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Determination of heparan sulphate in kidney tissues of patients with calcium nephrolithiasis

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Abstract While the pathogenic mechanisms responsible for calcium nephrolithiasis remain unknown, the influence of heparan sulphate proteoglycan (HSPG) on disease progression of other diseases, such as polycystic kidneys and diabetic glomerulosclerosis, makes it an important candidate for the study of stone formation. Using the indirect immunofluorescence assay and image analysis, we were able to quantify and visualize the loss of HSPG localized in the basement membrane of the glomerulus and the mucosa of ureter or renal pelvis in patients with recurrent calcium nephrolithiasis as compared to normal subjects. However, no significant change in HSPG was observed in the basement membrane of the tubular epithelium. The decreased HSPG in the glomerulus may reflect the potentially disrupted anion/neutral barrier for glomerular filtration, which would encourage the accumulation of stone solutes. The drop in HSPG staining intensity in the basement membrane of the mucosa of ureter/renal pelvis may suggest the tendency of adhesion of crystal to urothelial surfaces. Based on these immunological data, it appears that HSPG plays a modulatory role in the pathogenesis of this disease.

Key words Calcium nephrolithiasis · Heparan sulphate · Renal tissues

Calcium nephrolithiasis is a common disorder in men, with an estimated incidence rate in industrialized countries of 0.1–1% and a prevalence rate of 5–10%. Most patients suffer from stone recurrence, with the average rate of new stone formation found to be 2–3 years [10]. This disease is characterized by the presence of calcium oxalate and/or calcium phosphate stones in the urine.

An important prerequisite for stone formation is crystal aggregation and for this to occur the urine needs to be supersaturated with respect to calcium, oxalate and/or phosphate. All human urine is metastably supersaturated with stone solutes for most of the day. Fortunately, crystallization will not occur in normal urine because it possesses the ability to inhibit crystal development. Inhibitory activity has been ascribed to several high and low molecular weight urinary constituents and others such as Tamm-Horsefall uromucoid and acid glycosaminoglycan (GAG) have been described as both promoters and inhibitors of calcium oxalate crystallization. It is proposed that these inhibitors function by adsorption onto the crystal surface according to the principles of the Langmuir theory of adsorption equilibrium: the more negative they are, the better the inhibitory activity [2].

Heparan sulphate is the major acid GAG constituent of the glomerular basement membrane (GBM) [4]. It is present in tissues as a proteoglycan and in the urine as metabolic turnover product of the tissue proteoglycan [13]. It is apparent that in normal adults the growth-inhibitory activity of urinary heparan sulphate tends to balance out its promoting activity. In addition to this, in a study on the influence of urinary heparan sulphate on crystallization of urinary calcium oxalate, the growth inhibitory activity in stone formers was suggested to be generally lower than that in normal control subjects [12].

Heparan sulphate proteoglycan (HSPG) plays a modulatory role in cell adhesion, and is involved in variable types of cell behaviour such as cell migration, proliferation and differentiation [14]. Interest in it was further stimulated in recent years by the realization that it also plays an integral role in the pathology of the

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glomerulus. Its influence on disease progression was demonstrated previously when kidney tissues of patients with polycystic kidneys showed a lower level of HSPG in tubular basement membrane (TBM) than controls [3]; and changes in HSPG distribution were observed in membranous glomerulonephritis [7]. As no data are available at present regarding its influence on kidney stone formation, the aim of this study is to determine whether there is any difference in quantity and/or distribution of HSPG in the basement membrane of kidney tissues of stone formers.

Material and methods

Antibodies and reagents

A monoclonal antibody towards human HSPG and anti-mouse fluorescein isothiocyanate (FITC) conjugated immunoglobulin were purchased from Boehringer Mannheim (Singapore). Oct freezing medium was obtained from Mile Diagnostic Division and Fluoprep embedding medium was purchased from BioMerieus Laboratory Reagents and Instruments. All other organic solvents and reagents were obtained from BDH Laboratory Supplies (Poole, UK) and Sigma (St. Louis, USA).

Tissue sources

Kidney biopsies from the outer cortex and/or mucosa of the renal pelvis were obtained from nine patients (age range 24–58 years) with calcium nephrolithiasis who underwent percutaneous stone extraction. Thirty-two normal tissue samples were taken during transplantation at the time of bench surgery. Fine outer cortex and a short segment of ureter from these normal subjects were taken as controls (age range 22–63 years). Specimens were cut into approximately 0.5-cm^3 pieces immediately after being received from the operating theatre. They were snap frozen in Oct freezing medium in liquid nitrogen and kept at $-70\,°C$ until use.

Tissue processing

Specimens were sectioned into 5-μm thick slices using a Microm HM 500 cryostat at $-20\,°C$. They were then placed on poly-L-lysine-coated slides for haematoxylin and eosin staining for both light microscopy and immunohistochemistry. Those to be used for immnofluorescence studies were prefixed with acetone for 10 min at $-20\,°C$. Excess fixant was allowed to evaporate at room temperature. They were then wrapped in aluminium foil and stored at $-70\,°C$ until use.

Indirect immunofluorescence studies

Endogenous peroxidase was blocked by incubating with 0.6% hydrogen peroxide in phosphate-buffered saline (PBS), followed by non-specific protein blocking with bovine serum albumin (BSA) and serum. Tissue sections were then incubated overnight with HSPG antibody (1:200) in a moist chamber at 4 °C. FITC-labelled immunoglobulin (1:50) was then applied for 1 h at room temperature in the dark. Sections were temporarily preserved by mounting with Fluoprep embedding medium. Each procedural step was followed by PBS rinsing. For each individual patient and normal specimen, a respective negative control section was included in which primary antibody was omitted.

Computer-based densitometry analysis

Following the immunofluorescence assay, individual sections were then examined under the laser scanning microscope (Carl Zeiss, Germany) using a $\times 20$ objective with a zoom factor of 20. At least three different areas from each section, including the respective control, were chosen randomly and enlarged to an 7×6.5-in. print, with a final magnification of $\times 400$. The photographic images were captured immediately and were digitized with the Kontron image analyser (Carl Zeiss, Germany). One pixel represented 1.29 μm^2 of the digitized image. Vidas 2.1 software was used for grey-scale calibration of each photograph, for which the lightest pixel represented a grey-scale value of 255 and the darkest pixel represented a grey-scale value of 0. This allowed the full grey scale to be utilized, and an objective comparison was made between images of variable intensities. Any possible non-specific background of the sections was cancelled out by including the respective control in the analysis and calculation.

Analysis was carried out by segmenting the regions of interest. The staining density was then analysed and the average grey-scale value against the respective control was calculated for each image. Results were expressed in grey value per unit area with treated sections over controls.

For each study group (staining intensity of GBM, TBM or basement membrane of the mucosa of ureter/renal pelvis), the mean and standard deviation (SD) of the total number of cases studied were calculated. The unpaired Student's t-test was then used to determine P-values of the mean staining density differences between patients and normal adults of each group using SPSS statistical software.

Results

Reactivities for HSPG were observed in GBM, TBM, Bowman's capsule and basement membrane of the tubules and blood vessels in normal and patient specimens (Fig. 1a, b). A distinct and continuous staining pattern was also observed among the basement membrane of the normal ureter (Fig. 2a). Among the stone patients, the basement membrane of the pelvis mucosa was shown to have a diminishing discontinuous and uneven staining pattern though occasional residual distinct staining was also observed (Fig. 2b). Control staining was consistently negative.

Using computer-based densitometry and statistical analysis, the mean staining intensity and SD of each study group were calculated. By using the unpaired Student's t-test, differences between the staining intensities in GBM, TBM and basement membrane of the mucosa of ureter/renal pelvis in normal and stone patients were determined (Table 1).

Staining intensities demonstrated a significant loss of HSPG reactivities in GBM among the stone patients compared with that in the GBM among the normal subjects ($P < 0.05$). No significant difference was observed in TBM between these two groups ($P > 0.05$) (Fig. 1a, b). In the study of intensities of the mucosa basement membrane between that found in the normal ureters and patient pelvis, significant loss of HSPG

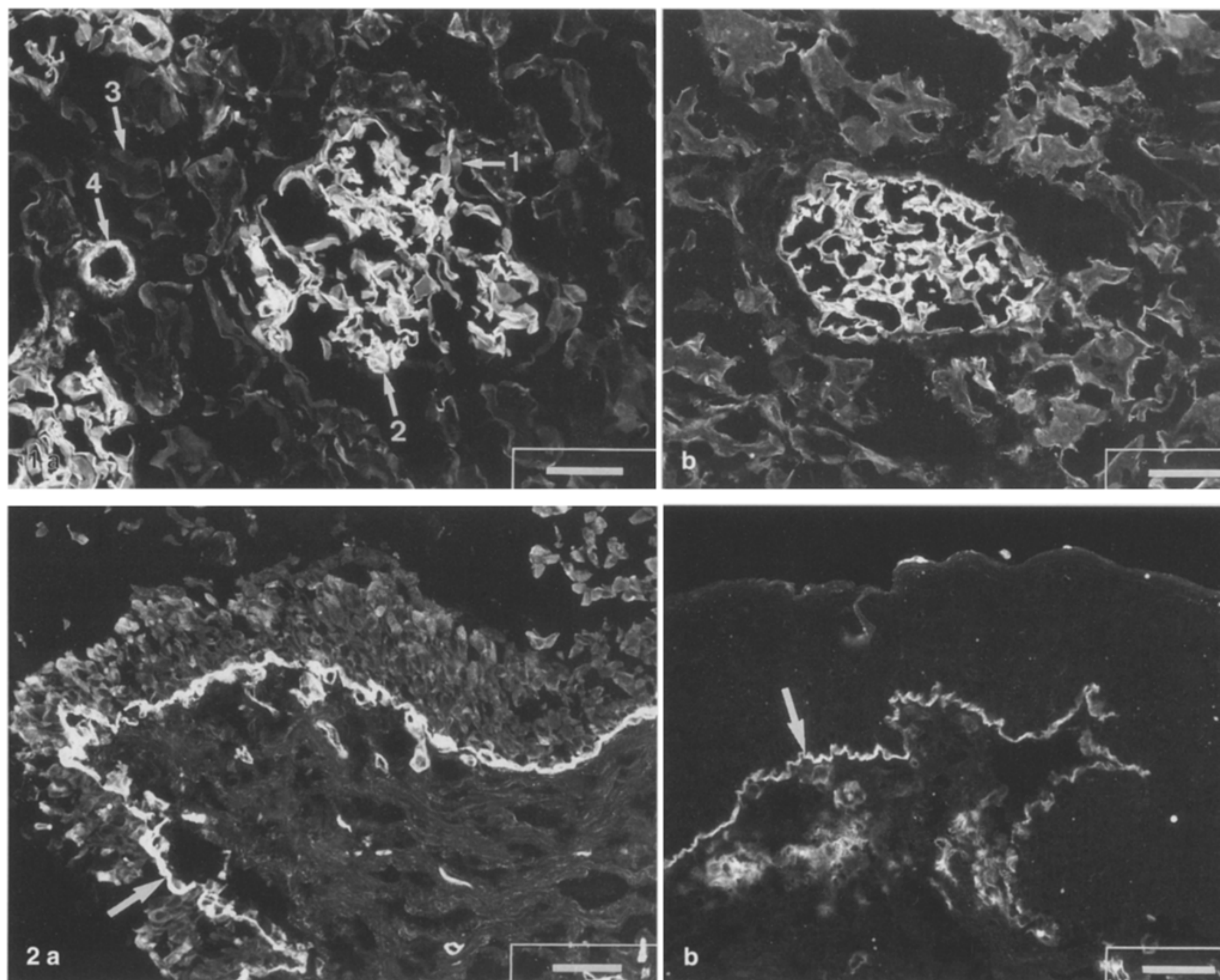

Fig. 1 **a** Indirect immunofluorescence of anti-HSPG antibody on cryostat sections of normal kidney. High staining intensity is observed in the GBM and less of TBM. *Arrow 1* Bowman's capsule, *arrow 2* GBM, *arrow 3* TBM, *arrow 4* basement membrane of blood vessel. *Bars* 50 μm. **b** Kidney section of a patient with calcium nephrolithiasis stained with the same antibody

Fig. 2 **a** Mucosa section of normal ureter stained with anti-HSPG antibody. Note the brilliant staining on the basement membrane (*arrow*). **b** Mucosa section of patient pelvis stained with the same antibody. Note the faint staining on the basement membrane (*arrow*) compared with that of the normal mucosa section

Table 1 Basement membrane HSPG intensities

Tissue	Normal	Stone formers	P value
Glomerulus	6.51 ± 2.56 ($n = 17$)	5.12 ± 0.20 ($n = 5$)	0.042 Significant
Tubules	1.77 ± 0.48 ($n = 20$)	1.56 ± 0.36 ($n = 6$)	0.342 Not significant
Mucosa, ureter/pelvis	2.59 ± 1.02 ($n = 19$)	1.82 ± 0.40 ($n = 7$)	0.009 Significant

reactivity among the stone patients was observed ($P < 0.05$).

Discussion

In this study the localization of HSPG in human kidney cortex and ureter was characterized using an indirect immunofluorescence assay. Under the laser scanning microscope, HSPG was observed within GBM, basement membrane of tubular epithelium, Bowman's capsule and basement membrane of the vessels in kidneys of both normal and stone patients. It has been shown that there are, at least, two antigenically different types of basement membrane HSPGs present in the kidney: an antibody that is directed to a small high-density HSPG produced prominent staining in the normal GBM with the TBM being stained less intensely, whereas an antibody towards the large

low-density HSPG stained TBM more intensely. The anti-human HSPG antibody used in our experiment was obtained through postimmunization with purified bovine glomeruli-HSPG. It has a similar distribution pattern to the anti-high-density HSPG antibody [1].

We have demonstrated a decreased staining of HSPG in GBM and basement membrane of the mucosa of ureter/renal pelvis in patients with recurrent calcium nephrolithiasis. It is not shown from our study whether this loss in HSPG staining is due to an altered antibody-epitope interaction, loss of the epitope or loss of HSPG. Nevertheless, decrease of HSPG staining in the GBM is not unique for calcium nephrolithiasis. In diabetic nephropathy, staining of HSPG was found to be decreased in GBM [8, 9]. In normal physiology, HSPG is known to be of importance for the integrity of the glomerular size and charge barrier. Its decrease in staining density in the glomerulus of stone patients may once again reflect a potential impairment in filtering capacity which encourages the aggregation of solutes and thus development of stones.

In our study, no significant difference in HSPG staining intensity was observed between normal and stone patients at the TBM. The possible role of HSPG in TBM, to our knowledge, has not been identified. It may be explained by the lower amount of HSPG detected by our type of antibody in the TBM, which makes the difference unnoticeable. However, the function of the tubules varies and further study is needed to quantify HSPG at the various segments of the long tubule.

The decrease in HSPG staining demonstrated in the basement membrane of the mucosa of ureter/renal pelvis in stone-forming patients may also be of great significance. The reason for this drop may be related to the fact that the cell-surface GAG secreted by the transitional epithelium play a role in maintaining urothelial impermeability and antibacterial adherence [6, 11]. Parsons et al. [11] postulated that the extreme hydrophilic nature of the sulphated moieties of this compound which bind several water molecules could place a water barrier between the transitional epithelial cells and urine, thereby masking highly charged moieties on the cell surfaces and impairing the binding of bacteria or crystals. Other evidence has also suggested that some GAGs can restore anticrystal adherence properties to injure urothelium [5]. It is apparent that HSPG acts as an antiadherence factor to protect the urinary system against its environment. Its loss in reactivities in the basement membrane of the mucosa of ureter/renal pelvis in stone-forming patients is suggestive of an alteration of this intrinsic anatomical protection which predisposes to and promotes adhesion of calcium crystals and other substances to urothelial surfaces.

In conclusion, our present study suggests that the decrease in HSPG staining in GBM and basement membrane of the mucosa of ureter/renal pelvis may have pathogenic relevance in, firstly, facilitating stone formation and, secondly, adherence to the urothelium. The role of HSPG in stone formation remains speculative. Further study will be carried out to correlate changes of other interacting extracellular matrix components such as chondroitin sulphate, collagen IV, laminin and fibronectin in these patients.

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