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Quantitative analysis on the localization of chondroitin sulfate proteoglycan in renal tissues of patients with calcium nephrolithiasis

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Abstract Previous studies have shown a significant decrease of heparin sulfate proteoglycan (HSPG) in the basement membrane of the glomerulus and the mucosa of the ureter/renal pelvis in patients with calcium nephrolithiasis. In this study, we looked at the localization of another influential proteoglycan, chondroitin sulfate (CSPG), using similar study groups by indirect immunofluorescence staining. Microscopic images were digitized and image analysis was used to quantitate the staining intensity of CSPG present in the basement membrane of the nephron. Our data showed significant loss of CSPG in the Bowman's capsule and the basement membrane of the mucosa of the ureter/renal pelvis using Mann-Whitney U-Wilcoxon Rank Sum W test with *P*-values of 0.0043 and 0.0041, respectively. However, absence of staining was noted in the basement membrane of the glomerulus and no significant change in the basement membrane of the tubular epithelium was observed. In conclusion, our results showed changes in the localization of CSPG in the basement membrane of the nephron, accompanied with HSPG, which may contribute to the pathological condition of calcium nephrolithiasis.

Key words Chondroitin sulfate · Renal tissues · Calcium nephrolithiasis · Immunofluorescence

Introduction

The events that initiate formation of a renal stone are not well defined, though disorders that raise supersaturation and promote heterogeneous nucleation of calcium oxalate monohydrate (COM) crystals are the presently accepted causes of nephrolithiasis [9]. Recent studies have shown COM, the most abundant crystalline component of kidney stones; to be bound and internalized into cultures of monkey renal epithelial cells [5, 10–12]. This provides a mechanism whereby crystals could be retained in the nephron and subsequently form calculi. The nucleation, growth, adhesion and aggregation of crystals to urothelial surfaces are believed to be critical for stone growth. However, such interactions could potentially be blocked by specific polyanions, and human urine normally contains compounds that can accomplish all three functions [e.g. citrate, glycosaminoglycans (GAG), uropontin, nephrocalcin] [11, 12].

GAG, one of the above-mentioned polyanions, occur in tissues in covalent bonds with proteins and in tissues as proteoglycans (apart from hyaluronic acid). Although present as minor components of basement membrane, proteoglycans have nevertheless been shown to be important in the assembly and function of the structure as a whole. GAG may have a role in preventing the cellular uptake of COM crystals. Kohijmoto and his co-workers [11] have demonstrated a significant reduction in the cellular uptake of COM crystals when Madin-Darby canine kidney cells were pretreated with each of the GAG (sodium pentosan polysulphate, heparin and chondroitin sulfate C). GAG has also been associated with idiopathic oxalate stone formation because of its promoting and inhibitory abilities in crystal growth and aggregation in all crystallization models studied. It has been shown that in stone patients, their urinary GAG have a lower level of growth-inhibitory activities than in normal people [2, 19].

Heparan- and chondroitin-sulfate proteoglycan are two of the GAG present in the renal basement membrane. Their exact composition in various renal diseases is not known. We have demonstrated, in our previous studies, loss of HSPG localization in the basement membrane of the glomeruli and the mucosa of ureter/renal pelvis in stone-forming patients using indirect

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immunofluorescence assay [3]. As the nature of GAG involved in stone-formation in urine is still uncertain, continued studies on the localization of CSPG may provide direction.

Materials and methods

Antibodies and reagents

A monoclonal antibody towards human CSPG was purchased from Sigma Immunochemical (St Louis, Mo.) and anti-mouse fluorescein isothiocyanate (FITC) conjugated immunoglobulin from Boehringer Mannheim (Singapore). Optimal cutting temperature (OCT) freezing medium was obtained from Mile Diagnostic Division (California, USA) rescent Mounting and Fluorescent Mounting medium was purchased from Dako Corporation, Carpinteia, USA.

Tissue sources

Twenty-four normal tissues 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 26–69 years). Since it is not practical surgically to take any segment of ureter from patients with calcium nephrolithiasis, kidney biopsies from the outer cortex and/or mucosa of the renal pelvis were obtained from 16 patients (age range 23–72 years) with calcium nephrolithiasis who underwent percutaneous stone extraction. Specimens were cut into approximately 0.5-cm^2 pieces immediately after being received from the operation theatre. They were snap-frozen in OCT freezing medium in liquid nitrogen and were kept at −70°C until use.

Tissue processing

Specimens were sliced into 5-μm thick sections using Microm HM 500 cryostat (Carl Zeiss, Germany) at −20°C. They were then placed on poly-L-lysine-coated slides and pre-fixed with acetone for 10 min at −20°C. Excess fixative was allowed to evaporate at room temperature. They were then wrapped in aluminium foil and stored at −70°C until use.

Indirect immunofluorescence studies

Non-specific protein binding was blocked by BSA and serum. Tissue sections were then incubated overnight with CSPG antibody

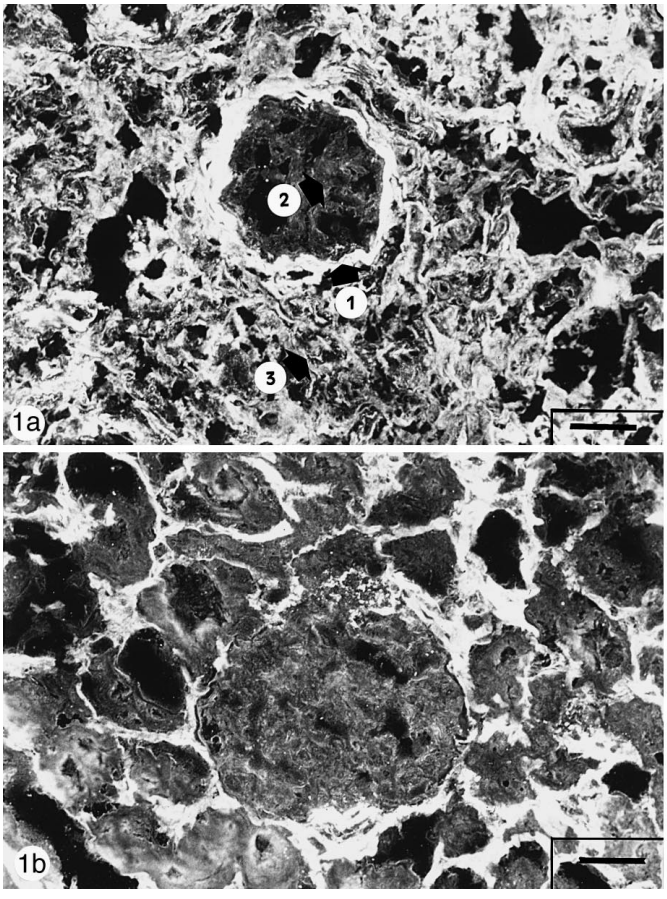

Fig. 1(a) A fluorescence micrograph of normal outer cortex stained with the monoclonal antibody chondroitin sulfate CSPG. Note that the basement membrane of the Bowman's capsule and tubules were both positively stained with this antibody except that of glomerulus. *Arrow 1* Bowman's capsule; *arrow 2* glomerulus basement membrane (gene), *arrow 3* tubules (TB). **(b)** Kidney section of a patient with calcium nephrolithiasis stained with the same antibody. (Bar = 50 μm)

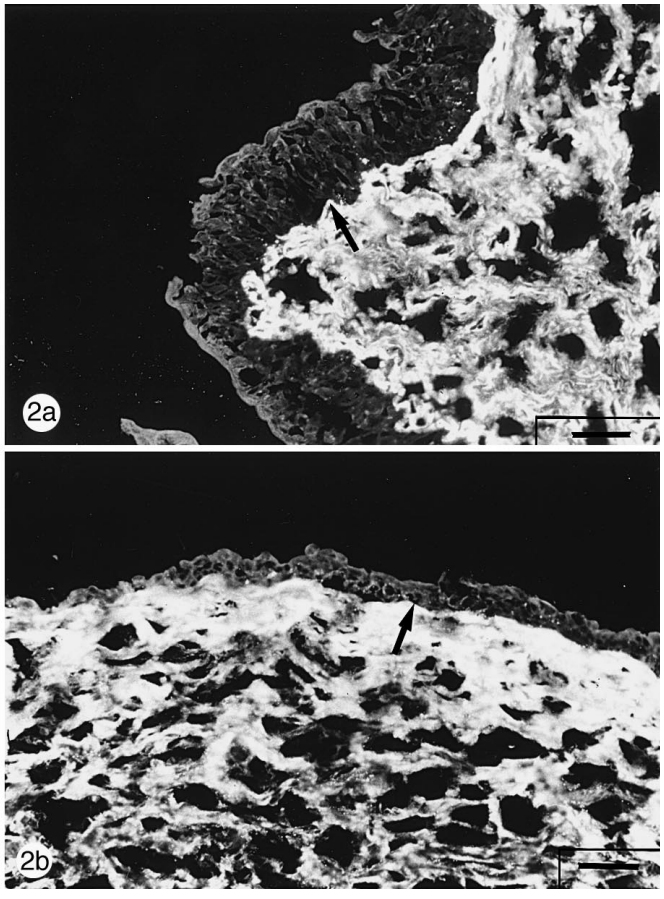

Fig. 2(a) A fluorescence micrograph of a section of normal ureter stained with anti-CSPG antibody. Note the intense staining of the basement membrane (*arrows*) and its surrounding tissues. **(b)** Mucosa section of patient pelvis stained with the same antibody. (Bar = 50 μm)

(1:200) in a moist chamber at 4°C. FITC-labeled immunoglobulin (1:20) was then applied for 1 h at room temperature in the dark. Sections were temporarily preserved by mounting with Fluorescent Mounting medium. Each procedural step was followed by PBS rinsing. For each individual test and normal specimen, a corresponding 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 laser scanning microscope (Carl Zeiss) using a ×20 objective lens with a zoom factor of 20. Analysis was carried out according to Chan et al. [3] and only the normal-looking glomeruli and tubules were examined. Results were expressed as ratios of grey values per unit area of treated over control specimens. For each study group [staining intensity of basement membrane of the glomerulus (GBM), tubules (TBM), Bowman's capsule or mucosa of ureter/renal pelvis], the mean and standard deviation of the total number of cases studied were calculated. As the sample sizes are less than 30 and contain data that do not fall under normal distribution, Mann-Whitney U-Wilcoxon Rank Sum W test was used to determine *P*-values of the mean staining density differences between stone patients and normal adults of each group using SPSS statistical software.

Results

Under normal laser scanning microscopy, CSPG staining was observed in the basement membrane of Bowman's capsule and in all surrounding tubules. No significant immunoreactivity was observed in the basement membrane of the glomerulus, although residual staining could be seen within the core occasionally (Fig. 1a, b). We suspect that the residual staining is due to the presence of CSPG in the basement membrane of the mesangial matrix, as reported by a number of laboratories which examined the tissues using electron microscopy [13, 14]. Strong immunostaining was also observed in the basement of the mucosa of the ureter/renal pelvis and its surrounding tissues (Fig. 2a, b). No staining was observed in any control tissues when primary antibody was replaced by normal serum.

Significant loss of CSPG immunoreactivity was observed in the Bowman's capsule and the basement membrane of the mucosa of the ureter/renal pelvis in stone patients using Mann-Whitney U-Wilcoxon Rank Sum W test with *P*-values of 0.0043 and 0.0041, respectively. However, no significant changes were noted in the basement membrane of the tubular epithelium (Table 1).

Table 1 Basement membrane Chondroitin sulfate (CSPG) fluorescent intensities

Tissue (basement membrane)	Normal (mean ± SD)	Stone-formers (mean ± SD)	*P*-value
Bowman's capsule	2.02 ± 0.59 (n = 21)	1.47 ± 0.19 (n = 10)	0.0043
Tubules	1.90 ± 0.91 (n = 21)	1.72 ± 0.56 (n = 10)	0.5123
Mucosa ureter/pelvis	6.63 ± 2.44 (n = 24)	4.57 ± 1.95 (n = 16)	0.0041

Discussion

CSPG is a constituent of most basement membranes, a major exception being the GBM. Because of its absence it has been suggested as having no important role in the assembly or maintenance of GBM structure (17). A connection between urolithiasis, GAG, and crystal adhesion to urothelial surfaces has long been suspected; however, the events that initiate stone formation are as yet unknown. Previous investigators have described the presence of CSPG in the basement membranes of developing tissues. CSPG may be involved in the mesenchymal–epithelial interactions that are required in the stabilization of branching morphogenesis [1, 6]. Yamagata and his co-workers [21] have shown CSPG to be able to interact with cell surfaces and modulate cell adhesion to extracellular matrix in chick embryo fibroblasts. Consistent with this, GAG has been suggested as having a role in maintaining urothelial impermeability and antibacterial adherence [4, 8, 18]. It does this by blocking the growth sites on the crystals, perhaps through crystal adhesion, thus preventing or delaying development of crystal [7]. The decreases in CSPG staining in the basement membrane of Bowman's capsule and the mucosa of ureter/renal pelvis in stone-forming patients suggest the possibilities of detachment or decrease of CSPG on urothelial surfaces caused by an imbalance in GAG production or tissue injuries. Alteration in the basement membrane component described above encourages stone crystals in urinary fluid along the nephron to anchor onto the basement membrane. This serves as a nidus for crystal aggregation and permits kidney stones to form [5, 7, 11].

In our study, all the specimens were collected from the surface of the outer cortex of the kidney due to surgical limitations. Most of the tubules present in this region of the kidney are the proximal tubules, surrounded with some distal, junctional tubules and collecting ducts. We did not expect to find no significant change in CSPG in the TBM. We suspect the insignificance may have been caused by the fact that the TBM we were examining were mostly the proximal tubules in the specimens collected. As the function of the tubules varies, we feel the need to quantify CSPG at various segments, especially the distal portion of the tubules.

While the pathogenic mechanisms responsible for calcium nephrolithiasis remain unknown, it is tempting to speculate that the increased tendency of stone formation and/or adhesion to urothelial surfaces is related primarily to an uncoordinated control mechanism in basement membrane turnover. Matrix metalloproteinases and their inhibitors are important regulators in the remodeling of basement membrane, therefore, we are currently investigating the gene expression of these proteins. Further studies to correlate changes of other

interacting basement membrane components such as collagen IV, laminin and fibronectin, in stone patients will also give us more insights on stone development.

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