sections (Figure 3A and B). When compared to group C, renal sections of obstructed mice (group UUO) showed a ~10-fold increase in myofibroblast accumulation (P < 0.05). Administration of DS to obstructed mice significantly reduced myofibroblast accumulation (group UUO+DS) to group C values (P < 0.05). No significant difference was observed between groups C and SH.

**Macrophage infiltration**

To investigate the effect of DS on macrophage infiltration, sections from control and obstructed kidneys were stained with the rat anti-mouse monoclonal antibody F4/80. Thirteen fields of renal cortex and medulla from F4/80-stained sections were captured from each animal and the results are reported as the number of macrophages per field. F4/80-positive cells increased from a median value of 0.816 for group C to 44.26 for group UUO (P < 0.05; Figure 4A and B). Administration of DS to mice after UUO decreased macrophage infiltration (median value = 19.8; P < 0.05). There was no significant difference between groups C and SH.

**Monocyte chemoattractant protein-1**

The recruitment of macrophages to the inflamed kidney depends on the release of several chemokines, including MCP-1. Therefore, we investigated the production of MCP-1 in obstructed kidneys after DS administration. The content of MCP-1 was estimated by immunohistomorphometry of renal tissue using an anti-MCP-1 antibody (Figure 5A and B). When compared to group C, renal sections of obstructed mice (group UUO) showed a drastic increase in MCP-1 content. Administration of DS to obstructed mice significantly reduced MCP-1 content (group UUO+DS) to control values (P < 0.05). No significant difference was observed between groups C and UUO+DS.

**Transforming growth factor-β**

TGF-β plays a crucial role in the progression of renal fibrosis associated with the model of UUO. The histomorphometry of stained sections, expressed as percent surface area, revealed, as expected, a ~6.5-fold increase, showing a uniform distribution within cortical
and medullary tubules in the obstructed kidneys of mice after UUO (group UUO; median value = 14.11) compared to group C (median value = 2.51; \( P < 0.05 \); Figure 4A and B). Group UUO+DS (median value = 6.38) showed a much lower content of TGF-\( \beta \) compared to group UUO (\( P < 0.05 \)) (Figure 6A and B). There was no significant difference between groups SH, C, and C+DS. Western blot analysis confirmed our immunohistochemical results for TGF-\( \beta \). There was an increase in the amount of TGF-\( \beta \) in obstructed kidneys compared to control. DS treatment drastically reduced TGF-\( \beta \) in obstructed kidneys (Figure 6C).
Kidney diseases can often lead to end-stage renal failure, which has a high morbidity and mortality. The prevalence of this condition is increasing at a rate of ~7% annually worldwide. Although several mechanisms involved in the process of fibrogenesis have been identified, no specific treatment is available that can efficiently block or reverse the progression of renal disease.

The UUO model of fibrosis has been well established and is currently used in an extensive manner (3). It is characterized by renal myofibroblast activation, tubular atrophy, and interstitial fibrosis. Myofibroblast accumulation is characterized by an increase in the content of extravascular α-SMA. This can be the result of either resident fibroblast transdifferentiation, bone marrow-derived cells or epithelial to mesenchymal transition (4). After UUO, TGF-β and its receptor are up-regulated in the renal tubular epithelium. Increased cellular infiltration, tubular atrophy and extracellular deposition are also present (3).

In the present study, we investigated the effect of DS on renal inflammation in mice submitted to UUO. The mice were treated by daily subcutaneous injection of DS (4 mg/kg) for 14 days. Ureteral obstruction produced the expected inflammatory response including morphological remodeling of the kidney, evidenced by tubular damage, interstitial edema and increased cellular recruitment. Increased TGF-β and MCP-1 production, collagen deposition, as well as myofibroblast accumulation, were also observed.
DS administration reduced tubular damage and fibrosis, as indicated by histological analysis of the obstructed kidney. This protection may be the result of combined effects of DS treatment, such as reduced TGF-β production, since this cytokine is involved in the synthesis of matrix components, including collagen, during the inflammatory process (22); reduced epithelial to mesenchymal transition, as indicated by the reduction in myofibroblast accumulation; reduced production of MCP-1 and macrophage recruitment.

The recruitment of mononuclear cells from blood to inflamed tissues requires several chemokines, such as MCP-1, macrophage inflammatory protein 1, and RANTES (3), and involves the interaction of endothelial P-selectin on the surface of activated endothelium, and P-selectin glycoprotein ligand-1 (PSGL-1), which is the common ligand of P-selectin on leukocytes. DS have been shown to bind P-selectin (16) and to inhibit P-selectin function in vitro (17). Therefore, in the context of the present study, exogenous DS that circulates in the peritubular capillary endothelium may act by inhibiting P-selectin-dependent mononuclear cell recruitment to the inflamed kidney. However, additional experiments are needed to confirm this hypothesis.

Inflammatory cells elaborate several molecules such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) during the fibrogenic-signaling phase of progressive renal disease (3). Binding of these signaling molecules to their cognate receptors expressed by resident kidney cells and infiltrating leukocytes induces the synthesis of TGF-β, which results in fibrogenesis. DS has been shown to bind several chemokines, including IL-1 and TGF-β, and

Figure 4. Macrophage infiltration into the kidney of mice submitted to different treatments. A, Macrophage infiltration was estimated by immunohistomorphometry of F4/80-positive cells in sections of kidneys of mice from group C (control kidney), group SH (sham-operated kidney), group unilateral ureteral obstruction (UUO), and group unilateral ureteral obstruction plus dermatan sulfate (UUO+DS). Data are reported as means ± SD for N = 6. The boxes represent the 25th and 75th percentiles, and the vertical bars represent the ranges, N = 30. B, The arrows point to strong F4/80-positive cells in the interstitial area compared with those with no staining in control and UUO+DS panels. g = glomerulum; i = interstitium. Scale bar = 20 µm. Magnification 400X. *P < 0.05 (ANOVA).
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Therefore, it is possible that exogenous DS from the plasma that eventually enter the renal interstitial space by the anion transport system (25) could attenuate the various chemokine-signaling events involved in the fibrinogenic process. For example, administration of DS could affect the binding of IL-1, TNF-α or MCP-1 to their receptors on the target cells, inhibiting matrix accumulation along the tubular basement membrane and within the interstitial space.

Overall, the results of the present study indicate that DS attenuates kidney inflammation and fibrosis by reducing macrophage recruitment, myofibroblast accumulation and TGF-β expression. Therefore, considering the lower blood clotting effects of DS compared to heparin, and its significant anti-inflammatory effect, this glycan is a potential therapeutic option for the treatment of renal inflammation and fibrosis.

**Acknowledgments**

We thank Alison R. Junior and Cesônia A. Martinsuso, Laboratório Multidisciplinar, Hospital Universitário Clementino Fraga Filho, UFRJ, for technical support. Research supported by CNPq, FAPERJ, the NIH Fogarty International Center (#R03 TW05775, to M.S.G. Pavão), and the Mizutani Foundation for Glycoscience (#RN:080002, to M.S.G. Pavão). M.S.G. Pavão is the recipient of a CNPq research fellowship.
Figure 6. Transforming growth factor beta (TGF-β) content in kidneys from mice submitted to different treatments. A, Immunohistomorphometry of anti-TGF-β-stained sections of kidneys of mice from group C (control kidney), group sham-operated kidney (SH), group unilateral ureteral obstruction (UUO), and group unilateral ureteral obstruction plus dermatan sulfate (UUO+DS). The horizontal bars represent medians, the boxes represent the 25th and 75th percentiles, and the vertical bars represent the ranges, N = 30. B, The arrows in the UUO panel show strong TGF-β-stained areas that seem to be near epithelial tubular cells. Note the less intense staining in control and UUO+DS panels. g = glomerulum; i = interstitium. Magnification 400X. C, The content of TGF-β in rat kidney was analyzed by Western blot using anti-TGF-β. The amount of the load was normalized by the content of actin, as described in Material and Methods. Scale bar = 20 µm. *P < 0.05 (ANOVA).

References

7. Rosenberg JS, Rosenberg RD. Advances in the understand-
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