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Low-Molecular-Weight Variants of Osteopontin Generated by Serine Proteinases in Urine of Patients With Kidney Stones

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Abstract Osteopontin (OPN) is a multifunctional glycosylated phosphoprotein found in body fluids, including urine, and has been implicated in urinary stone formation. We tested the hypothesis that OPN levels in urine of patients with kidney stones differed from normal individuals. To quantify OPN levels in the urine, we developed an ELISA using a combination of a mouse monoclonal and rabbit polyclonal antibodies raised against a recombinant glutathione-S-transferase-human OPN fusion protein. In a group of 34 patients diagnosed with kidney stones compared with a control group of 23 normal individuals, we found that OPN levels in urine of the patient and control groups ranged from 0.01 to 2.7 μg/ml, with no significant difference in their medians ($P > 0.8$, Mann-Whitney test). OPN in urine was qualitatively assessed by Western blotting using a biotinylated monoclonal antibody to detect various molecular forms. The urine of most individuals contained OPN species within in the 55- to 66-kDa electrophoretic mobility range. However, a significantly higher proportion of individuals in the patient group (13 of 34) was found to have aberrant urine OPN species (≤ 40 kDa) compared to 2 of 23 for the control group ($P < 0.03$, χ^2 test). Mixing experiments indicated that urine samples with aberrant OPN contain proteases inhibitable with phenylmethylsulfonyl fluoride. Such proteases could break down normal urine OPN in vitro. Therefore, urine from a high frequency of kidney stone patients contains serine proteases that contribute to proteolytic cleavage of OPN.

Key words: sialoglycoproteins, ELISA for urine osteopontin, kidney stones, phenylmethylsulfonyl fluoride, proteolytic cleavage

Urinary stone formation is a complex process involving the generation of a crystalline (mineral) phase and a noncrystalline (organic) phase. About 80% of renal stones are of the calcium oxalate (CaOx) and calcium phosphate type [Yoshida and Okada, 1990]. The organic matrix is thought to be an important component in the nucleation and growth of the renal calculi [Boyce, 1968], but its role in stone formation remains poorly understood. In vitro crystal formation studies have demonstrated that urine contains proteins with inhibitory properties for growth of CaOx crystals [Berland and Dussol, 1994]. One such inhibitor is the glycoprotein nephrocalcin [Nakagawa et al., 1983], which is thought to be synthesized in the kidney [Nakagawa et al., 1990]. More recently, osteopontin (OPN) has been identified as another potential inhibitory protein for CaOx kidney stone formation in an in vitro system [Shiraga et al., 1992; Worcester et al., 1992]. OPN is a noncollagenous, phosphorylated sialoprotein originally found in bone [reviews Denhardt and Guo, 1993; Patarca et al., 1993]. Its role in urinary stone formation is controversial, since in vivo studies using a rat model for CaOx stone formation suggests a stimulatory, as opposed to inhibitory, role for OPN [Kohri et al., 1993].

In this report, urine samples of individuals who had been clinically diagnosed with urinary stones were quantitatively evaluated by enzyme-linked immunosorbent assay (ELISA) to test the hypothesis that levels of OPN in these patients differ from those of normal individuals. We demonstrate that the levels of urine OPN in individuals with kidney stones and in healthy individuals did not differ significantly. However, examination of urine OPN by immunoblotting using

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OPN-specific monoclonal antibodies (mAbs) revealed that low-molecular-weight OPN variants were present in the urine and occurred in a significantly higher proportion in individuals with kidney stone disease compared with normal individuals. The aberrant OPN species are cleavage products of inhibitable serine proteases present in urine from some kidney stone patients.

METHODS

Patient and Control Group Characteristics

Thirty-four caucasian individuals (20 males, 14 females, aged 24–82 y), who demonstrated a radiopaque renal or ureteral calculus on X-ray were recruited into the patient group. All patients were referred for either extracorporeal shock wave lithotripsy or ureteroscopy for stone disease. Eighteen of these patients had recurrent disease or multiple stones. The control group consisted of 23 healthy caucasian individuals (10 males, 13 females, aged 26–79 y) who had never been diagnosed with any form of kidney stone disease. There was no significant difference between these groups with respect to age. Midstream urine samples were collected during the day and tested free of bacterial contamination. All samples were frozen and stored at −20°C and thawed at room temperature as needed.

SDS–PAGE and Immunoblotting

For analysis by gel electrophoresis, urine samples were mixed with 1/10th vol of 1.0 M Tris–HCl pH 8.0 prior to mixing with an equal volume of 2× gel loading buffer. The equivalent of 3 μl urine was fractionated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), then electrotransferred to a nylon membrane for immunodetection. Biotinylated mouse monoclonal antibody mAb53 [Bautista et al., 1994] at 0.2 mg/ml was used to detect OPN in immunoblots in combination with streptavidin–horseradish peroxidase (HPO) conjugate. The enhanced chemiluminescent system (Amersham, Oakville, ON) was used to develop the signal. Film exposure time was 10–20 s. For quantification of immunoblots, volume densitometry of autoradiograms was made using the Personal Densitometer SI and the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). OPN-specificity test by thrombin cleavage of samples was as described [Xuan et al., 1994]. In mixing experiments designed to detect proteolytic activity in urine, unprocessed samples were incubated at 37°C with or without the serine-protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma Canada).

Quantification of Urine OPN by ELISA

We developed a "capture" format ELISA to measure OPN in plasma using a combination of a high-affinity mouse monoclonal antibody mAb53 [Bautista et al., 1994] for the capture step, and a rabbit polyclonal antiserum for amplification, both of which were raised against a recombinant glutathione-S-transferase-human OPN fusion protein (GST-hOPN) [Xuan et al., 1994]. For the assay, Maxisorp immunoplates (Life Technologies, Burlington, ON) were coated with mAb53 (100 μl/well, 10 μg/ml in 0.1 M sodium bicarbonate, pH 9.0, 4°C, 16 h), then blocked with 1% bovine serum albumin (BSA) in ST-Tween buffer (10 mM Tris pH 8.0, 150 mM NaCl, and 0.05% Tween-20 [BioRad, Mississauga, ON]). Plates were then washed extensively with ST-Tween buffer. Urine samples were diluted into wells containing 1% BSA/ST–Tween buffer as diluent and incubated at 37°C for 1 h. At least four twofold dilutions of each sample (starting at 1/10th dilution) were tested. Subsequent incubations were performed at 37°C in 100 μl for 1 h, followed by three washes in ST-Tween buffer. In order to amplify and detect captured OPN, wells were sequentially incubated with (1) 1,000-fold diluted rabbit anti-OPN antiserum; (2) 2,000-fold diluted biotinylated goat antirabbit Ig antibody (Life Technologies, Burlington, ON); and (3) 2,000-fold diluted streptavidin conjugated with alkaline phosphatase (Jackson Immunological Laboratories, West Grove, PA) for 0.5 h. For color development, wells were incubated with 100 μl of p-nitrophenyl phosphate (1 mg/ml in 100 mM Tris pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$), and the signal was allowed to develop at room temperature for a duration of 4–6 min. To stop the reaction, 50 μl of 0.2M Na $_2$EDTA (pH 8.0) was added. A BioRad plate reader was used to quantify the signal. The standard used was the recombinant GST–OPN fusion protein, prepared and purified as previously described [Xuan et al., 1994].

Statistical Analyses

The standard curve for the ELISAs was determined by the curve-fitting feature of SigmaPlot software (Jandel Scientific, San Rafael, CA).

Experimental data points were fitted to the exponential rise function. In these assays, the correlation coefficient for the standard was consistently > 0.99. Appropriate statistical tests using parametric and nonparametric methods were done with SigmaStat software (Jandel Scientific).

RESULTS

Quantification of OPN in Urine by ELISA

We developed an antigen "capture" ELISA to facilitate the measurement of OPN levels in urine. In the assay, we used as the standard an affinity column-purified recombinant GST–human OPN fusion protein (GST–hOPN). To validate the standard, we compared the dilution profiles of a urine sample and that of GST–OPN in an ELISA. Figure 1 shows the fitting of the data points for the standard (●, absorbance versus amount of protein per assay) to the exponential rise function ($r > 0.999$). In order to compare the dilution profile of the urine sample with that of the standard, an estimate of the amount of OPN in the urine sample was made for the highest concentration by interpolating from the recombinant OPN standard curve. The estimate was then used to generate a series of protein amounts predicted by the twofold dilution used. These amounts were plotted against their corresponding absorbance values obtained by ELISA (□). The results (Fig. 1) showed that the dilution profile of the urine sample was superimposable to that of the recombinant standard, suggesting that the recombinant OPN may be used to substitute native OPN for quantifying urine OPN in ELISA.

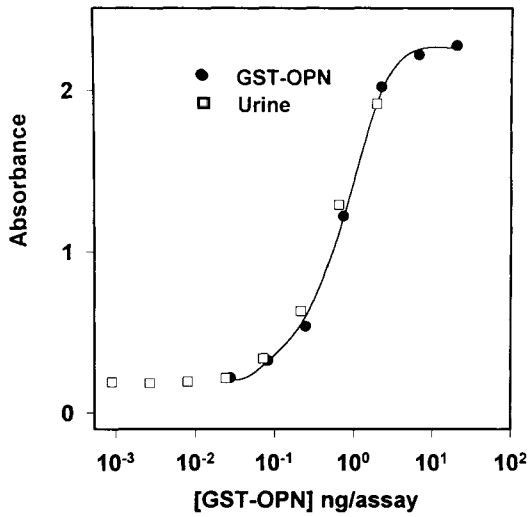

Fig. 1. Determination of urine OPN levels using a recombinant GST–OPN standard. The recombinant GST-human OPN used as standard in the "capture" ELISA was serially diluted by 3-fold, each step starting from 20 ng/assay. The data points (●) of [GST-OPN] in ng/assay versus $Absorbance_{405nm}$ are shown on a semilog scale. The best-fit curve on the exponential rise function was derived using the curve-fitting feature of SigmaPlot ($r > 0.999$). For comparison, a urine sample (□) was used in the assay as a 10-fold dilution then serially diluted by 2-fold each step. An initial estimate of the amount of OPN for the highest concentration in the series was made using the standard curve and then the other amounts were derived from the initial estimate based on the 2-fold dilution used.

Levels of Urine OPN in Renal Stone Patients Versus Control

We determined the levels of OPN in urine samples from the group of patients who had been diagnosed with urinary stones and compared them with those from the control group of disease-free individuals. Frequency distributions of OPN levels in patient and control groups are shown in Figure 2. The patient group had a mean OPN level of 0.76 μg/ml ± 0.71 (SD) and a median of 0.57 μg/ml. The control group mean level was 0.79 μg/ml ± 0.76 (SD) and a median of 0.60 μg/ml. The differences in the mean ($P > 0.70$, *t*-test) and in the median ($P > 0.8$, Mann-Whitney test) were not statistically significant. Thus, we found no evidence to support the idea that the urine OPN levels of individuals with renal stones were significantly different from those of normal individuals. In addition, correlation tests showed that the levels of urine OPN in either patient ($P > 0.10$, Spearman rank-order correlation method) or control group ($P > 0.10$) were not influenced by age. Also, the sex of the individual was not a major determining factor for level of urine OPN in either patient ($P > 0.40$, Mann-Whitney rank-sum test) or the control group ($P > 0.40$).

Western Blot Analysis of OPN in Urine

To confirm the levels of urine OPN as determined by our ELISA and to examine the molecular forms of OPN detected by the antibodies used, we tested all urine samples from both patient and control groups by Western blotting using a mAb specific to human OPN. Figure 3A shows an immunoblot of urine from kidney stone patients (lanes 1–7) and from normal individuals (lanes 8 and 9) using mAb53 as probe. First, we observed that the intensity of OPN-specific bands was highly variable between individuals, ranging from undetectable at this film exposure (lanes 4 and 7) to intensely dark (e.g., lanes 2

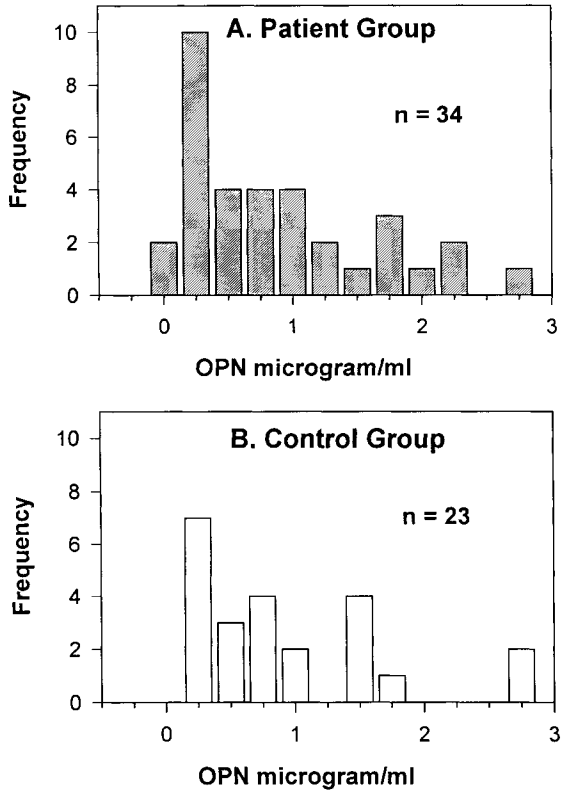

Fig. 2. Frequency distribution of OPN levels in urine. Frequency distributions of the renal stone patient (**A**) and control (**B**) groups are shown as histograms. Normality test using the Kolmogorov–Smirnov method indicated that the control group had a normal distribution ($P > 0.44$, n = 23) and the patient group did not ($P < 0.004$, n = 34). For the patient group, the mean OPN level was 0.76 μg/ml ± 0.71 (SD), median of 0.57 μg/ml, and a range of 0.01–2.52 μg/ml. For the control group, the mean was 0.79 μg/ml ± 0.76 (SD), median of 0.60 μg/ml, and a range of 0.01–2.69 μg/ml.

and 8). The immune-specific bands were quantified by volume densitometry to measure OPN. Above each lane for the urine samples are the results of the ELISA and densitometry determinations. Trend-line analysis (Fig. 3B) indicated that a linear relationship existed between ELISA quantities and densitometric measurements ($r = 0.87, P < 0.002$), suggesting that the quantities measured by ELISA were representative of the levels of OPN in urine by Western blotting.

Examination of the immune-specific bands in the Western blots such as one in Figure 3A indicated that most individual urine samples contained OPN species in the 55- to 66-kDa mobility range (lanes 1, 2, 3, 5, 8, 9), while several others contained distinctly different species such as found on lane 6. This particular urine sample had a predominant OPN-specific band of about 40 kDa in size and four other minor bands distributed over a broad molecular size range.

We confirmed the identity of the immunoreactive bands by thrombin cleavage and detection in Western blots with mAb53, as thrombin cleavability is a good indicator of the identity of OPN on SDS–PAGE [Xuan et al., 1994] and on immunoblots [Bautista et al., 1994]. We previously showed that mAb53 recognizes a thrombin-labile epitope in OPN [Bautista et al., 1994]. Figure 4 shows the effect of thrombin cleavage on three urine samples. Lane 1 shows a low-molecular-weight variant identical to that in Figure 3A (lane 6), while lanes 2 and 3 are samples with the common OPN species. Thrombin cleavage

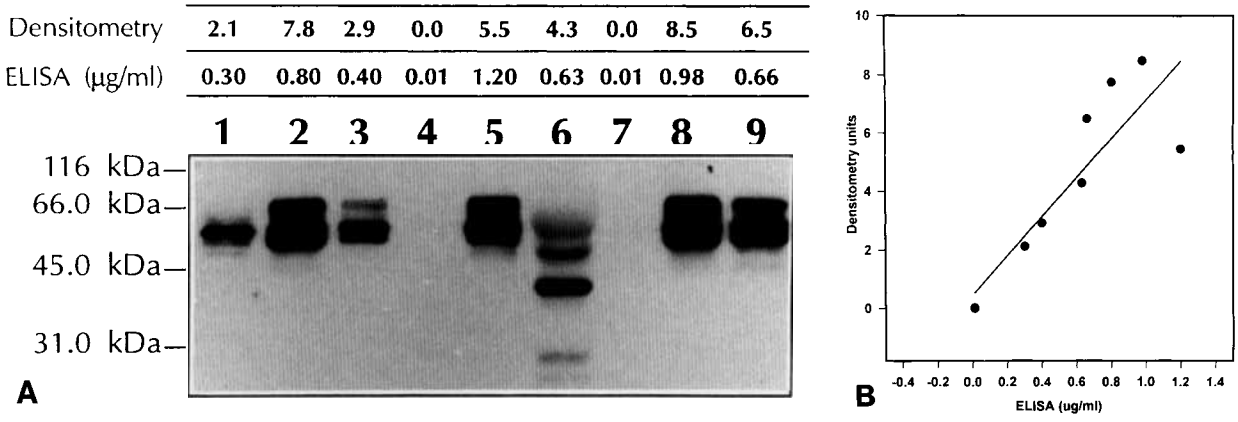

Fig. 3. Western blot analysis of urine OPN and comparison between ELISA and densitometry for quantification of urine OPN levels. **A:** Urine samples from seven renal stone patients (*lanes 1–7*) and two normal individuals (*lanes 8, 9*) were analyzed by 12% SDS–PAGE and immunoblotting as described in Materials and Methods. The corresponding concentrations of OPN as measured by ELISA for each sample are indicated above the lanes. Quantification of the bands on the blot was done by volume densitometry and expressed in pixel count units per 1,000. **B:** Linear regression analysis of the relationship between determination of OPN levels by volume densitometry and ELISA. Pearson correlation coefficient of the regression line was 0.99.

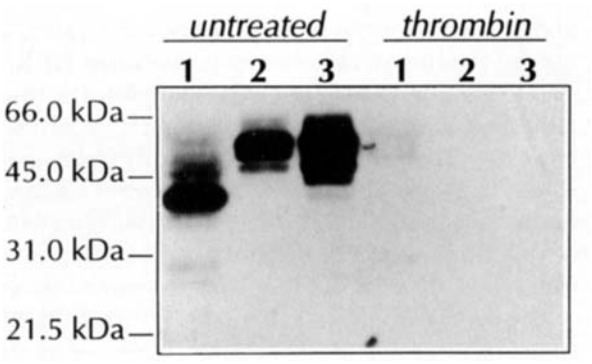

Fig. 4. Analysis of urine OPN by immunoblotting and thrombin digestion. Three representative urine samples were included for analysis: one with an aberrant OPN (*lane 1*) and two with the common OPN types (*lanes 2, 3*). Urine samples incubated at 37°C with thrombin or without (untreated) were fractionated by 12% SDS–PAGE and OPN was detected by immunoblotting using biotinylated mAb53.

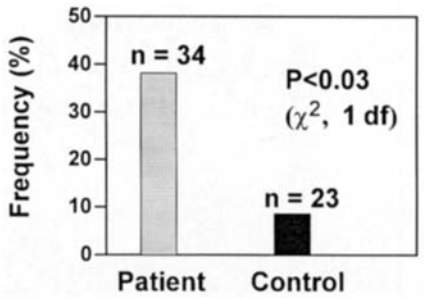

Fig. 5. Comparative proportions of urine samples with aberrant OPN in patient and control groups. Individuals in the patient and control groups were scored for the presence of aberrant OPN in their urine as detected by Western blotting. In the patient group, 13 of 34 individuals were positive for aberrant OPN and 2/23 for the control group. The difference in these proportions between the two groups was significant (χ^2, 1 degree of freedom, $P < 0.03$).

resulted in the complete disappearance of bands detectable by mAb53, indicating that the immunoreactive bands detected by Western blotting were authentic OPN species.

Association Between Frequency of Aberrant OPN and Incidence of Renal Stone Disease

We statistically tested if the proportion of individuals with aberrant OPN species in their urine was significantly different between the renal stone patient and control groups (Fig. 5). Among the 34 individuals comprising the patient group, 13 were found to have aberrant <40-kDa urine OPN; only two of the 23 individuals in the control group had the aberrant form. The differences in the proportion of individuals with the aberrant OPN were significantly different between the patient and control groups ($P < 0.03$, z- and χ^2-tests with Yate's correction; $P < 0.02$, Fisher's exact test, using a two-tailed probability). Thus, we found a strong observational association between patients with kidney stones and the presence in their urine of aberrantly migrating OPN species on SDS–PAGE.

We then tested whether gender bias was present in the two groups we surveyed, since it is known that renal stones occur more frequently among males than females in the human population. When we compared the proportion of individuals with aberrant OPN between the normal control and patient groups following subgrouping by sex, we found that eight of 20 male patients had aberrant urine OPN, compared to 5 out of 14 female patients. Examination of the normal group showed that the two individuals with aberrant OPN were females (2 out of 10); all 13 males had normal urine OPN. Statistical test showed that the proportion of male patients with aberrant urine OPN (8 of 20) was significantly higher than the proportion in the control group ($P = 0.01$, Fisher's exact test). However, the proportions in the female control and patient groups were not significantly different ($P > 0.65$, Fisher's exact test). These results suggest that males with kidney stones are more likely to have aberrant urine OPN species than are their female counterparts.

Further examination of the patient group indicated that 6 of 16 of primary stone formers and 7 of 18 of recurrent or multiple stone formers had the aberrant low-molecular-weight OPN species. These ratios do not differ significantly ($P > 0.78$, χ^2-test with Yate's correction). Thus the aberrant OPN species occur in both recurrent and primary stone patients with similar frequencies.

Urine Samples With Aberrant OPN Contain Inhibitable Proteases That Degrade Normal OPN

As a first step toward characterizing the aberrant OPN species, we tested the hypothesis that some urine samples in which they were detected contain a proteolytic activity that breaks down OPN, and that the aberrant OPN is a proteolytic cleavage product. Figure 6 shows the results of a Western blot for a mixing experiment designed primarily to determine whether a urine sample with aberrant OPN species contains a proteolytic activity. Lane 1 contains a urine sample identified as having the aberrant form of urine

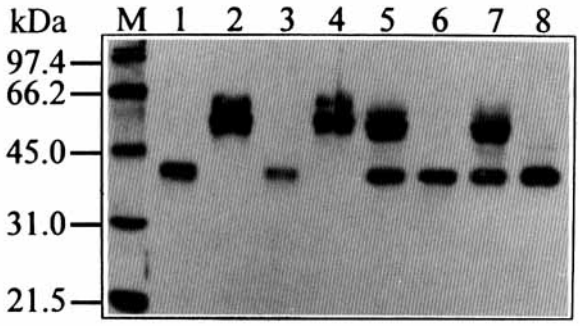

Fig. 6. Mixing experiment showing a urine sample with aberrant OPN contains inhibitable proteases. *Lane 1,* Urine sample with aberrant OPN from a kidney stone patient; *lane 2,* urine sample with normal OPN; *lane 3,* same sample as in *lane 1,* incubated at 37°C, 2 h; *lane 4,* same sample as on *lane 2,* incubated at 37°C, 2 h; *lane 5,* samples with aberrant (from *lane 1*) and normal OPN (from *lane 2*) combined without incubation at 37°C; *lane 6,* same as *lane 5,* with incubation at 37°C, 2 h; *lane 7,* same as *lane 6,* with 1 mM PMSF in methanol; *lane 8,* same as *lane 7,* with methanol without PMSF. All urine samples were directly used from frozen stock and mixed with gel loading buffer with or without prior incubation at 37°C. Samples were run on 12% SDS–PAGE and transferred onto nylon membrane; bands were immunodetected with biotinylated mAb53.

OPN that was collected from an individual with kidney stones, while lane 2 contains a sample with normal OPN from an individual in the control group. Incubation of the patient urine sample at 37°C resulted in diminished intensity of the aberrant OPN (lane 3) suggesting that this urine sample contained OPN that was subject to further degradation under these conditions. Similar incubation at 37°C of the normal urine sample alone (lane 4) also resulted in a detectable reduction in the intensity of the 55- to 60-kDa OPN-specific bands (cf. lane 2) but only to a lesser extent than that in lane 3. In addition, lane 4 showed no detectable OPN-specific bands with mobilities in the 40-kDa range, indicating that the breakdown products of the normal OPN in the urine sample of a healthy individual after in cubation at 37°C could not be detected by mAb53.

The mixed urine samples containing both normal and aberrant OPN species are shown on lane 5. Note that the normal 55- to 66-kDa species show slightly altered mobility, perhaps due to change in ionic strength in the mixture. Incubation of the mixed urine samples at 37°C resulted in the complete disappearance of the normal OPN species (lane 6), apparently from degradation into some form which was not immunodetectable. The band representing the incubated aberrant OPN in lane 6 did not diminish in intensity compared to the corresponding unincubated band in lane 5, but relative to that in lane 3 it appeared to have gained intensity. Since lane 3 shows that incubation of the kidney stone patient urine sample alone resulted in further breakdown of the aberrant OPN species, then the corresponding relatively darker aberrant band on lane 6, in which both urine samples were incubated together, suggests that degradation products of the normal OPN accumulated into the aberrant form. Therefore, we conclude that the aberrant 40 kDa was derived from proteolytic degradation of the normal 55- to 60-kDa OPN species.

To characterize the proteolytic activity in urine with aberrant OPN, we used the protease inhibitor PMSF in the mixing experiment. We found that PMSF completely blocked degradation of the normal OPN (Fig. 6, lane 7). Similar mixing experiments were carried out using a combination of an aberrant OPN-containing urine sample from an individual in the normal control group and one normal OPN-containing sample from an individual in the patient group. We found that the PMSF-inhibitable proteolytic activity was associated with the urine with aberrant OPN (results not shown).

DISCUSSION

The role of OPN in kidney stone formation is controversial. The observations that OPN is a potent inhibitor of CaOx crystal growth in vitro led to the hypothesis that OPN functions as a crystal growth inhibitory protein in mouse urine [Worcester et al., 1992] and in human urine [Shiraga et al., 1992]. Within this context, OPN has also been shown to inhibit hydroxyapatite crystal formation from metastable calcium phosphate solutions in vitro [Hunter et al., 1994; Boskey et al., 1993]. By contrast, a role in promotion of stone formation by OPN is suggested by an animal model of stone disease in which glyoxylic acid induction of stone formation in rats coincided with an increase in OPN mRNA levels in proportion to dosage and duration of the inducer [Kohri et al., 1993]. On the one hand, a presumptive inhibitory role by OPN in renal stone formation predicts that high OPN levels in urine prevent stone formation and, on the other hand, a role in promotion by OPN predicts that elevated levels promote stone formation. One expects that the levels in patients with urinary stones might differ from those of normal individuals. Thus, we tested the hypothesis

that OPN levels in a group of kidney stone patients differ from those of a group consisting of normal individuals.

To accurately compare the levels of OPN in urine of individuals with or without kidney stones, we developed a quantitative "capture" ELISA. We showed that a recombinant GST–hOPN fusion protein can be used as standard in the assay. Since purification of native OPN is often difficult, recombinant OPN offers a convenient source. The availability of a batch-prepared standard should provide the consistency needed to measure OPN levels over several experiments. Furthermore, comparison between ELISA and densitometric quantification of Western blots showed a high correlation between the two methods. However, the sensitivity of the ELISA to detect and measure low levels (at ng/ml) of urine OPN occurs at a much wider range than immunoblotting, the latter being often limited by the exposure range of autoradiographic films. In this study, we used an ELISA to test urine samples of 34 patients who had been diagnosed with kidney stone disease and compared them with the group of 23 normal individuals. We observed that the levels of urine OPN did not differ significantly between patients with kidney stones and normal individuals.

By Western blotting, we have shown that the predominant urine OPN species have an electrophoretic mobility in the range of 55–60 kDa, in agreement with the size of native OPN from various sources. However, we discovered that some urine samples have predominant OPN species that aberrantly migrated at around 40 kDa, and still others have both the normal and the aberrant forms. That these aberrant OPN species are likely generated by proteases found in urine was demonstrated in mixing experiments. First, we found that urine samples containing aberrant OPN were able to degrade normal urine OPN during co-incubation of urine samples with either normal or aberrant OPN. Second, cleavage products of the normal OPN that were detectable in Western blots co-migrated and were immunologically indistinguishable from the aberrant species, suggesting that the urine proteases have a preferential cleavage site on OPN. Third, although we detected proteolytic activity in urine samples with normal OPN, this activity was lower than that found in samples with aberrant OPN and, more importantly, did not give rise to cleavage products corresponding to the aberrant form. The proteolytic activity in the urine with normal OPN may therefore be distinct from that found in the urine with aberrant OPN.

To initially characterize the specific protease that cleaves OPN in urine, we used the inhibitor PMSF, which is active against serine proteases such as chymotrypsin and trypsin. The strong activity of this inhibitor to prevent breakdown of urine OPN in the mixing experiments suggests that the protease associated with the aberrant OPN species belongs to the same class. We therefore propose that serine proteases that are present in urine may play a role in modulating the activity of OPN in inhibiting the formation of urinary stones. Analysis of the primary sequence of OPN shows that there are several potential cleavage sites of serine proteases such as trypsin. Cleavage of normal OPN by such proteases could occur at any of the sites. It appears, though, that OPN contains a preferred cleavage site (or two very close to each other), giving rise to an easily detectable form referred to here as the aberrant OPN species. However, the aberrant OPN can be degraded further by incubating at 37°C into a form not detected by our antibody as demonstrated on lane 4, suggesting that other sites are also available for cleavage.

Frequency distribution analysis of OPN levels in urine indicated no significant differences between the patient and control groups. This suggests that the level of OPN in the urine may not be a good diagnostic indicator of kidney stone disease; however, the size of both the control and patient groups may not have been sufficient to detect a statistical difference. We have demonstrated instead that the frequency of aberrantly migrating, low-molecular-weight, OPN-specific bands ($\leq$40 kDa) in the urine of kidney stone patients (13 of 34; 38%) was significantly higher than that found in normal individuals (2 of 23; 8.7%). Both recurrent and primary stone patients had similar frequencies of aberrant OPN. Thus, our study associates the presence in urine of this previously undefined class of OPN molecules with clinical manifestations of kidney stone disease. Other groups have shown that various molecular forms of OPN exist and are products of differential RNA splicing, glycosylation, phosphorylation, sulfation, and even possibly susceptibility to proteases [reviews Denhardt and Guo, 1993; Patarca et al., 1993].

Kidney stone formation is a complex process in which several factors such as OPN are be-

lieved to be involved in its inhibition [reviewed in Berland and Dussol, 1994]. We speculate that the aberrant, low-molecular-weight OPN has reduced inhibitory activity relative to the intact molecule. Our findings suggest the hypothesis that individuals with low urine OPN levels, or those whose levels are normal but with diminished inhibitory activity as a result of proteolysis, might be more susceptible to kidney stone disease.

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REFERENCES

Bautista DS, Xuan JW, Hota C, Chambers AF, Harris JF (1994): Inhibition of Arg-Gly-Asp (RGD)-mediated cell adhesion to osteopontin by a monoclonal antibody against osteopontin. J Biol Chem 269:23280–23285.

Berland Y, Dussol B (1994): New insights into renal stone formation. Curr Opinion Nephrol Hypertension, 3:417–423.

Boskey AL, Maresca M, Ullrich W, Doty SB, Butler WT, Prince CW (1993): Osteopontin-hydroxyapatite interactions in vitro: Inhibition of hydroxyapatite formation and growth in a gelatin-gel. Bone Miner 22:147–159.

Boyce WH (1968): Organic matrix of human urinary concretions. Am J Med 45:673–683.

Denhardt DT, Guo X (1993): Osteopontin: A protein with diverse functions. FASEB J 7:1475–1482.

Hunter GK, Kyle CL, Goldberg HA (1994): Modulation of crystal formation by bone phosphoproteins: Structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochem J 300:723–728.

Kohri K, Nomura S, Kitamura Y, Nagata T, Yoshioka K, Iguchi M, Yamate T, Umekawa T, Suzuki Y, Sinohara H, Kurita T (1993): Structure and expression of the mRNA encoding urinary stone protein (osteopontin). J Biol Chem 268:15180–15184.

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 256:12594–12600.

Nakagawa Y, Netzer M, Coe FL (1990): Immunohistochemical localization of nephrocalcin to proximal tubule and thick ascending limb of Henle's loop of human and mouse kidney: resolution of a conflict. Kidney Int 37:474.

Patarca R, Saavedra RA, Cantor H (1993): Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene. Crit Rev Immunol 13:225–246.

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 89:426–430.

Worcester EM, Blumenthal SS, Beshensky AM, Lewand DL (1992): The calcium oxalate crystal growth inhibitor protein produced by mouse kidney cortical cells in culture is osteopontin. J Bone Miner Res 7:1029–1036.

Xuan JW, Hota C, Chambers AF (1994): Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. J Cell Biochem 54:247–255.

Yoshida O, Okada Y (1990): Epidemiology of urolithiasis in Japan: A chronological and geographical study. Urol Int 45:104–111.