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# Expression of heparan sulfate proteoglycan mRNA in rat kidneys during calcium oxalate nephrolithiasis

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**Abstract** This study used reverse transcription polymerase chain reaction (RT-PCR) to examine heparan sulfate proteoglycan (HS-PG) mRNA expression levels during stone formation in the rat kidney. Total RNA in kidneys was extracted and converted to cDNA. PCR products were resolved by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide. Fragment intensity and area were measured using an image analyzer. Control cyclophilin and HS-PG mRNAs were expressed in all samples examined as 235 bp and 506 bp bands, respectively. Cyclophilin expression in the normal group was not significantly different from expression in the group that formed stones. However, the level of HS-PG mRNA expression apparently increased in calcium oxalate (CaOx) microlith. The findings suggest an association between CaOx nephrolithiasis and expression of HS-PG in the rat kidney.

**Key words** Calcium oxalate stone · Heparan sulfate proteoglycan · Heparan sulfate · Rat kidney · Reverse transcription polymerase chain reaction

## Introduction

Urinary stones contain an organic matrix that accounts for 2%–3% of their weight [3]. Nishio et al. [16], as well as Roberts and Resnick [21], reported that the glycosaminoglycans (GAGs) in the calcium stone matrix consist mainly of heparan sulfate (HS) and hyaluronic acid. Recently, Yamaguchi et al. [26] and Suzuki et al. [24] reported that HS is selectively included in calcium oxalate (CaOx) crystals. However, the reason for this remains unclear. Chan and Tan [5] investigated the levels of heparan sulfate proteoglycan (HS-PG) in tissues from calcium nephrolithiasis patients and in those from normal controls. They found that HS-PG levels apparently did not change in the tubular basement membrane. However, their immunological study suggested that a decrease in HS-PG in the basement membrane of the glomerulus and upper urinary tract tends to increase the adhesion of CaOx crystals to urothelial surfaces. In contrast, Nishio et al. [17] reported that HS production increases in rat renal tubules during microlith formation. Their study indicated why HS was selectively included in the CaOx stone matrix. However, it remains unclear whether the changes observed in HS levels are absolute or relative. In the present study, we examined HS-PG mRNA expression levels during stone formation in the rat kidney using a reverse transcription polymerase chain reaction (RT-PCR). Moreover, we quantified the PCR products by means of image analysis.

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## Materials and methods

### Formation of CaOx microlith in the rat kidney and tissue preparation

Eighteen male Wistar rats weighing 200–250 g were divided into three groups (six rats each) and administered 0.5% ethylene glycol daily and 0.5 μg of 1α-OH-$D_3$ every other day by gavage [18]. Animals were killed after 1 (SF-1), 2 (SF-2), and 3 weeks (SF-3) and both kidneys were removed. The right kidney was stored in liquid nitrogen and the left was fixed in 20% formalin for histochemical studies. Twelve normal control rats were divided into three groups (four rats each), then killed, and their kidneys processed in the same manner.

### First strand cDNA synthesis

Total RNA was extracted from rat kidney tissues using RNAzolB (Tel-test, Tex.). Tissue samples (50 mg) were minced and shaken in 1000 μl of RNAzolB. The mRNA in the total RNA was converted to cDNA using reverse transcriptase (Superscript II RT, Life Technologies). The total RNA concentration in each sample was adjusted to 5 μg in 12 μl of diethyl pyrocarbonate (DEPC)-treated

water. Thereafter, 50 ng of random primer (random hexamer) and 40 U of RNase inhibitor (Wako Pure Chemical, Osaka, Japan) were added. The RNA sample mixture was incubated at 70°C for 10 min, then quick-chilled on ice for at least 1 min. The RNA sample mixture was then mixed with 2 μl of 10 × PCR buffer, 2 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mixture, 2 μl of 0.1M DTT and 200 U of Superscript II RT (Gibco BRL, Life Technologies). The RNA sample mixture was incubated at 25°C for 10 min, at 42°C for 50 min, at 90°C for 5 min, then placed on ice for 10 min.

### PCR analysis

We developed primers specific to rat HS-PG (syndecan) core protein mRNA as follows: sense primer, 5′-TGAAGAAGAAGG-ACGAAGGC-3′ corresponding to nucleotides 965–985 of rat HS-PG (syndecan) core protein mRNA; anti-sense primer, 5'-CCGGATGATGTTGAATTGG-3′ corresponding to nucleotides 1451–1470 of rat HS-PG (syndecan) core protein mRNA [4]. HS-PG mRNA expression was investigated by RT-PCR using the specific HS-PG primer. To assess variability in the mRNA content, cyclophilin mRNA was amplified simultaneously [9] because it is expressed in virtually all types of tissues and seems to have been highly conserved throughout mammalian evolution [6]. The DNA sequences of primers for cyclophilin were as follows: sense primer, 5′-TTTATGTGTCAGGGTGGTGACTTCA-3′ corresponding to nucleotides 192–216 of human cyclophilin mRNA; antisense primer, 5′-TATTCATGCCTTCTTTCACTTTGC-3′ corresponding to nucleotides 403–426 of human cyclophilin mRNA [9]. The PCR reaction mixture (25 μl) included 2 μl of the cDNA product, 0.65 U of recombinant *Taq* DNA polymerase (Takara Shuzo, Kyoto, Japan), reaction buffer (10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM of each deoxyribonucleoside triphosphate (dNTPs), and 1.0 μM of each primer. Cyclophilin was amplified by 35 cycles of denaturation at 93°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 90 s. HS-PG was amplified by 35 cycles of denaturation at 93°C for 30 s, annealing at 57°C for 60 s and extension at 72°C for 60 s using the Program Temp Control System PC-700 (Astec, Tokyo, Japan). PCR products were resolved by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The intensity and area of each fragment were measured using an image analyzer (Gel Doc 1000, Bio-Rad, Calif.). DNA size standards (AmpliSize Standard No. 170–8200, Bio-Rad) were also electrophoresed as controls. Samples were analyzed three times by PCR and the results are expressed as average values. We also sequenced the PCR products and compared them with those of cDNA clones.

### Histochemical study

The left kidneys from the normal and experimental groups were stained with hematoxylin-eosin (HE).

### Statistical analysis

Data are expressed as means ± standard deviation. Differences between data sets were analyzed by the Mann-Whitney test. A level of $P < 0.05$ was considered to be statistically significant.

Principles of laboratory animal care and Japanese law were followed.

## Results

### PCR analysis

Cyclophilin and HS-PG mRNAs were expressed in all normal and experimental rat kidneys as single bands of 235 bp and 506 bp, respectively (Fig. 1). Cyclophilin bands were stained at an equal intensity, whereas HS-PG bands from normal kidney were less intensely stained than those from the stone-forming (SF) group. The sequences of the PCR products matched those of the cDNA clones (data not shown). We also quantified the stained bands using an image analyzer. The relative expression levels of cyclophilin were not significantly different between the normal and SF groups. However, the level of HS-PG mRNA appeared to increase in rats with CaOx stone formation. (Table 1).

### Histochemical study

Figure 2 shows photomicrographs obtained using cross-polarizing light in each group. Figure 2A shows proxi-

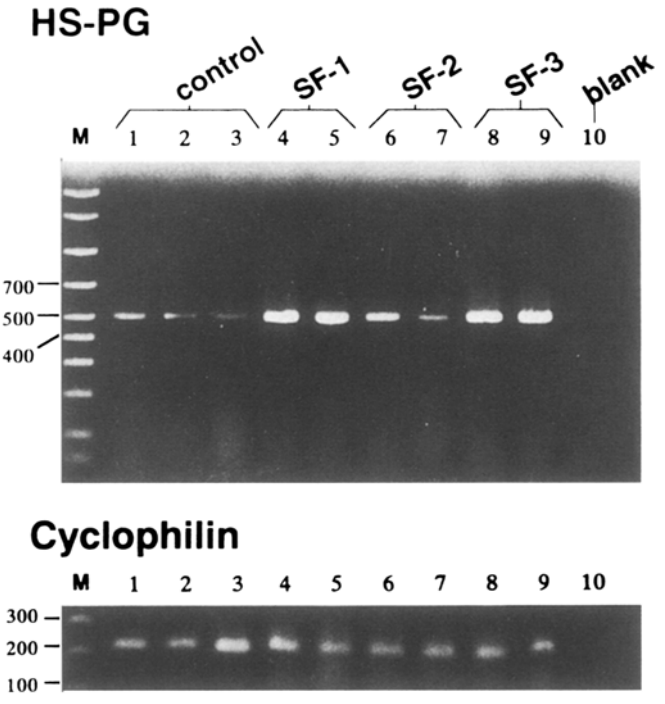


**Fig. 1** Heparin sulfate proteoglycen (HS-PG) analysis by means of reverse transcription polymerase chain reaction (RT-PCR). These PCR products were run indivdually on 1.5% agarose gel. Amplified HS-PG or cyclophilin cDNA from the normal control group and the stone-forming (SF) group. *Lanes 1–3* normal control ($n = 4$ in each group, *lane 1* killed after 1 week, *lane 2* killed after 2 weeks, *lane 3* killed after 3 weeks), *Lanes 4 and 5* SF group 1 ($n = 6$, killed after 1 week), *lanes 6 and 7* SF group 2 ($n = 6$, killed after 2 weeks), *lanes 8 and 9* SF group 3 ($n = 6$, killed after 3 weeks). *Lane 10* is a blank control obtained without cDNA

**Table 1** Relative mRNA levels of heparin sulfate proteoglycan (HS-PG) and cyclophilin in rat kidneys. Values are means ± SD. *SF* stone forming group

| | HS-PG | Cyclophilin | |
|---|---|---|---|
| Normal 1 ($n = 4$) | 92.00 ± 44.75 | 141.97 ± 43.18 | NS |
| SF 1 ($n = 6$) | 288.56 ± 155.43* | 103.21 ± 27.17 | |
| Normal 2 ($n = 4$) | 83.70 ± 66.98 | 184.52 ± 97.56 | NS |
| SF 2 ($n = 6$) | 187.71 ± 42.04* | 147.86 ± 34.63 | |
| Normal 3 ($n = 4$) | 88.57 ± 21.38 | 218.56 ± 66.63 | NS |
| SF 3 ($n = 6$) | 233.61 ± 54.41** | 242.46 ± 49.90 | |

significantly different from normal, $^*P < 0.05$, $^{**}P < 0.01$

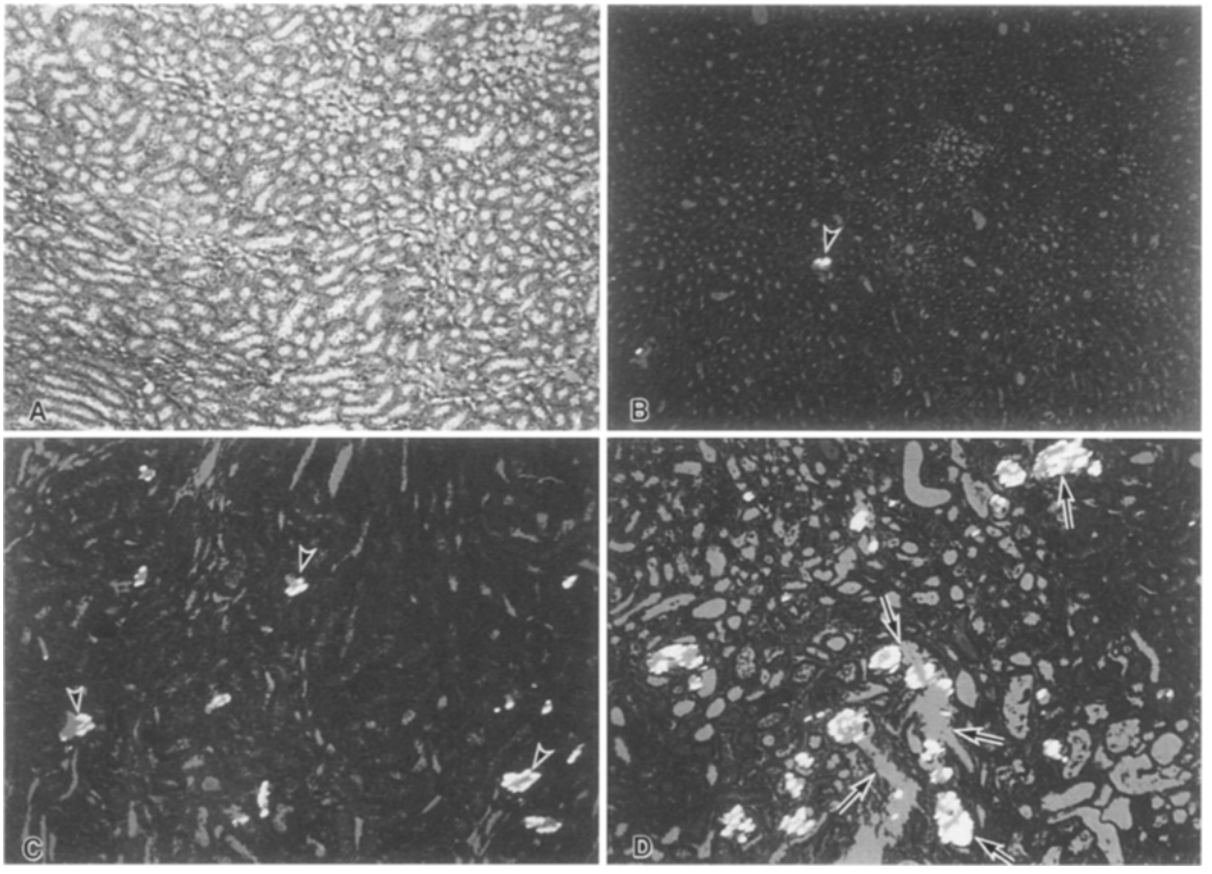


**Fig. 2A–D** Polarized light photomicrograph of HE-stained rat kidneys (original magnification: × 40). **A** Distal tubules in normal rat kidney. **B** Several CaOx crystals are apparent in the distal tubules of an SF-1 rat. **C** CaOx crystals are evident in the kidney of an SF-2 rat. **D** Numerous crystals are apparent in the dilated and partially destroyed distal tubules of an SF-3 rat

mal and distal tubules in a normal rat kidney. In the SF-1 group, CaOx microlith formation was rare and tubular cells did not appear to change (Fig. 2B). In the SF-2 and SF-3 groups, however, CaOx microlith formation and epithelial cell damage tended to increase (Fig. 2C, D).

## Discussion

HS is a major matrix GAG component in CaOx stones, but is also a potent inhibitor of CaOx crystallization [24, 26]. HS is present in tissues as a proteoglycan and in the urine as a metabolic turnover product of tissue proteoglycan. In the kidney, HS is the major acid GAG constituent of the glomerular and tubular basement membranes (GBM and TBM), respectively [10]. HS-PG in GBM is important for maintaining the integrity of glomerular size and the charge barrier [7, 19]. HS-PG levels in GBM are seen to be lower in diabetic nephropathy [25] and in membranous glomerulonephritis [12]. A decrease in negatively charged molecules within the GBM is thought to be responsible for the albuminuria that arises under these conditions [7, 12, 19, 25].

On the other hand, the localization and function of other types of HS-PG, such as syndecan [2], remains unclear in the rat kidney. Farquhar et al. [8] identified two antigenically distinct types of heparan sulfate proteoglycans in rat glomeruli, one of which is associated with cell membranes and the other with basement membranes. Kiefer et al. [14] isolated cell surface fibroblast growth factor (FGF)-binding protein from baby hamster kidney cells, and showed that its cDNA sequence is highly homologous with that of murine syndecan. These two studies implied that there are at least two types of HS-PG in the kidney. Syndecan is localized on the surface of a wide variety of mammalian epithelial cells, where it is involved in maintaining epithelial morphology and stabilization [2].

In the present study, we used primers specific for rat HS-PG core protein mRNA (*N*-syndecan) [4] for PCR. This syndecan was isolated from a neonatal rat Schwann cell and is similar to a known transmembrane heparan sulfate proteoglycan [4]. *N*-syndecan is a transmembrane proteoglycan that differs from other basement membrane proteoglycans. It is present in peripheral nerves, newborn rat brain, heart, aorta, and epithelial cell layers of other neonatal tissues [4].

Our study using PCR demonstrated that kidney tissues from normal and SF rats contain HS-PG. However, the level of HS-PG mRNA expression was significantly higher in SF rats than in normal rats. Basement membrane HS-PG levels in urolithiasis were investigated by

Chan and Tan [5], who reported a significant decrease of HS-PG in the GBM and upper urinary tract. However, they did not find significant changes in HS-PG in the TBM between patients with stones and normal controls [5]. Nishio et al. [17] also reported that HS levels increased in rat renal tubules during microlith formation. They concluded that CaOx microlith formation may accelerate the production of HS in rat renal tubules. These two reports suggest that changes in the levels of HS or HS-PG expression are associated with CaOx stone formation.

The results of the present study suggest that the following mechanism may be involved in HS-PG mRNA expression during CaOx crystal formation. CaOx crystals formed in the tubuler lumen injure the epithelial layer containing HS-PG. Moreover, this condition may induce increased HS-PG mRNA expression in order to produce more HS-PG to repair damaged urothelium. In fact, Stein and Parsons [22] reported that syndecan mRNA is expressed in bladder biopsy tissue taken from interstitial cystitis (IC) patients. IC may thus reflect the inability of the bladder to repair its protective surface coating (GAGs and proteoglycans). They concluded that a defect in these protective substances on the urothelial surface is implicated in the expression of syndecan. Their report supports our hypothesis.

In conclusion, our preliminary data suggest CaOx nephrolithiasis in our model, with the expression of HS-PG mRNA in rat kidneys, and may be associated with urothelial damage. HS-PG modulates cell adhesion, and is involved in various cellular properties such as migration, proliferation and differentiation [2, 11, 13]. Many extracellular matrix adhesive proteins such as laminin [15], fibronectin [1] and heparin-binding growth factors [20] are correlated with HS-PG. Further studies are needed to clarify the effects of HS-PG and other cell adhesion molecules upon CaOx stone formation.

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