Urol Res (1999) 27:238–242

ORIGINAL PAPER

Toshiyasu Tawada · Keiji Fujita · Takeshi Sakakura
Toshiaki Shibutani · Toshihiko Nagata
Masanori Iguchi · Kenjiro Kohri

Distribution of osteopontin and calprotectin as matrix protein in calcium-containing stone

Received: 24 February 1998 / Accepted: 28 January 1999

Abstract We recently reported that osteopontin (OPN) and calprotectin (CPT) are present in the matrix of urinary calcium stones, and that OPN mRNA is expressed in the renal distal tubular cells. In the present study, we examined the immunohistochemical distributions of OPN and CPT in urinary stones. The stones used in this study were passed spontaneously from the upper urinary tract. One half of each of the stones was analyzed with an infrared spectrophotometer, and were shown to be comprised of calcium oxalate, calcium phosphate, uric acid and cystine. The other half of each stone was immersed in tetrasodium ethylenediaminetetraacetate (EDTA) solution. The half-stones were embedded in paraffin and cut into 5-μm sections. The avidin-biotin-peroxidase complex technique was employed. A monoclonal antibody to human milk-derived OPN and a monoclonal antibody to human granulocyte-derived CPT were used as primary antibodies. The immunochemical study using the OPN and CPT antibodies showed positive staining of the matrix of the urinary calcium stones. The stones showed staining in two distinct zones: a core area was stained with randomly aggregated OPN and CPT, and peripheral layers were stained in concentric circles. On the basis of our observations, it is reasonable to presume that OPN and CPT play roles as the matrix in the structure of urinary calcium stones.

T. Tawada · K. Kohri (✉) · K. Fujita · T. Sakakura
Department of Urology,
Nagoya City University School of Medicine, Nagoya, Japan

T. Shibutani
Department of Periodontology,
Asahi University School of Dentisty, Gifu, Japan

T. Nagata
Department of Periodontology and Endodontology,
Tokusima University School of Dentisty, Tokushima, Japan

M. Iguchi
Department of Urology, Kaizuka City Hospital, Osaka, Japan

Key words Urinary stone · Matrix · Osteopontin · Calprotectin

Introduction

Urinary stones consist of a crystalline or mineral phase and a noncrystalline or organic phase. Urinary stone contain 1%–5% protein, and several reports have suggested the importance of protein in urinary stone formation. However, little is known about urinary stone proteins. We have demonstrated in molecular biology studies that the proteins osteopontin (OPN) and calprotectin (CPT) are present in the matrix of urinary stones [6, 7].

OPN is a 44-kDa secreted phosphoprotein that is highly negatively charged and frequently associated with mineralization processes. It is produced by many types of epithelial cell and is found both in normal plasma and in a variety of body secretions including urine, milk, and bile [1]. Transformed cells, particularly *ras*-transformed cells, express OPN at elevated levels. Via the interaction of an Arg-Gly-Asp (RGD) sequence [11] in OPN with an integrin (probably the α_v β_3 integrin), OPN is able to promote cell adhesion and to activate a signal transduction pathway. One consequence of OPN signaling is an alteration in the intracellular calcium concentration $[Ca^{2+}]_i$; both a decrease [9] and an increase in osteoclasts have been observed. In the mouse kidney, high-level focal expression is observed in a subset of the nephrons, mostly in the epithelial cells of the thick ascending limb of the long loop of Henle, and in sclerosing glomeruli [8].

CPT is a major leukocyte protein, initially separated from the cystosol of human granulocytes and also present in macrophages and epithelial cells. It is also known as L1 antigen or macrophage migration inhibitory factor-related proteins 8 and 14; the subunits are members of the S-100 protein family. Although the physiological functions of CPT are unclear, this protein shows calcium-binding properties and antimicrobial activity against several bacteria, and its tissue level

is markedly increased in many infections and malignant diseases.

Few studies have examined the distribution of matrix proteins in urinary stones. In this study, we made serial sections of calcium oxalate stones, calcium phosphate stones, uric acid stones and cystine stones, and examined the immunohistochemical distributions of OPN and CPT in them.

Materials and methods

Preparation of tissue

The stones used for this study were passed spontaneously from the upper urinary tract. These stones were fractured through their center with a razor blade. Half of each of the stones was analyzed with an infrared spectrophotometer, and they were proven to be calcium oxalate, calcium phosphate, uric acid, and cystine stones. The other half of each stone was fixed with 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. These halves were immersed in 10% (weight/volume) tetrasodium ethylenediaminetetraacetate (EDTA) solution adjusted to pH 7.4 at 4°C for 2 weeks. The stones were then washed with PBS, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. Five-μm sections were cut on a microtome, and the sections were mounted on glass slides precoated with 0.1% poly-L-lysine (Sigma Chemicals, St Louis, Mo.) and dried overnight at 37°C.

Preparation of anti-OPN antibody

All experimental procedures were performed in accordance with protocols approved by the Animal Care Committee of the Faculty of Medicine, Nagoya City University. BALB/c mice were immunized subcutaneously with 20 μg of human milk-derived osteopontin [14] emulsified with an equal volume of complete Freund's adjuvant. The mice were boosted twice at 10-day intervals with the antigen emulsified with incomplete adjuvant. The immunization was finalized with an intravenous injection of 10 μg antigen in PBS. Three days after the third booster, the mice were killed, the spleen was removed and the splenocytes were hybridized with murine myeloma cells (P3x63Ag8.653) and subcloned according to standard procedures. Monoclonal antibodies were purified from ascitic fluid by using Affi-Gel protein and a monoclonal antibody purification system (Bio-Rad, Hercules, Calif.). The enzyme-linked immunosorbent assay (ELISA) was performed using 96-well, U-bottomed polyvinylchloride microtiter plates. Each well was filled with 50 μl of 2.5 μg/ml osteopontin in 0.1 M bicarbonate buffer, pH 9.0, and incubated at room temperature for 2 h. After three washes with 0.1% Tween 20 (Sigma) in PBS (TBS), 200 μl of 3% bovine serum albumin (BSA) in PBS was added to the plates, which were then allowed to stand for 30 min. After the plates were washed with TBS, 100 μl of hybridoma supernatant or serial dilutions of purified antibody were added, followed by incubation at 4°C for 60 min. After three washes with TBS, 100 μl of an appropriate dilution of a horseradish peroxidase-conjugated anti-mouse Ig xenoantibody was added to each well. The plates were then incubated for 60 min at 4°C and washed three times with TBS and once with PBS. Then 200 μl of freshly prepared substrate solution containing 0.05% 0-phenylenediamine and 0.0075% hydrogen peroxide in McIlvaine's buffer, pH 6.0, was added to each well. After 20 min of incubation in the dark at room temperature, absorbance was read at 405 nm with a plate reader (Titertech Multiscan).

Preparation of anti-CPT antibody

CPT purified from human granulocytes and antihuman CPT rabbit immunogloblin were generously supplied by Dr M.K. Fagerhol (Ulleval Hospital, Oslo, Norway) [2].

Immunohistochemical procedure

Before immunostaining, the paraffin sections were de-waxed and rehydrated for three 5-min periods in PBS. The sections were incubated with 0.01% actinase in distilled water and washed three time in PBS for 3 min. The nonspecific binding of immunoglobulins was prevented by incubating the sections with 1% skimmed milk (Yukijirushi, Tokyo, Japan) and 0.5% NaN_3 in PBS for 30 min. The section were the incubated with the primary antibody for 2 h at room temperature. Sections were incubated with PBS containing 1% BSA as a negative control. The sections were then washed three times in PBS for 5 min. To remove endogenous peroxidase, the sections were incubated with 3% hydrogen peroxide in methanol and washed three times in TBS for 5 min and then incubated for 30 min with biotinylated mouse antimouse IgG diluted in PBS. The sections were rinsed in TBS and incubated for 30 min with diluted ABC reagent, washed in PBS and finally incubated in a freshly prepared solution of 0.02% diaminobenzidine tetrahydrochloride (DAB, Sigma) in 0.1 M TRIS/HCl buffer, pH 7.2, containing 0.005% hydrogen peroxide until optical staining was obtained. The slides were then washed in cold running water for 10 min, dehydrated in graded ethanol, cleared in xylene and sealed with Entellan (Merck, Darmstadt, Germany).

Results

The immunostaining with OPN antibody and CPT antibody revealed staining of the calcium oxalate stones. Two distinct zones were stained: the core area in the center and the concentrically laminated peripheral layer. The core areas showed stains for randomly aggregated OPN and CPT. The peripheral layer stains were concentric circles.

Figure 1 shows the distribution of OPN on a calcium oxalate stone. The peripheral layers were strongly stained for OPN is concentric circles. CPT was more strongly and clearly stained in the peripheral layers (similar to concentric circles) than was OPN (Fig. 1b).

The distributions of OPN and CPT in a small calcium oxalate stone, shown in Fig. 2, made concentric circles in the peripheral layers. OPN was most positive on the outer edge of the stone.

The OPN and CPT distributions in a calcium phosphate stone are shown in Fig. 3. The matrix of this stone extended radially from a central area and this radial extension takes the form of concentric circles. The OPN and CPT were distributed in the matrix correspondingly. OPN was more strongly stained than CPT. Figures 4 and 5 show a uric acid stone and cystine stone, respectively. Both these types of stone were negative for anti-OPN monoclonal antibody. The matrix of these stones was structured in layers.

Discussion

The present findings show the distributions of OPN and CPT on both calcium oxalate stones and calcium phosphate stones. OPN is a recently discovered non-collagenous bone matrix protein, and a 44-kDa phosphorylated glycoprotein containing the amino acid sequence Arg-Gly-Asp [11], which elicits the binding of

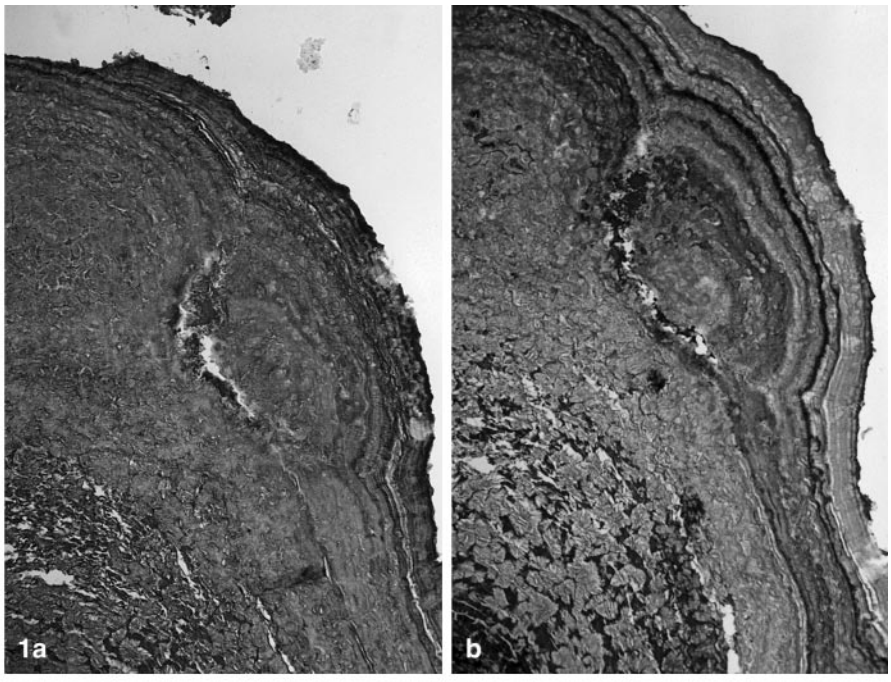


Fig. 1a The distribution of osteopontin (OPN) in a calcium oxalate stone. Peripheral layers are strongly stained in concentric circles. **b** The distribution of calprotectin (CPT) in a calcium oxalate stone. CPT is more strongly and clearly stained that OPN in the peripheral layers, in concentric circles. (×100)

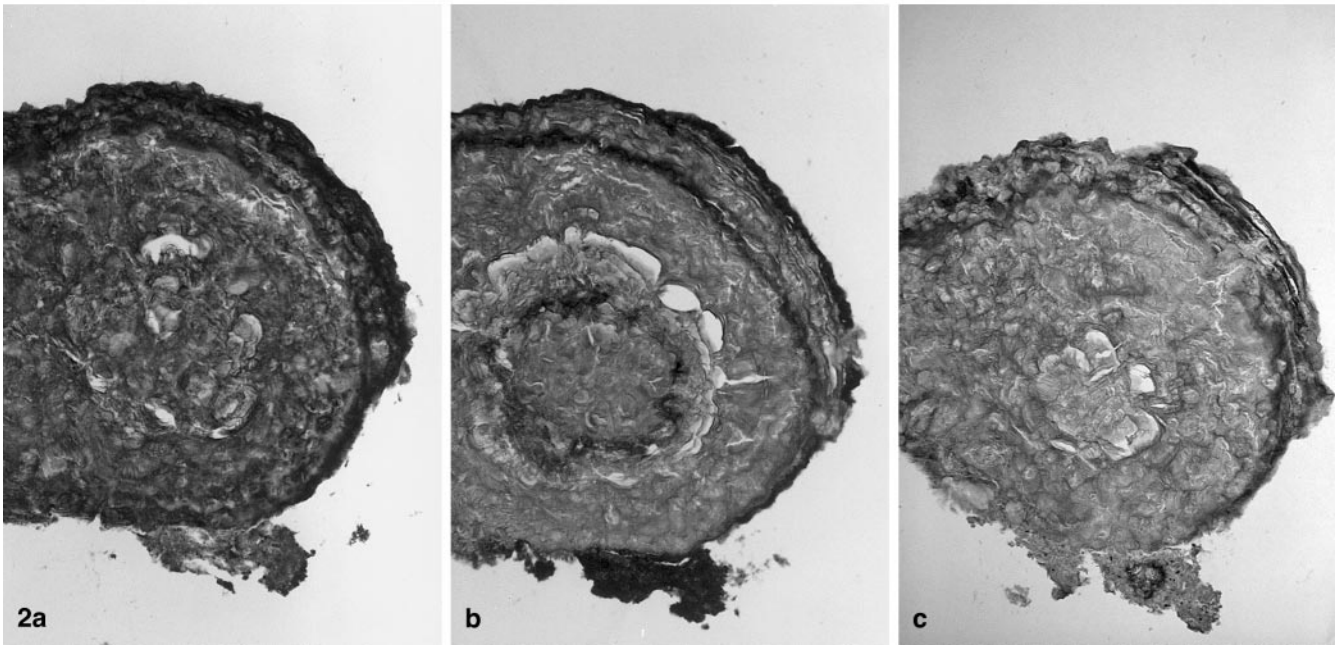
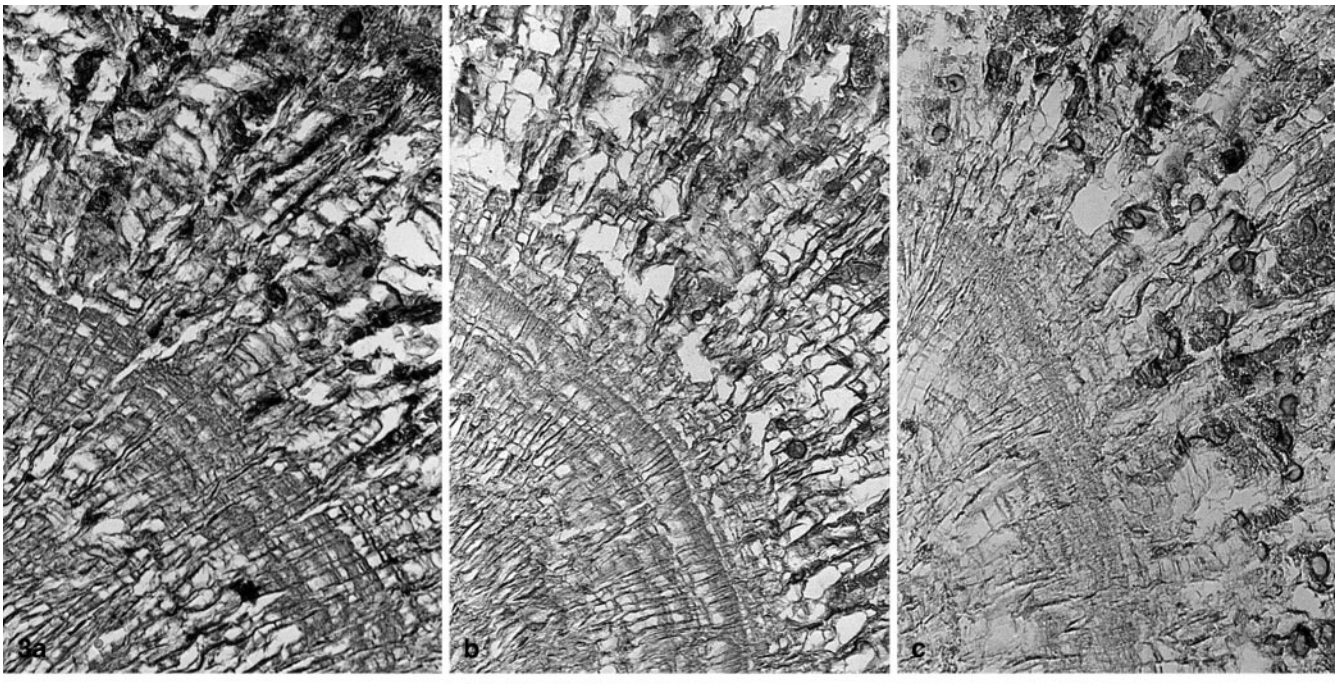


Fig. 2 The distributions of **a** OPN and **b** CPT in a small calcium oxalate stone. OPN and CPT were stained in concentric circles in the peripheral layers. OPN was the most positive on the outer edge of the stone. **c** Negative control. (×200)

integrin. OPN has a high affinity for hydroxyapatite [11] and seems to play a role in modulating the mineralization of calcifying tissues [10, 11]. The function of OPN is not fully elucidated, although it is postulated to have roles in mineralization and/or bone resorption. OPN enhances fibroblast attachment in vitro [16–18] and is thought to anchor osteoclasts to bone [15] via its Arg-Gly-Asp tripeptide, which is postulated to bind to a member of the integrin receptor family [11]. It is not known whether OPN binds to the extracellular matrix components that are also present in the bone matrix. It is known, however, that OPN binds extremely tightly to hydroxyapatite [11]. Which region or regions in the OPN molecule are involved in this binding is not known. The bone-specific protein osteocalcin interacts with hydroxyapatite most likely via γ-carboxyglutamic acid residues [12]. A potential region for mineral binding in OPN is the region consisting of nine consecutive aspartic acid residues. Perhaps this region can assume a conformation with carboxyl-group arrangements similar to the γ-carboxyglutamic acid residues in osteocalcin, and bind to hydroxyapatite via a similar mechanism. Another possibility is that the numerous sialic acid-containing oligosaccharides confer on OPN an affinity for hydroxyapatite [14].

Interestingly, OPN and mRNA is observed mainly in bone and kidney [5, 20]. A possible relationship between OPN and calcium metabolism is indicated by the tissue



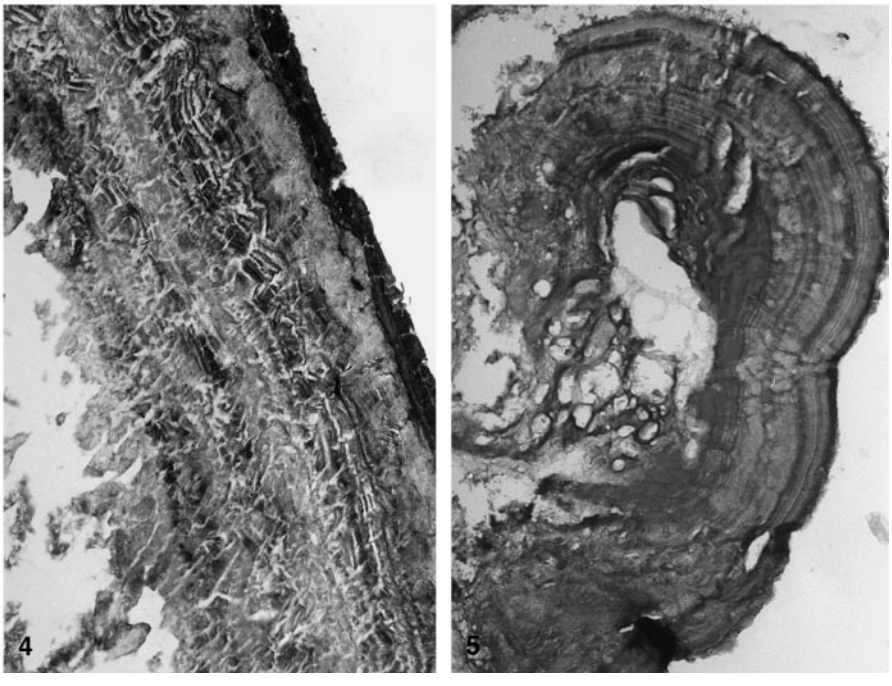


Fig. 3 The distribution of **a** OPN and **b** CPT in a calcium phosphate stone. The matrix of this tone extended radially from a central area, and the radial extension took the form of concentric circles. The OPN and CPT were distributed in the matrix correspondingly. OPN was more strongly stained than CPT. **c** Negative control. (×200)

Fig. 4 Uric acid stone. This type of stone was negative for anti-OPN monoclonal antibody. The matrix was structured in layers. (×200)

Fig. 5 Cystine stone. This type of stone was negative for anti-OPN monoclonal antibody. The matrix was structured in layers. (×200)

distribution of the protein and by the regulation of OPN gene expression by 1,25-dihydroxyvitamin D_3 [13, 20, 22], and by its presense in milk [9]. 1,25-dihydroxyvitamin D_3 is strongly related to calcium metabolism and is activated in the kidneys. Calcium-containing stones such as calcium oxalate and calcium phosphate stones account for 80% of renal stones [21]. Urinary stone matrix contains an abundance of acidic amino acids (glutamic acid and aspartic and acid), which are the main amino acids in OPN [12, 19, 21].

CPT is a major leukocyte protein, initially separated from the cystosol of human granulocytes, and also present in macrophages and epithelial cells. Although the physiological functions of CPT are unclear, this protein shows calcium-binding properties and antimicrobial activity against several bacteria, and its tissue level is markedly increased in many infections and malignant diseases.

The presence of OPN and CPT was demonstrated in the matrix of urinary stones [6, 7], but the distributions of OPN and CPT in urinary stones was not known. In this study, immunostaining with OPN antibody and CPT antibody revealed the staining of calcium oxalate stones in two distinct zones: a core area in the center and the concentrically laminated peripheral layers. The core area was stained with randomly aggregated OPN and

CPT. The peripheral layers were stained in concentric circles. CPT is more strongly and clearly stained than OPN in the peripheral layers. It is speculated that the expression of CPT is increased after stone formation because CPT presents in leukocytes as well as renal epithelial cells.

The distributions of OPN and CPT in calcium phosphate stones were also identified. The matrix of these stones extends radially from a central area, and the radial extension takes the form of concentric circles. OPN and CPT are distributed in the matrix correspondingly. OPN was more strongly stained than CPT. It is important to study whether OPN and CPT are specific or nonspecific in stone formation. We speculate that both proteins play a specific role in stone formation, because they bind tightly with calcium. The uric acid stones and cystine stones were negative for anti-OPN monoclonal antibody. The matrix of these stones is structured in layers. Hashimoto et al. [4] recently reported that an immunoglobulin heavy chain and a κ light chain were clearly detected in uric acid stones by Western blotting.

Recently, using high-resolution colloidal gold immunocytochemistry Mckee et al. [23] have shown OPN and osteocalcin to be major components in ghosts of excreted renal calculi and intratubular and papillary crystals. de Bruijn et al. [24] have studied in situ ultrastructural OPN localization in papillary stones induced in rats, and demonstrated that the presence of OPN was confirmed inside crystal ghosts of larger stones in the interstitium and at the outer surface of the papilla [24].

On the basis of these observations and the present data, we conclude that it is reasonable to presume that OPN and CPT each play a role as the matrix in urinary calcium stone formation.

References

1. Brown LF, Berse B, Van De Water L, Papadopoulos-Sergiou A, Perruzzi CA, Manseau EJ, Dvorak HF, Senger DR (1992) Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. Mol Biol Cell 3:1169
2. Fagerhol MK, Andersson KB, Naess-Andresen C-F, Brandtzaeg P, Dale I (1990) Calprotectin (the L1 leukocyte protein). In: Smith VL, Dedman JR (eds) Stimulus response coupling: the roll of intracellular calcium-binding protein. Boca Raton: CRC Press, p187
3. Gotoh Y, Pierschbacher MD, Grzesiak JJ, Gerstenfeld L, Glimcher MJ (1990) A comparison of two phosphoproteins in chicken bone and their similarities to the mammalian bone proteins, osteopontin and bone sialoprotein II. Biochem Biophys Res Commun 173:471
4. Hashimoto Y, Kohri K, Hayashi Y, Moriyama A, Iguchi M, Asai K, Kato T (1997) Specific detection of κ light chain in uric acid stones. Life Sci 61:249
5. Kerr JM, Fisher LW, Termine JD, Young MF (1991) The cDNA cloning and RNA distribution of bovine osteopontin. Gene 108:237
6. Kohri K, Suzuki Y, Yoshida K, Yamamoto K, Amasaki N, Yamate T, Umekawa T, Iguchi M, Shinohara H, Kurita T (1992) Molecular cloning and sequencing of cDNA encoding urinary stone protein, which is identical to osteopontin. Biochem Biophys Res Commun 184:859
7. Kohri K, Nomura S, Kitamura Y, Nagata T, Yoshioka K, Iguchi M, Yamate T, Umekawa T, Suzuki Y, Shinohara H, Kurita T (1993) Structure and expression of the mRNA encoding urinary stone protein (osteopontin). J Biol Chem 268:15180
8. Lopez CA, Hoyer JR, Wilson PD, Waterhouse P, Denhardt DT (1993) Heterogeneity of osteopontin expression among nephrons in mouse kidneys and enhanced expression in sclerotic glomeruli. Lab Invest 69:355
9. Miyauchi A, Alvarez J, Greenfield EM, Teti A, Grano M, Colucci S, Zambonin-Zallne A, Rose FP, Teitelbaum SL, Cheresh D, Hurska KA (1991) Recognition of osteopontin and related peptides by an α_v β_3 integrin stimulates immediate cell signals in osteoclasts. J Biol Chem 266:20369
10. Nomura S, Wills AJ, Edwards DR, Heath JK, Hogan BLM (1988) Developmental expression of 2ar (osteopontin) and SPARC (osteonectin) RNA as revealed by in situ hybridization. J Cell Biol 106:441
11. Oldberg A, Franzen A, Heinegard D (1986) Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp Cell-binding sequence. Proc Natl Acad Sci USA 83:8819
12. Poser JW, Price PA (1987) A method for decarboxylation of γ-carboxyglutamic acid in proteins. J Biol Chem 254:431
13. Prince CW, Butler WT (1987) 1,25-dihydroxyvitamin D_3 regulates the biosynthesis of osteopontin, a bone-derived cell attachment protein, in clonal osteoblast-like osteosarcoma cells. Collagen Relat Res 7:305
14. Reinholt FP, Hultenby K, Oldberg A, Heinegard D (1990) Osteopontin–a possible anchor of osteoclasts to bone. Proc Natl Acad Sci USA 87:4473
15. Senger DR, Perruzzi CA, Papadopoulous A, Tenen DG (1989) Purification of a human milk protein closely similar to tumor-secreted phosphoproteins and osteopontin. Biochem Biophys Acta 996:43
16. Somerman MJ, Prince CW, Sauk JJ, Foster RA, Butler WT (1987) Mechanism of fibroblast attachment to bone extracellular matrix: role of a 44-kilodalton bone phosphoprotein. J Bone Miner Res 2:259
17. Somerman MJ. Fisher LW, Foster RA, Sauk JJ (1989) Human bone sialoproteins I and II enhance fibroblast attachment in vitro. Calcif Tissue Int 43:50
18. Somerman MJ, Prince CW, Butler WT, Foster RA, Moehring JM, Sauk JJ (1989) Cell attachment activity of the 44-kilodalton bone phosphoprotein is not restricted to bone cells. Matrix 9:49
19. Spector AR, Gray A, Prien EL (1976) Kidney stone matrix: differences in acidic protein composition. Invest Urol 13:387
20. Yoon Y, Buenaga R, Rodan GA (1987) Tissue specificity and development expression of rat osteopontin. Biochem Biophys Res Commun 148:1129
21. Yoshida O, Okada Y (1990) Epidemiology of urolithiasis in Japan: a chronological and geographical study. Urol Int 45:104
22. Wrana JL, Kubota T, Zhang Q, Overall CM, Aubin JE, Butler WT, Sodek J (1991) Regulation of transformation-sensitive secreted phosphoprotein (SPPI/osteopontin) expression by transforming growth factor-β. Biochem J 273:523
23. Mckee MD, Nanci A, Khan SR (1995) Ultrastructural immunodetection of osteopontin and osteocalcin as major matrix components of renal calculi. J Bone Miner Res 10:1913
24. de Bruijn WC, de Water R, van Run PRWA, Boeve ER, Kok DJ, Cao L, Romijn HC, Vekoelen CF, Schroder FH (1997) Ultrastructural osteopontin localization in papillary stones induced in rats. Eur Urol 32:360