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EFFECTS OF URINARY PROTHROMBIN FRAGMENT 1 IN THE FORMATION OF CALCIUM OXALATE CALCULUS

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ABSTRACT

Purpose: We investigated the effects of urinary prothrombin fragment 1 in the formation of calcium oxalate urolithiasis.

Materials and Methods: Fresh urine and renal parenchyma from patients with calcium oxalate calculus and normal controls were collected. Urinary prothrombin fragment 1 was isolated and purified from urine. It was identified by sodium dodecyl sulfide-polyacrylamide gel electrophoresis and analysis of its first 13 N-amino acids. The inhibitory activity of urinary prothrombin fragment 1 on calcium oxalate crystal growth was tested by the seeded crystallization technique. Meanwhile, the γ-carboxyglutamic acid composition of urinary prothrombin fragment 1 was analyzed by a previously described method and genetic mutation of the γ-carboxyglutamic acid domain of urinary prothrombin fragment 1 from renal parenchyma was detected by polymerase chain reaction-single strand conformational polymorphism sequencing.

Results: The γ-carboxyglutamic acid composition of urinary prothrombin fragment 1 was significantly decreased from normal (24.4 to 1.7 mol/1,000 amino acids) in patients with calcium oxalate calculus. The mean growth index ± SD of urinary prothrombin fragment 1 to calcium oxalate crystals was 42.3 ± 4.2 compared with the normal index of 19.2 ± 2.8 ($p < 0.01$). The polymerase chain reaction-single strand conformational polymorphism sequencing technique revealed no genetic mutation of the γ-carboxyglutamic acid domain of urinary prothrombin fragment 1 in patients with calcium oxalate calculus.

Conclusions: The γ-carboxyglutamic acid composition of urinary prothrombin fragment 1 as well as its ability to inhibit calcium oxalate crystal growth was significantly decreased in patients with calcium oxalate calculus. This was not caused by genetic mutation of the γ-carboxyglutamic acid domain of urinary prothrombin fragment 1. It is important to elucidate the mechanisms of calcium oxalate stones in view of urinary prothrombin fragment 1.

KEY WORDS: calculus, 1-carboxyglutamic acid, prothrombin fragment 1, calcium oxalate, kidney

About 70% of urinary stones are composed of calcium oxalate (CaOx). This common disease affects 10% and 5% of men and women, respectively, with a lifetime prevalence in industrialized nations. Conventional and contemporary therapies available for clinically evident calculus are well described but the mechanisms that influence stone formation remain comparatively poorly understood. Apart from its crystalline component every calculus contains organic matrix, of which approximately 64% is protein.[1] The organic matrix of CaOx crystals freshly generated from human urine in vitro, formerly described as crystal matrix protein (CMP), a 31 kD glycoprotein, is now known to be urinary prothrombin fragment 1 (UPTF1), as tested by analysis of its N-terminal amino acid sequence. UPTF1 has been shown to be one of the most potent macromolecular inhibitors of CaOx crystallization in undiluted human urine in vitro. It contains 10–12 γ-carboxyglutamic acid (Gla) residues near its N-terminal, called the Gla domain, which confer on it the ability to attract and bind calcium ions in solution. Another fragment of prothrombin, namely prethrombin 1, lacks Gla and has markedly decreased inhibitory activity, suggesting that the Gla domain of UPTF1 is responsible for its inhibitory effect.[2–4] UPTF1 has been detected by immunohistochemistry and found to exist in tubule cells of the distal convoluted tubule and the thick ascending limb of loop of Henle in several kinds of animals.[5] Recently prothrombin mRNA was also identified in human kidney, implying that the protein is present in human kidney and could potentially have a role in CaOx renal calculous formation.[6] We elucidated the effects of UPTF1 in the pathogenesis of CaOx calculus.

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For another article on a related topic see page 271.

MATERIALS AND METHODS

Preparation of CMP. Total 24-hour urine samples were collected from 12 male patients 24 to 40 years old with CaOx renal calculus and 8 healthy men 28 to 43 years old with no history of urinary stones. Samples were preserved with 0.02% weight per volume NaN_3. Patients with CaOx stones were identified by infrared spectrophotometry. The CaOx

content was more than 70% of the stone and specimens showed no blood and were confirmed by urinalysis.

The samples were centrifuged at 8,000 × gravity for 15 minutes at 20C. Supernatants were stored at −20C. CaOx crystals were generated by adding sodium oxalate and shaking in a water bath at 37C for 4 hours. Crystals were harvested by filtration through 0.22 μm filters and washed thoroughly with 0.1 mol/l sodium hydroxide, followed by distilled water. They were then dried, weighed and demineralized with 0.25 mol/l ethylenediaminetetraacetic acid, pH 8.0. Specimens were lyophilized and stored at −80C for later use.

Electrophoresis of CMP with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). CMP samples were electrophoresised in 10% gradient gels with a Mini-Protean II apparatus (BioRad Laboratories, Hercules, California). The gels were stained with silver.

Purification of UPTF1. UPTF1 was isolated and purified by ion exchange and hydroxyapatite chromatography.[7] Specimen solutions were dialysed against 7 mol/l urea and 10 mol/l tris-HCl buffer, pH 6.8, with 5 l × 3 changes in 24 hours. The solutions were then centrifuged at 100,000 × gravity for 30 minutes at 4C. The supernatants were applied to 1.0 × 2.0 cm DEAE-Sepharose CL-6B, which had been equilibrated with the same buffer. The collected eluates were again dialysed against 10 mmol/l Na_3PO_4 and 250 mmol/L NaCl with 5 l × 3 changes in 24 hours. The proteins were further purified by applying to hydroxyapatite column. The protein concentration was measured by the bicinchoninic acid method and the specimens were lyophilized and stored at −80C for later use.

Analysis of sequence and amino acid constitution of UPTF1. The first 13 N-terminal sequences of samples were detected by an automated Edman degraded apparatus. The Gla amino acid composition of UPTF1 was detected by the method described by Hauschka.[8]

Determination of inhibitory activity of UPTF1. The inhibitory activity of UPTF1 to calcium oxalate crystals growth was tested using a seeded crystallization technique.[9] The growth index (GI) was calculated using the formula, $GI = (A_0 - A_t)/A_0 \times 100$ with A_0 representing the spectrum value of the solution without seeded crystals and A_t representing that in experimental subjects with seeded crystals.

Polymerase chain reaction (PCR)-single strand conformational polymorphism (SSCP) sequencing of UPTF1 Gla domain. Renal parenchyma was obtained from 14 male patients 30 to 56 years old who had stones and poor renal function, and from 7 male patients 42 to 65 years with renal tumors who underwent nephrectomy. The renal parenchyma was obtained from the noncancerous portions of the kidney. The primers were 5′-cgg cga gcc aac acc ttc tt-3′ and 5′-tca ccg tag ccg tgg agg ac-3′, specifically designed according to the known DNA sequence of human prothrombin.[10] The Gla domain of UPTF1 was specifically amplified by PCR in patients with CaOx calculus and normal controls. Denatured PCR products (20 μl) mixed with loading buffer were run on 8% nondenaturing polyacrylamide gel to detect genetic mutation of the products. The results were further verified by DNA sequencing. PCR products (50 μl) were purified and submitted for sequencing.

Statistical analysis. Results are expressed as the mean ± SD. Statistical analysis was determined using the Student t test with significant considered at $p < 0.05$.

RESULTS

Figure 1 shows electrophoresis results of crystal-surface binding substance samples by SDS/PAGE. There were mainly 2 kinds of macromolecules purified from urine in the 2 groups, that is bands 67 and 31 kD, respectively. The latter was less visible in the CaOx group, suggested that this protein may be not as abundant in urine in the CaOx calculus group. To identify the 31 kD protein the samples were submitted to DEAE-Sepharose CL-6B (Amersham International, Arlington, Illinois)

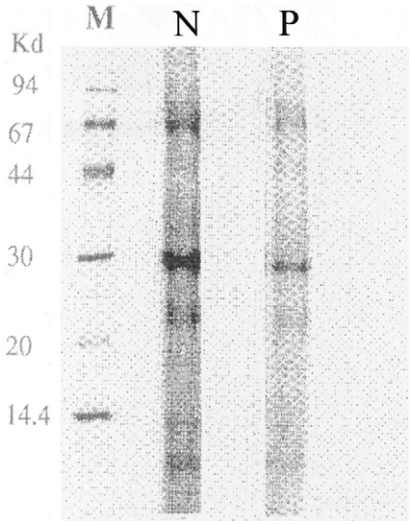

FIG. 1. SDS/PAGE of CSBS purified from urine shows that 31 kDa band was less visible than 67 kDa band in CaOx group vs normal (*N*) controls. *P*, patient. M, marker.

UPTF1 amino acid composition

Amino Acid	Normal (mol/1,000 amino acids)	CaOx (mol/1,000 amino acids)
Thr	47.2	47.7
Asp	102.0	83.6
Ser	60.8	46.9
Glu	89.6	101.2
Gly	45.5	43.1
Ala	43.7	64.1
Val	38.0	51.5
Met	0.4	7.6
Leu	52.2	75.1
Tyr	15.1	22.7
Phe	19.3	32.9
Lys	23.3	51.6
His	16.4	15.3
Arg	18.9	39.9
Pro	34.2	37.5
Gla*	24.4	1.7

* Significantly decreased.

to further purification as designed. The result of amino acid sequencing of the purified 31 kD protein showed that the first 13 N-terminal amino acids were Ala-Asn-Thr-Phe-Leu-Xxx-Xxx-Val-Arg-Lys-Gly-Asn-Leu-, sharing the same amino acid sequence of prothrombin and indicating that it was a fragment of prothrombin, namely prothrombin fragment 1, as expected.[11] Meanwhile, amino acid composition analysis revealed that its Gla composition was significantly decreased from normal in the CaOx group (24.4 to 1.7 mol/1,000 amino acids, see table). This was meaningful in terms of the function of prothrombin fragment 1 in patients with CaOx calculus.

It was then clear that the inhibitory activity of UPTF1 to CaOx crystallization depends on Gla to attract and bind calcium ions in urine, fulfilling its inhibitory function in the pathogenesis of CaOx calculus. The result was in agreement with the data obtained by the seeded crystallization technique, namely that the GI of UPTF1 was 42.3 ± 4.2 in the CaOx group and 19.2 ± 2.8 in controls (fig. 2). This indicated that the Gla content of UPTF1 as well as its inhibitory activity on the nucleation, aggregation and growth of CaOx crystals was remarkably decreased in patients with CaOx calculus, so that it might have an important role in the pathogenesis of urinary CaOx stones.

To explore further the causes that led to the remarkable decrease in the Gla content of UPTF1 PCR-SSCP sequencing was performed specifically to amplify the Gla domain of UPTF1 from renal parenchyma and investigate whether any genetic mutation led to the lower content of UPTF1 Gla and