Urol Res (1999) 27:225–230

RAPID COMMUNICATION

Takahiro Yasui · Keiji Fujita · Yutaro Hayashi
Kousuke Ueda · Shigeyuki Kon · Masahiro Maeda
Toshimitsu Uede · Kenjiro Kohri

Quantification of osteopontin in the urine of healthy and stone-forming men

Received: 4 September 1998 / Accepted: 1 March 1999

Abstract Osteopontin (OPN) is one of the most important components in calcium stone matrix, but its role in stone formation is not clear. Since quantitative data regarding the excretion of OPN are necessary to assess its role, we have developed a quantitative enzyme-linked immunosorbent assay (ELISA) for OPN, and measured the urinary OPN concentrations in urolithiasis patients. Forty-seven men with urinary stones composed chiefly of calcium oxalate participated in the study. The controls were 13 normal healthy male volunteers. Urine samples were collected early in the morning and analyzed by a quantitative ELISA employing purified polyclonal antibodies to synthesized OPN aminopolypeptides. The urinary ratio of the concentrations of OPN and creatinine (OPN/Cre) in the urolithiasis patients (0.039 ± 0.029) was significantly lower than that in the control subjects (0.062 ± 0.030) ($P < 0.05$). Single stone formers ($n = 26$; 0.050 ± 0.020) had significantly higher OPN/Cre ratios compared with recurrent stone formers ($n = 21$; 0.031 ± 0.021) ($P < 0.05$). The results show that OPN excretion in urolithiasis patients was lowered, presumably because of the incorporation of OPN by kidney stones.

Key words Osteopontin · Urolithiasis · ELISA · Calcium oxalate · Stone formation

T. Yasui (✉) · K. Fujita · Y. Hayashi · K. Ueda · K. Kohri
Department of Urology,
Nagoya City University Medical School,
1 Kawasumi, Mizuho-cho, Mizuho-ku,
Nagoya 467-8601, Japan

M. Maeda
Immuno Biological Laboratories,
1091-1 Naka, Fujioka, Gunma 375-0005, Japan

S. Kon · T. Uede
Section of Immunopathogenesis,
Institute of Immunological Science, Hokkaido University,
Kita-15, Nishi-7, Kita-ku, Sapporo 060, Japan

Introduction

Urine excreted by normal individuals, as well as that excreted by stone formers, is often supersaturated with respect to calcium oxalate. Stone formation is induced rather than inhibited by a rise in the concentration of calcium and oxalate. The complex process of stone formation is retarded by substances in the urine that inhibit crystal-crystal as well as crystal-cell interactions [5, 7, 15, 18]. Although small molecules such as magnesium, pyrophosphate and citrate have been shown to be inhibitory [8], the major contribution to the inhibition of calcium oxalate crystallization in urine appears to be provided by the protein constituents of urine [5, 19]. A variety of inhibitory activities have been reported for several human urinary proteins, including nephrocalcin, Tamm-Horsfall protein and osteopontin (OPN) [1, 15, 19, 23]. At present, however, the relative importance of these activities is not known.

The majority of urinary stones are composed of calcium salts, and their predominant constituent is calcium oxalate [5, 11]. Biochemical analyses of urinary stones have revealed that 1–5% of their weight consists of proteinaceous ingredients, and several reports have suggested the importance of proteins in stone formation [2]. We previously extracted the proteinaceous fraction from calcium oxalate and calcium phosphorus stones with ethylenediamine tetraacetic acid (EDTA) and identified OPN as one of the major components [13]. A strong expression of OPN mRNA by distal tubular cells in the kidneys of both urolithiasis patients and stone-forming animal models was also demonstrated [12, 16]. OPN has also been shown to be a potent inhibitor of nucleation and of the growth of calcium oxalate crystal formation and the binding of calcium oxalate crystals to renal epithelial cells in vitro [23]. The reasons for this difference in the effects of OPN between in vivo and in vitro conditions are not known. Splicing variants and various glycosylated and phosphorylated forms of human OPN (hOPN) have diverse functions [6]. OPN is

efficiently cleaved by thrombin, producing two fragments of approximately equivalent size, in a reaction of unknown physiological importance but one that may affect the interaction of OPN with $\alpha_v\beta_3$ integrin [6]. Different functions of OPN have been suggested for the renal tubules and interstitial tissue [12, 17]. The predominance within the distal nephron may reflect mild subclinical tubular injury, since several forms of renal injury induce a marked up-regulation of OPN expression in this site [16]. With respect to calcium oxalate stone disease, it is noteworthy that osteopontin expression has been found to be markedly increased in distal nephrons of rats given ethylene glycol to induce hyperoxaluria [12] and that OPN production by cultured primate renal epithelial cells is stimulated by exposure to calcium oxalate crystals [14].

Since quantitative information concerning the normal rates of OPN excretion and the effects of factors that influence its excretion is required to precisely define the role of OPN in stone formation, we have developed a quantitative enzyme-linked immunosorbent assay (ELISA) employing purified polyclonal antibodies to OPN aminopolypeptides. In the present study, this assay was used to characterize urinary OPN excretion in both urolithiasis patients and healthy controls.

Methods

Subject selection

Forty-seven men (age 29–73 years, mean 53 years) with urinary stones composed chiefly of calcium oxalate participated in the study. None had primary hyperparathyroidism, renal tubular acidosis or other conditions known to be associated with abnormal metabolism. Individuals with a urinary tract infection or renal insufficiency were excluded. All gave their informed consent to this study, according to the Helsinki Declaration. The 13 healthy adult male volunteers (age 24–40 years, mean 30 years) who acted as controls were free of any history of kidney stone disease or radiographic evidence of stone formation. Serum calcium and creatinine and urinary calcium and creatinine excretion per 24 h were measured prior to entry; individuals with abnormal values (>300 mg calcium per 24 h; >4 mg calcium per kilogram body weight per 24 h) were excluded from the study. All urine samples for ELISA were collected early in the morning before any exercise or work. Urinary calcium, magnesium, phosphorus, sodium and potassium were measured with an automatic analyzer (Hitachi Model 705) after centrifugation.

Fig. 1 Schematic illustration of human osteopontin (hOPN). The GRGDS domain is boxed and the thrombin cleavage site is indicated by an *arrow*. The sequences of peptides used as immunogens for antibody production are shown for hOPN3 (^{170}Lys-^{187}Glu) and hOPN4 (^{296}Lys-^{314}Asn)

Characterization of antibodies

The synthetic peptides used as immunogens were as follows (Fig. 1):

hOPN3: ^{170}KSKKFRRPDIQYPDATDE

hOPN4: ^{296}KHLKFRISHELDSASSEVN

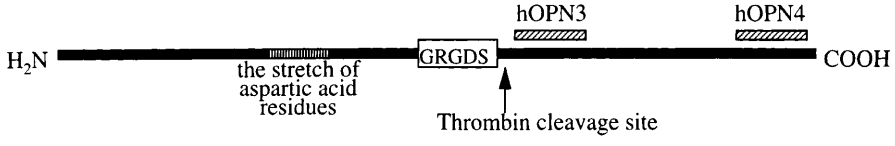
These were synthesized with a peptide synthesizer (Millipore 9050 plus, Millipore Bedford, Mass.), coupled with thyroglobulin and injected twice with complete Freund's adjuvant (0.5 mg of each dry synthesized polypeptide in 1 ml) into subcutaneous and intracutaneous tissues of rabbits weighing 3 kg. One milliliter aliquots of incomplete Freund's adjuvant were subsequently given 5 times weekly. Antisera against the peptides were obtained 1 week later, purified by affinity column chromatography with the peptides used as immunogens, and stored at −20°C.

Cloning of hOPN cDNA and generation of the expression vector

Total RNA was prepared [4] from NRC-12 cells (a renal cancer cell line). RNA (5 μg/sample) was reverse-transcribed using oligo-dT(15) and Superscript II reverse transcriptase (Gibco-BRL, Rockville, Md.) for 50 min at 42°C. The cDNA was amplified by the polymerase chain reaction (PCR) with 2.5 U *Taq* polymerase (Boehringer Mannheim Biochemica, Mannheim, Germany) in 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP-mix, and 50 pmol oligonucleotide primers: hOPN-5′ (5′-actaccatgagaattgcagtgatttgc-3′) and hOPN-3′ (5′-ttaattgacctcagaagatgcactatc-3′) based upon the published hOPN sequence [24]. The cycling parameters were as follows: 94°C for 30 s, 55°C for 60 s, 72°C for 60 s over 30 cycles, followed by 72°C for 3 min using a DNA Engine Model PTC-200 thermal cycler (MJ Research, Westertown, Mass.). The amplified products were ligated with T4 DNA ligase (Gibco-BRL) into pBlueScript-KS vector (Stratagene Cloning Systems, La Jolla, Calif.). Plasmids were subjected to DNA sequencing. The cDNA of hOPN was subcloned into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, Calif.) and transfected (10 μg per 60 mm dish) into CHO-K1 cells, which were seeded at a density of 10^5 per 60 mm culture dish, using a Cellphect transfection kit (Pharmacia, Uppsala, Sweden) following the manufacturer's instructions. After 6 h the cells were treated for 1 min with 15% glycerol in phosphate-buffered saline (PBS) (0.073 M NaCl, 0.018 M KH_2PO_4, 0.057 M Na_2HPO_4, pH 7.2) at room temperature and washed, and regular culture medium was added. Twenty-four hours later, G418 (Gibco-BRL) was added to the culture medium at a concentration of 400 μg/ml. The selection medium was changed every third day to remove dead cells, and this process was continued for 14 days after transfection. G418-resistant colonies were isolated, subcultured in regular growth medium, and periodically switched to selection medium to eliminate revertant cells.

Purification of recombinant hOPN protein from hOPN/CHO transcripts

The culture supernatant of hOPN/CHO transfectants was collected and concentrated to 1/10 volume by ultrafiltration on hollow fiber cartridges (H1P10–20, Amicon, Beverly, Mass.), mixed with urea to 6 M (final concentration) and applied to DEAE-sepharose CL-6B



hOPN3:^{170}KSKKFRRPDIQYPDATDE

hOPN4:^{296}KHLKFRISHELDSASSEVN

columns (Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 7.2) containing 6 M urea and eluted with 0.7 M NaCl in 50 mM Tris-HCl buffer (pH 7.2) containing 6 M urea after washing with 0.14 M NaCl in 50 mM Tris-HCl buffer (pH 7.2) containing 6 M urea. The eluted proteins were pooled and then reapplied to the DEAE-sepharose CL-6B column described above. The eluted pool was concentrated with polyethylene glycol, subjected to gel filtration chromatography on an ULTROGEL AcA44 column equilibrated with 50 mM Tris-HCl buffer (pH 7.2) containing 6 M urea and eluted with the same buffer. The OPN fraction was subjected to reverse-phase column chromatography on a RESOURCE RPC column and eluted with a 150 ml linear gradient from 0 to 80% acetonitrile (each containing 0.1% TFA) using a high-performance liquid chromatography (HPLC) system (Waters, Milford, Mass.). The main peak containing OPN was lyophilized, reconstituted with PBS and affinity chromatographed on a formylcellulofine column (Seikagakukougyo, Tokyo, Japan) conjugated with anti-hOPN4 antibody and eluted with 0.2 M glycine-HCl (pH 2.5). The eluted OPN fraction was neutralized with Tris-HCl buffer, added to a Sephadex G-25 column with a buffer change to 1/10 × PBS and lyophilized.

The identity of the purified recombinant (r)-hOPN was confirmed as follows. After electrophoresis on 12.5% SDS-polyacrylamide gels under reducing conditions, staining was performed with Coomassie brilliant blue or the cationic carbocyanine dye "Stain all" (Nakalai Chemicals, Tokyo), which stains sialoglycoproteins and phosphoproteins blue and other proteins red. Purification by the above method resulted in much clearer-hOPN immunostaining with 50 kDa of anti-hOPN3 and anti-hOPN4 antibodies (data not shown). The digestion of purified r-hOPN (18 μg) by incubation with or without thrombin (0.2 U, 0.1 U) at 37°C for 2 h was confirmed by Western blotting with anti-hOPN3 and anti-hOPN4 antibodies (data not shown).

Establishment of an ELISA system for hOPN

The sandwich ELISA was established when the anti-hOPN4 antibody was used as the capture antibody (coated on the wells) and the anti-hOPN3 antibody labeled with horseradish peroxidase (HRP) was used as the detection antibody. Recombinant hOPN purified from hOPN/CHO transfectant was used as the standard.

Measurement procedure

Wells of a Nunc maxisorp plate (Nunc, Roskilde, Denmark) were coated with 1 μg anti-hOPN4 polyclonal antibody in 50 μl PBS for 2 h at room temperature. Pre-coated wells were washed with PBS and then incubated with blocking solution [2% bovine serum albumin (BSA), 1% normal rabbit serum in PBS]. After washing, 100 μl aliquots of diluted samples or a known concentration of purified standard hOPN in PBS containing 1% BSA and 0.05% Tween20 were added to each well and incubated for 1 h at 37°C or overnight at 4°C. After extensive washing with PBS, 100 μl (200 ng) of HRP-labeled anti-hOPN3 polyclonal antibody was added to each well, followed by incubation for 30 min at 37°C. After washing 9 times, tetramethyl benzidine buffer (TMB) as substrate (100 μl) was added to each well and incubated for 30 min at room temperature in the dark. Color development was stopped with 100 μl of stop solution (1 N H_2SO_4), and absorbance was measured at 450 nm within 30 min. The concentrations of OPN in the samples were calculated using the curve derived from the optical densities of standards run on the same plate. Each sample was measured in triplicate at four dilutions with the initial urine dilution being 1:1000. If the mean optical densities of at least two dilutions of a urine sample did not fall within the linear portion of the standard curve for that plate, the assay for that sample was repeated using an appropriate series of dilutions. As shown in Fig. 2, this sandwich ELISA was quantitative with a linear range spanning from 1 to 40 ng/ml per well.

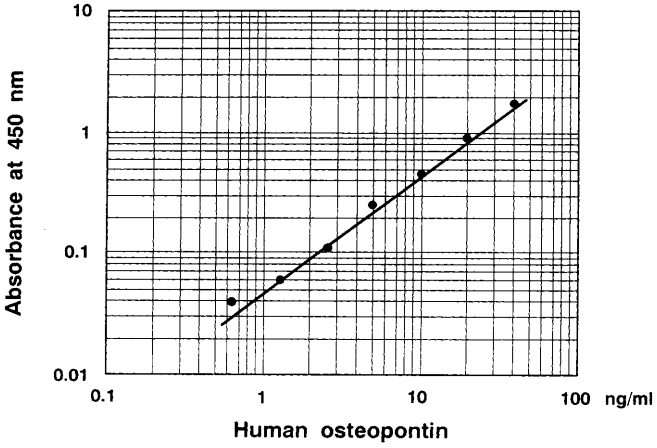


Fig. 2 The ELISA standard curve using the hOPN4 polyclonal antibody as the capture antibody and the hOPN3 polyclonal antibody as the detection antibody. Purified recombinant hOPN protein from hOPN/CHO transfectant was used as the standard antigen. Each *point* represents the average of duplicate assays

Statistical analysis

The data are presented as mean ± standard deviation (SD), and Student's *t*-test for paired values was applied to assess the significance of differences. The paired *t*-test for paired values was applied for the same individuals.

Results

The ratios of the concentrations of urinary OPN and creatinine (OPN/Cre) for the urolithiasis patients (n = 47, 0.039 ± 0.029) were significantly lower than those of the healthy controls (n = 13, 0.062 ± 0.030) ($P < 0.05$) (Fig. 3). This was also the case for urinary OPN: 35.77 ± 27.92 μg/ml as compared with 88.79 ± 35.28 μg/ml ($P < 0.05$).

Patients with urolithiasis were divided into two groups: "single" with no history of urolithiasis and "recurrence" cases. The OPN/Cre values for the single cases (n = 26, 0.050 ± 0.020) were significantly higher than those of the recurrence cases (n = 21, 0.031 ± 0.021) ($P < 0.05$) (Fig. 4). The same was true for urinary OPN (46.61 ± 28.88 μg/ml and 22.40 ± 20.30 μg/ml; $P < 0.05$). The OPN/Cre value for the single cases (n = 26, 0.050 ± 0.020) were slightly lower than those of the healthy controls (n = 13, 0.062 ± 0.030).

Patients referred to hospital with colic or stone symptoms and in whom the existence of urinary stones was confirmed were considered "active," while those without stones for over 1 year after treatment for urinary stones were termed "stable." Four of the 47 urolithiasis patients were examined because of a change between active and stable conditions; their respective OPN/Cre ratios of 0.042 ± 0.047 and 0.070 ± 0.032 were not significantly different (P = 0.41) (Fig. 5). There was also no significance in the lower concentration of urinary OPN in the active (15.28 ± 14.75 μg/ml) compared with the stable condition (50.92 ± 34.16 μg/

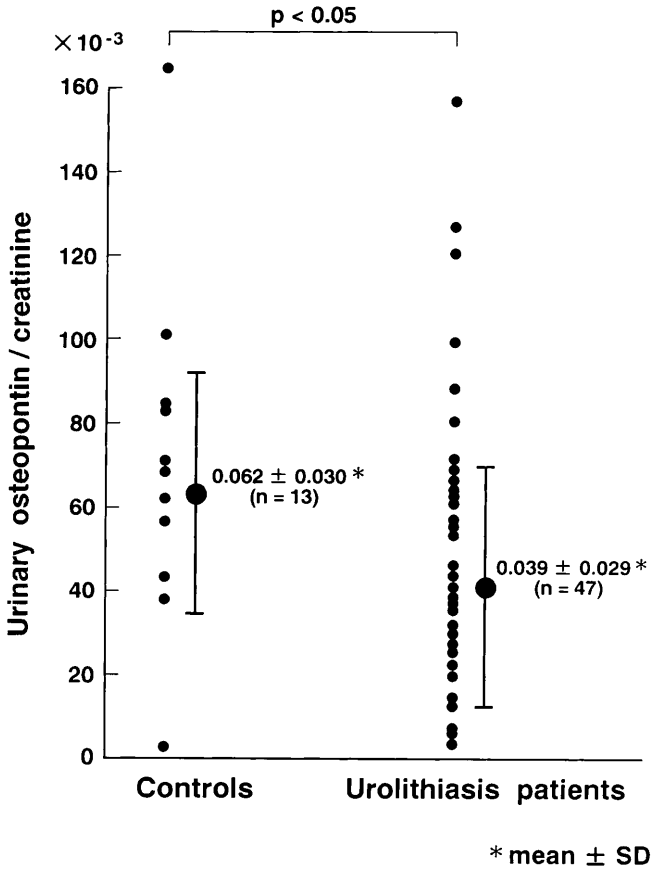


Fig. 3 The osteopontin/creatinine (OPN/Cre) concentration ratios of the urolithiasis patients (n = 47, 0.039 ± 0.029) were significantly lower than those of the healthy controls (n = 13, 0.062 ± 0.030) ($P < 0.05$)

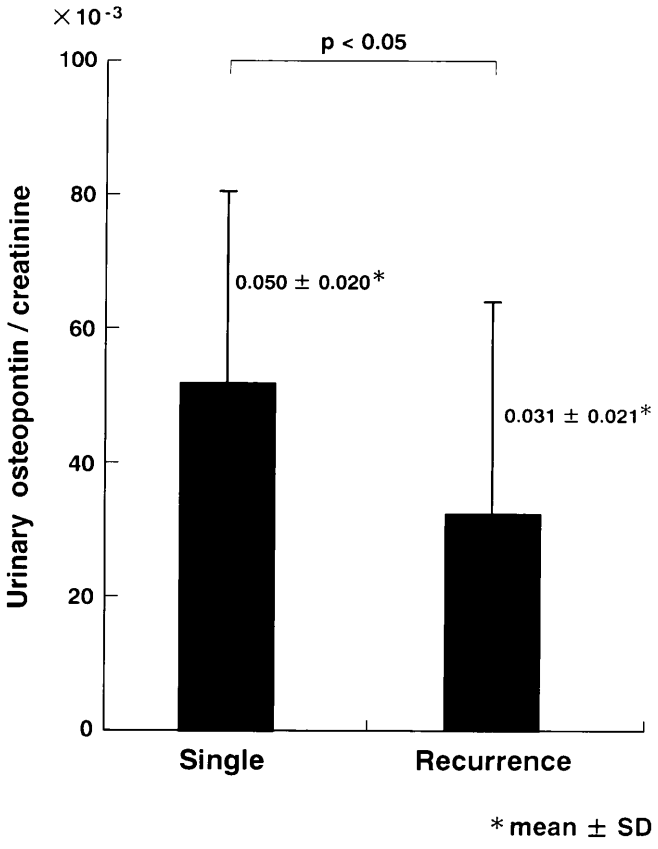


Fig. 4 The urolithiasis patients were divided into two groups: "single" with no urolithiasis history and "recurrence" cases. The OPN/Cre ratios of the single cases (n = 26, 0.050 ± 0.020) were significantly higher than those of the recurrence cases (n = 21, 0.031 ± 0.021) ($P < 0.05$)

ml) (P = 0.12). This might have been due to the small number of cases.

Overall, no significant relationship between the OPN/Cre and calcium/Cre ratios was seen (Fig. 6); the regression line, $y = 0.14 + 0.037x$, for the 47 samples of urolithiasis patients had an r value of only 0.197. Comparison of the urinary excretion rates also showed no significant link between OPN and calcium, magnesium, phosphorus, sodium or potassium (data not shown).

Discussion

The present results demonstrate that the excretion of urinary OPN in urolithiasis patients or stone-forming patients might be lower than in controls with no history of urolithiasis or in stable (non-stone-forming) urolithiasis patients. In this study, stone-forming individuals were older than the controls. However, in the stone-forming individual, OPN excretion showed no relationship with age. The role of OPN in stone formation is quite controversial. OPN expression has been localized mainly to the cells of the descending thin limb of Henle's loop and papillary surface epithelium [12, 16]. Since OPN is excreted from these cells, there is a possibility that its urinary concentration becomes reduced because of its combination with other materials. The present finding of reduced OPN excretion in urolithiasis patients suggests two possible explanations. First, the synthesis of OPN might be reduced, and the final OPN concentration is a potent inhibitor of the nucleation, growth and aggregation of calcium oxalate crystals, and would thus be expected to increase stone formation. The second possibility is that the OPN synthesis is maintained but its urinary excretion is decreased because of its incorporation into growing stones. The latter possibility seems more likely, because of the previous finding that OPN expression in renal tissue was increased in a stone-forming animal model [12]. It is known that OPN binds extremely tightly to hydroxyapatite [22], the most abundant component of bone-calcified matrix, through its nine consecutive aspartic acid residues [6]. OPN is a highly acidic, 44 kDa phosphorylated glycoprotein with an amino acid sequence of Arg-Gly-Asp (RGD) that elicits the binding of integrin [21]. OPN is associated with the transformation process, and is markedly up-regulated upon the transformation of cells [3, 10], playing a role in cell attachment and pathological calcium deposition such as that seen in urinary tract stones [9, 12, 13, 20, 21].

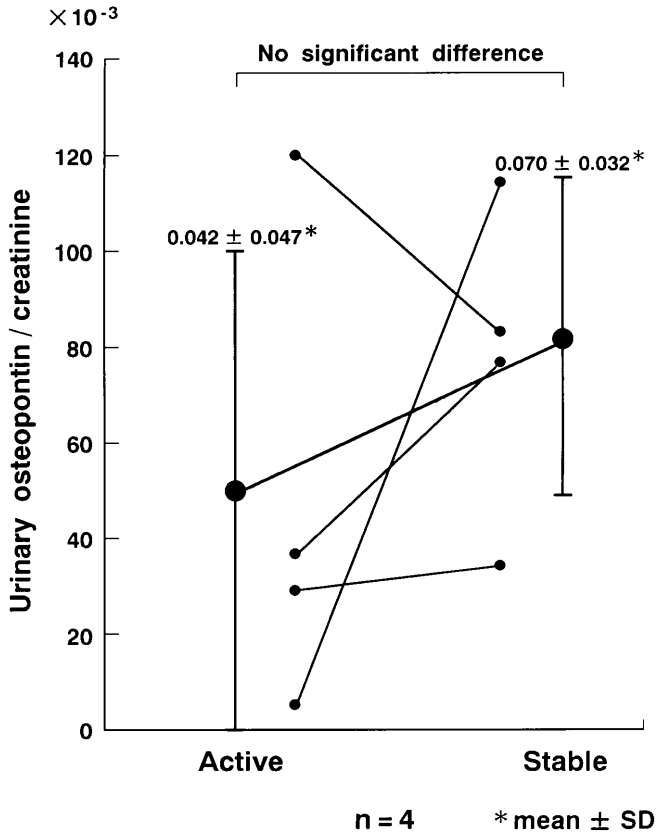


Fig. 5 The OPN/Cre ratios for the "active" and "stable" conditions of the same four patients. Patients referred to hospital with colic or stone symptoms and in whom the existence of urinary stones was confirmed were considered "active", while those without stones for over 1 year after treatment for urinary stones were termed "stable". The respective OPN/Cre ratios of 0.042 ± 0.047 and 0.070 ± 0.032 were not significantly different ($P = 0.41$)

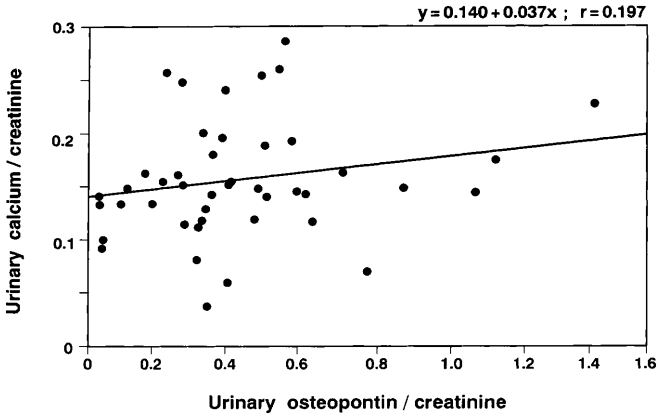


Fig. 6 Comparison of urinary osteopontin/creatinine and calcium/creatinine concentrations for 47 urine samples of male urolithiasis patients. The regression line, $y = 0.140 + 0.037x$, had an r value of only 0.197

In summary, we have developed a quantitative ELISA employing purified polyclonal antibodies to synthesized OPN aminopolypeptides. The application of this assay in the present study revealed that OPN excretion in urolithiasis patients is reduced, presumably because of the incorporation of OPN into kidney stones. Future use of this ELISA may help to estimate the initiation of urolithiasis.

Acknowledgements This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (nos. 0947035 and 10877251), and a grant from the National Space Development Agency of Japan (NASDA).

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