Urol Res (1994) 22:257–260

ORIGINAL PAPER

F. Atmani · B. Lacour · P. Jungers · T. Drüeke
M. Daudon

Reduced inhibitory activity of uronic-acid-rich protein in urine of stone formers

Received: 25 February 1994/Accepted: 16 June 1984

Abstract We recently reported that human urine contains a newly identified urinary glycoprotein acting as a potent inhibitor against calcium oxalate crystallization. This inhibitor is a uronic-acid-rich protein (UAP) with a molecular weight of approximately 35 kDa. In the present study, UAP was isolated from urine of stone formers and of subjects without a stone history, and its inhibitory activity was tested in a calcium oxalate crystallization system in vitro. Our results show a weaker inhibitory activity of UAP extracted from the urine of stone formers than that extracted from the urine of healthy subjects. Preliminary analyses of amino acid and carbohydrate content showed some differences between the two groups. The main difference was the reduction in sialic acid in UAP isolated from the urine of stone formers. We suggest that UAP contributes significantly to total urinary inhibitory activity of calcium oxalate crystallization and that the decrease in this activity in the urine of recurrent stone formers is due, in part, to the weak inhibitory activity of UAP. A structural abnormality of UAP could explain the diminution of its inhibitory activity in the urine of stone formers.

Key words Calcium oxalate crystallization · Nephrolithiasis · Uronic-acid-rich protein · Glycosaminoglycans · Chromatography

Numerous studies have shown that normal urine contains macromolecular substances capable of preventing and reducing calcium oxalate crystal formation [8, 22, 23]. The urine of recurrent stone formers appears to exert less inhibitory activity in this crystallization process, due to the reduced activity of several macromolecular inhibitors [3, 10, 15]. The nature of all these compounds has not yet been well established. Bowyer et al. [5] isolated two isomers of chondroitin sulfate from normal urine and claimed that these glycosaminoglycans (GAGs) are the major inhibitors of calcium oxalate crystal growth and aggregation. Nakagawa et al. [19] have isolated a glycoprotein named nephrocalcin which, according to the authors, accounts for 90% of the macromolecular inhibitory activity. Tamm-Horsfall protein (THP) was also proposed as a macromolecular inhibitor of crystal aggregation [12]. A structural abnormality of GAGs [10, 14], nephrocalcin [20], and THP [13] has been implicated in lithogenesis. Finally, Sorensen et al. [25] reported that the main inhibitory activity in normal urine is supported by a protein with a molecular weight of about 40000 Da which seems to be analogous to the inter-α-trypsin inhibitor. Finally, uropontin has been isolated from human urine by monoclonal antibody immunoaffinity chromatography and seems to play a role in calcium oxalate crystal growth [24]. Recently, we have isolated from the urine of healthy subjects a uronic-acid-rich protein (UAP) with a molecular weight of about 35000 Da and reported evidence that UAP, GAGs and a nephrocalcin-like glycoprotein all are effective inhibitors of calcium oxalate crystallization in vitro [1]. We have also determined the preliminary structure of UAP extracted from the urine of healthy man [2].

The aim of the present study was to compare the inhibitory activity of UAP isolated from the urine of recurrent stone formers with that of UAP isolated from the urine of healthy control subjects.

F. Atmani (✉)[1] · B. Lacour · P. Jungers · T. Drüeke · M. Daudon
Unité 90 de l'INSERM, Département de Néphrologie,
Hôpital Necker-Enfants Malades,
161 rue de Sèvres, F-75743 Paris Cedex 15, France

[1] *Present address*: University of Florida, College of Medicine, Department of Pathology and Medicine. Gainesville, FL 32610-0275, USA; Fax: 1 (904)3926249

Subjects and methods

We collected 24-h urine samples from five healthy male subjects (aged 25–43 years) and from five recurrent calcium oxalate male stone formers (aged 27–52 years) in polyethylene bottles containing sodium azide as preservative (300 mg). None of the subjects were

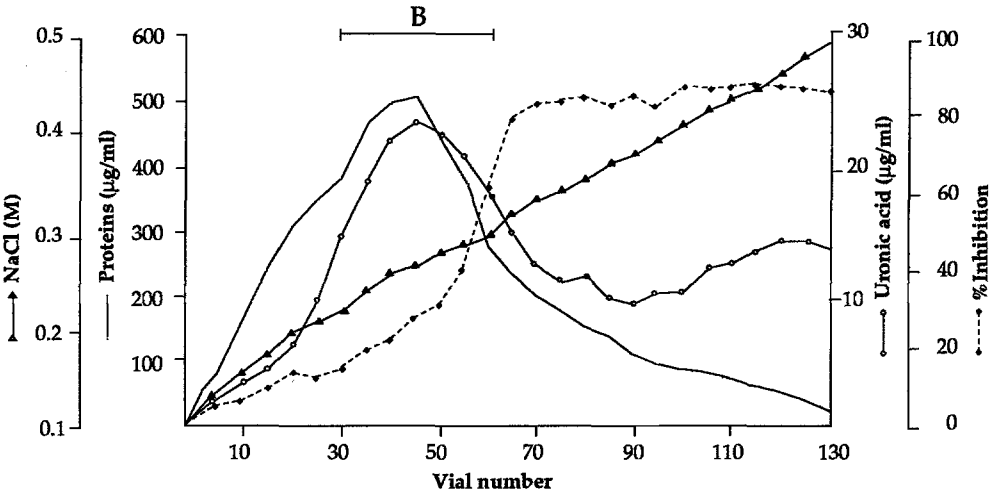

Fig. 1 Anion exchange chromatography on DEAE-Sephacel of a 24-h urine sample from a helathy man. Elution was carried out using an NaCl gradient from 0.1 to 0.5 in 0.05 M TRIS-HCl, pH 7.3. Protein concentration was measured by Lowry's method. Uronic acid was determined by carbazole reaction, and salt concentration by flame spectrophotometry. Inhibition was assayed in a calcium oxalate crystallization system. Fraction B was used for subsequent prurification

taking medications. Diethylaminoethanol (DEAE)-Sephacel gel, Sephacryl S-300 gel and Mono Q column were purchased from Pharmacia (Sweden) and recycled according to the manufacturer's procedure. Columns and electrophoresis equipment were obtained from LKB (Sweden). A Beckman 6300 amino acid analyzer and a Varian gas chromatograph were used. All other reagents were purchased from Sigma (Germany).

UAP was isolated from each urine sample according to a method previously reported in detail [1], including three chromatography steps. Briefly, each urine sample was dialyzed against 10 l deionized water, adjusted to pH 7.3, NaCl 0.1 M and mixed in a DEAE-Sephacel bath for 30 min. The gel was eluted by a buffer solution of TRIS-HCl 0.05 M, NaCl 0.5 M, pH 7.3. This sample was dialyzed, adjusted to pH 7.3, NaCl 0.1 M and applied to the DEAE-Sephacel column. The B fraction eluted between 0.15 and 0.3 M was dialyzed and incubated with ethylenediamine tetraacetic acid (EDTA) at a final concentration of 0.05 M, pH 8, for 4 days with stirring at 4°C. The fraction was dialyzed again, lyophilized and Sephacryl S-300 gel chromatography performed. The fractions containing UAP were finally purified using fast protein liquid chromatography (FPLC) (Mono Q) column. Fractions obtained from the DEAE-Sephacel column likely to contain GAGs were used for cellulose acetate electrophoresis with the procedure described by Renzo et al. [21]. Thus, GAGs were precipitated in the first place by cetylpyridinium methylammonium bromide (CTMB) according to Diferrante's method [9]. Protein and uronic acid concentrations were determined according to Lowry's method [16] and the carbazole procedure [4] respectively.

Proteins were assayed in a calcium oxalate crystallization system recently developed in our laboratory [1]. It is a simple reproducible model, needing only a small amount of proteins. Moreover, we tested several inhibitors with results comparable to those in the literature [1]. A range of 2.5 to 20 µg protein samples were dissolved with 50 µl 0.15 M NaCl in a tube containing 1 ml 2 mM calcium chloride solution (0.05 M TRIS-HCl, 0.15 M NaCl, pH 7.3 with a trace of ^{45}Ca). The assay was initiated by adding 1 ml 2 mM ammonium oxalate solution (0.05 M TRIS-HCl, 0.15 M NaCl, pH 7.3). After 1 h of stirring at room temperature, tubes were centrifuged at 2500*g* for 5 min and 500 µl aliquots of the supernatant assayed for ^{45}Ca radioactivity in a liquid scintillation counter.

Gel electrophoresis was performed to follow the purity of proteins using from 8% to 20% linear polyacrylamide in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol. The protein bands were stained with coomasis blue R or using the silver staining procedure [18].

Carbohydrate content was analyzed by gas chromatography after hydrolysis of samples in 0.5 N HCl-methanol at 80°C for 24 h, acetylation, and trimethylsilylation as described by Chambers and Clamp [7].

Amino acid analysis was performed after hydrolysis of samples in 6 M HCl in sealed vacuum vials at 110°C for 24, 48 and 72 h. The number of γ-carboxyglutamic acid (Gla) residues was determined after alkaline hydrolysis at 110°C for 22 h [11]. Statistical analysis was performed using the ANOVA test and values are means ±SD.

Results

A typical chromatographic pattern obtained from one healthy subject is shown in Fig. 1. In the first half of fractions (vials 1–65), the inhibitory activity increased in an exponential manner and was still marked in the last fractions. The last half of fractions (vials 65–130) contained proteins as well as chondroitin sulfate and dermatan sulfate as determined by cellulose acetate electrophoresis. These results are in agreement with another study which demonstrated that the last fraction contained GAGs and showed maximum inhibitory activity toward calcium oxalate crystallization [17]. Moreover, in our crystallization system we have only considered the amount of proteins (5 µg/ml). Therefore, GAGs would be found in large amounts in the test. Thus, we are interested in the B fraction, which is rich in glycoproteins only. The urine of stone former patients treated in the same way exhibited a clearly different chromatographic pattern, as illustrated in Fig. 2. Although the protein and uronic acid profiles were analogous to those observed with normal urine, the inhibition pattern showed a markedly depressed activity compared with that obtained from control urine, with the inhibitory activity being tested at the same protein concentrations in both cases. Even though the last fractions also contained GAGs, their inhibitory activity was lower in recurrent stone formers than in healthy controls. Fractions B and B' which contain UAP and other macromolecules were applied to Sephacryl S-300 gel, followed by FPLC on a Mono Q column. The purity of UAP was confirmed by polyacrylamide gel electrophoresis using silver staining. The average inhibition exerted by UAP isolated and purified from the five healthy subjects was compared with the pure UAP extracted from the five calcium oxalate stone formers. As shown in Fig. 3, UAP isolated from stone formers exerted significantly less

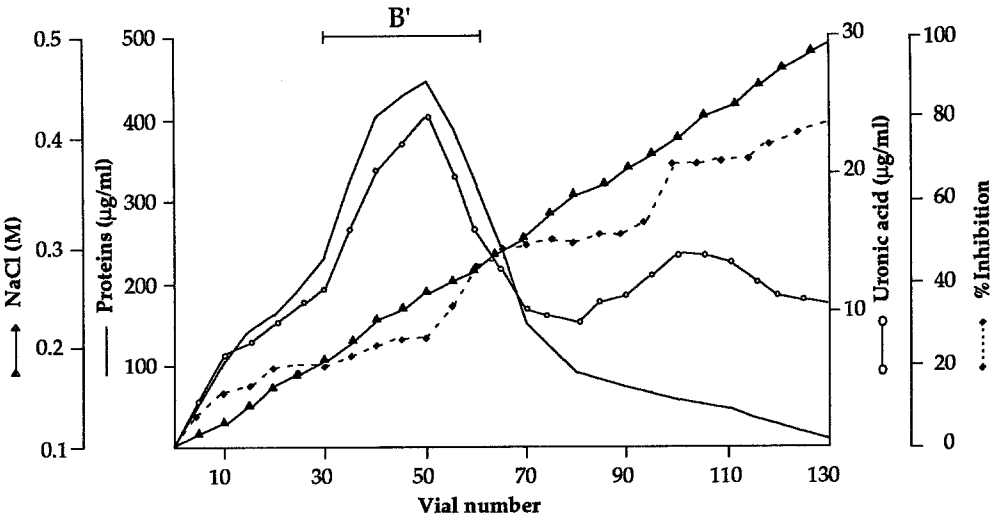

Fig. 2 Anion exchange chromatography on DEAE-Sephacel of a 24-h urine sample from a recurrent stone former. The urine sample was treated as described for Fig. 1

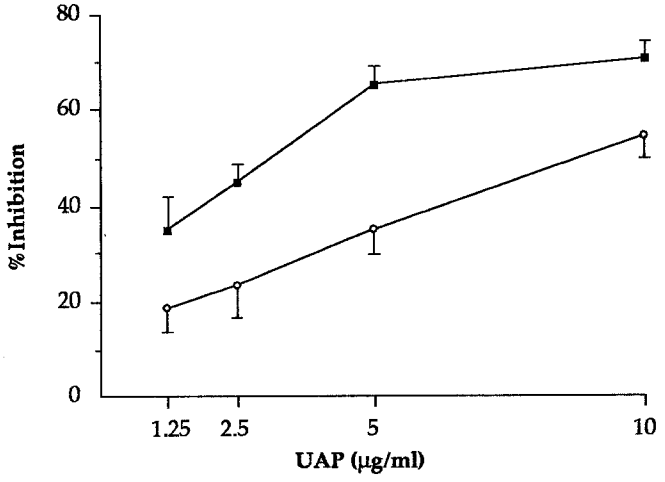

Fig. 3 Inhibitory activity of UAP isolated from healthy subjects (■—■) and from stone formers (○—○) in a calcium oxalate crystallization system. A significant difference was noted at each concentration between both groups ($P < 0.02$ for 1.25 μg/ml and $P < 0.001$ for 2.5, 5, and 10 μg/ml

inhibitory activity than that obtained from healthy subjects at each concentration used ($P < 0.02$ or less).

Amino acid and carbohydrate of UAP extracted from the urine of healthy humans has been reported in a recent study [2]. The same analyses performed on UAP extracted from the urine of stone formers showed some changes. Glutamic acid residue values are decreased and half-cysteine values were increased. Differences were not significant. However, the sialic acid content was dramatically diminished in UAP purified from the urine of stone formers. No γ-carboxyglutamic acid residues were detected in either case.

Discussion

The UAP recently purified by our group has been shown to strongly inhibit calcium oxalate crystallization in vitro and therefore takes its place among the other macromolecular inhibitors previously described [5, 19, 24, 25].

Our results show that UAP purified from the urine of stone formers exhibits less inhibitory activity than that isolated from normal urine. This behavior could be due to a structural abnormality of UAP, as observed in a preliminary structural analysis of UAP isolated from the urine of healthy subjects and from recurrent stone formers. Indeed, values of glutamic acid, half-cysteine and sialic acid residues are different in UAP extracted from the urine of stone formers. As already known, glutamic acid and sialic acid are highly charged. Thus, they could be adsorbed efficiency on the crystal surface blocking their growth sites. Half-cysteine amino acid might contribute to the molecular conformation by disulfide bridges. Currently, the partial structure of UAP purified from the urine of a large number of patients and healthy subjects is being elucidated. Similarly, structural abnormalities of other urinary macromolecular substances have been reported in the literature. Foye et al. [10] demonstrated that GAGs extracted from stone formers were more sulfated than those extracted from healthy subjects. Moreover, Itatani et al.[14] have also reported that sulfated GAGs could bind calcium oxalate crystals and form crystal aggregates. Nakagawa et al. [20] reported that nephrocalcin isolated from the urine of stone formers was deficient in γ-carboxyglutamic acid residue and exerted less inhibitory activity than nephrocalcin isolated from the urine of control subjects. In another recent study, Hess et al. [13] suggested that THP extracted from calcium oxalate stone formers might present a molecular abnormality. Thus, evidence is accumulating that several urinary macromolecular substances may be qualitatively abnormal in stone formers and thereby lose their normal inhibitory activity in opposing calcium oxalate crystallization.

Our results also show that the last fractions eluted from the DEAE-Sephacel column, and containing a mixture of chondroitin sulfate, dermatan sulfate and other glycoproteins, exhibit less inhibitory activity in stone formers than in healthy subjects. Accordingly, it has become clear in

recent years that the decrease in inhibitory activity frequently observed in the urine of recurrent stone formers cannot be supported by a single inhibitor alone but that a number of inhibitory macromolecular substances must be involved such as GAGs, nephrocalcin, THP, inter-α-trypsin inhibitor, uropontin, and UAP.

The reason why the inhibitory activity of several macromolecular substances may be simultaneously altered is not clear. As suggested by Hess et al. [13], genetic factors probably play a role and must be elucidated. Some reports have already emphasized the possibility of modifying the urinary excretion of GAGs by dietary manipulation [6]. Specific physiologic conditions are also able to change the amount of glycoproteins excreted, as has been reported for nephrocalcin [26].

In conclusion, the decrease in inhibitory activity in the urine of stone formers must be attributed to the altered structure of one or several inhibitory macromolecular substances, and UAP has now been shown to be one of them. The factors responsible for impaired inhibitory activity of such macromolecules require continued research effort.

References

1. Atmani F, Lacour B, Drüeke T, Daudon M (1993) Isolation and purification of a new glycoprotein from human urine inhibiting calcium oxalate crystallization. Urol Res 21:61
2. Atmani F, Lacour B, Strecker G, Parvy P, Drüeke T, Daudon M (1993) Molecular characteristics of uronic-acid-rich protein, a strong inhibitor of calcium oxalate crystallization in vitro. Biochem Biophys Res Commun 191:1158
3. Azoury R, Goldwasser B, Wax Y, Perlberg S, Garti N, Sarig S (1985) Evaluation of the relative inhibitory potential of fractionated urinary macromolecules. Urol Res 13
4. Bitter T, Muir HM (1962) A modified uronic acid carbazole reaction. Anal Biochem 4:330
5. Bowyer RC, Brockis JG, McCulloch RK (1979) Glycosaminoglycans as inhibitors of calcium oxalate crystal growth and aggregation. Clin Chim Acta 95:23
6. Caudarella R, Rizzolie E, Martilli G, Berveglieri F, Malavolta N (1988) Urinary excretion of glycosaminoglycans in calcium lithiasis: the role of protein intake. In: Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds) Urolithiasis Plenum, Vancouver, p 249
7. Chambers RE, Clamp JR (1971) An assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials. Biochem J 125:1009
8. Dent CE, Sutor DJ (1971) Presence or absence of inhibitor of calcium-oxalate crystal growth in urine of normals and stone-formers. Lancet II: 775
9. Diferrante N, Rich C (1956) The determination of aminopolysaccharide in urine. J Lab Clin Med 48:491
10. Foye WO, Hong HS, Kim CM, Prien EL (1976) Degree of sulfation in mucopolysaccharide sulfates in normal and stone-forming urines. Invest Urol 14:33
11. Hauschka PV (1977) Quantitative determination of γ-carboxyglutamic acid in proteins. Anal Biochem 80:212
12. Hess B, Nakagawa Y, Coe FL (1989) Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. Am J Physiol 257: F99
13. Hess B, Nakagawa Y, Parks JH, Coe FL (1991) Molecular abnormality of Tamm-Horsfall glycoprotein in calcium oxalate nephrolithiasis. Am J Physiol 260: F569
14. Itatani H, Itoh H, Yoshioka T, Namiki M, Koide T, Okuyama A, Sonoda T (1981) Renal metabolic changes relating to calculogenesis in an experimental model of calcium containing renal stone formation in rabbits. Invest Urol 19:119
15. Kitamura T, Zerwekh JE, Pak CYC (1982) Partial biochemical and physicochemical characterization of organic macromolecules in urine from patients with renal stones and control subjects. Kidney Int 21:379
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265
17. Matsumoto A, Terado T, Kin M, Nishio S, Iwata H, Takeuchi M (1990) Inhibitory factor of calcium oxalate crystal growth in urinary macromolecules. Nippon Hinyokika Gakkai Zasshi 81:883
18. Morrissey JH (1981) Silver stain for protein in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307
19. Nakagawa Y, Abram V, Kezdy FJ, Kaiser ET, Coe FL (1983) Purification and characterization of the principal inhibitor of calcium oxalate monohydrate crystal growth in human urine. J Biol Chem 258:12594
20. Nakagawa Y, Abram V, Parks JH, Lau HSH, Kawooya JK, Coe FL (1985) Urine glycoprotein crystal growth inhibitors. Evidence for a molecular abnormality in calcium oxalate nephrolithiasis. J Clin Invest 76:1455
21. Renzo C, Mario DR, Vincenzo PC (1979) A new electrophoretic method for the complete separation of all known animal glycosaminoglycans in a monodimensional run. Anal Biochem 99:311
22. Robertson WG, Peacock M (1972) Calcium oxalate crystalluria and inhibitors of crystallization in recurrent renal stone-formers. Clin Sci 43:499
23. Sallis JD (1987) Glycosaminoglycans as inhibitors of stone formation. Mineral Electrolyte Metab 13:273
24. Shiraga H, Min W, Vandusen WJ, Clayman MD, Miner D, Terrell CH, Sherbotie JR, Foreman JW, Przysiecki C, Neilson EG, Hoyer JR (1992) Inhibition of calcium oxalate crystal growth in vitro by uropontin: another member of the aspartic acid-rich protein superfamily. Proc Natl Acad Sci USA 89:426
25. Sørensen S, Hansen K, Bak S, Justesen SJ (1990) An unidentified macromolecular inhibitory constituent of calcium oxalate crystal growth in human urine. Urol Res 18:373
26. Wabner C, Sirivongs D, Maikranz P, Nakagawa Y, Coe FL (1987) Evidence for increased excretion in pregnancy of nephrocalcin (NC), a urinary inhibitor of calcium oxalate (CaOx) crystal growth. Kidney Int 31:359