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Antibodies to Different Peptides in Osteopontin Reveal Complexities in the Various Secreted Forms

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Abstract We have generated synthetic peptides corresponding to various portions of human osteopontin (OPN) and have immunized rabbits and mice with these peptides to generate polyclonal and monoclonal antibodies specific to human OPN. We then generated six distinct sandwich enzyme-linked immunoabsorbent assay (ELISA) systems by using different pairs of polyclonal and monoclonal antibodies against human OPN. These systems allowed us to detect not only various isoforms and truncated forms of recombinant OPN, but also the glycosylated form of native urinary OPN. Most importantly, tumor-derived OPN was differentially detected by the six ELISA systems. The ELISA systems that we have developed will be useful for clarifying the functional roles for OPN in vivo in various physiologic and pathologic conditions. J. Cell. Biochem. 77:487–498, 2000.

Key words: osteopontin; isoforms; urine; tumor cells; ELISA

Osteopontin (OPN) is a highly acidic calcium-binding glycosylated phosphoprotein secreted by many cell types, including osteoblasts, kidney tubule cells, macrophages, activated T cells, and vascular smooth muscle cells. Osteopontin expression has been shown to associate closely with tumorigenesis and metastasis [Chambers et al., 1993; Oates et al., 1996; Oates et al., 1997]. Development of glomerulonephritis in the kidney and granuloma in various tissues is accompanied by upregulation of OPN expression [Carlson et al., 1997; Nau et al., 1997; Lan et al., 1998]. Evidence that OPN is involved in the formation and calcification of atherosclerotic plaques has accumulated in recent years [Hirota et al., 1993; Ikeda et al., 1993; O'Brien et al., 1994; Fitzpatrick et al, 1994]. Thus, OPN has now received considerable attention for its potential role in several disease processes.

One important feature of OPN is the presence of various molecular forms in vivo due to differential RNA splicing, glycosylation, phosphorylation, sulfation, and susceptibility to proteases [Denhardt and Guo, 1993; Patarca et al., 1993]. Both OPN and thrombin are likely to be localized together at the site of injury, inflammation, and angiogenesis and in tumor tissues. Osteopontin is susceptible to proteolytic fragmentation, and this process may have physiologic importance. Thrombin cleavage of OPN has been reported to destroy arginine-glycine-aspartic acid (RGD)-mediated cell adhesion [Xuan et al., 1994]. Another report demonstrated that thrombin treatment enhanced OPN cell adhesive activity [Senger et al., 1994], suggesting that cleavage of OPN by thrombin exposes a cryptic adhesive sequence. More recently, it was shown that an amino-terminal OPN fragment contains a cryptic

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binding site that can be recognized by α9β1 integrin [Smith et al., 1996]. Furthermore, OPN contains multiple cell binding sites and interacts with various receptors; these interactions may have distinct functional consequences [Yue et al., 1994; Liaw et al., 1995; Katagiri et al., 1996; Katagiri et al., 1999]. Therefore, systems for detecting various forms of OPN are essential to better understand the role of OPN in vivo. In this report, we demonstrate that various forms of recombinant OPN can be measured by the six distinct enzyme-linked immunoabsorbent assay (ELISA) systems we developed. The specificities of the polyclonal and monoclonal antibodies used to establish these ELISA systems were established. We then demonstrated that these ELISA systems can differentially measure various forms of native human OPN.

MATERIALS AND METHODS

Cloning of Human OPN cDNA

Total RNA was prepared from human NRC-12 renal carcinoma cells, and first-strand cDNA was generated with reverse transcriptase and random primers. Oligonucleotides used in this study were synthesized based on the published human OPN cDNA sequence [Saitoh et al., 1995]. The OPN cDNA was then amplified by polymerase chain reaction (PCR) using an OPN-5 (5′-CGGGATCCACTACCATGAGAATTGCAGTGATTTGC-3′) primer complementary to the human cDNA sequence of the first seven amino acids (M_1–C_7) with the signal peptide and the *Bam* HI restriction site, and an OPN-3 (5′-CCGCTCGAGTTAATTGACCTCAGAAGATGCACTATC-3′) primer complementary to human cDNA sequences at the carboxy-terminal amino acid (D_{307}–N_{314}) and the stop codon with a *Xho* I restriction site. The amplified products were digested with *Bam* HI and *Xho* I and ligated into pBlueScript-KS vector (Stratagene, La Jolla, CA). By this method, we cloned OPN-a and OPN-b cDNA (Fig. 1). The OPN-a and OPN-b cDNA cloned by PCR were completely sequenced.

The cDNA of OPN-c, which lacks 28 amino acids from Q31 to Q58, was cloned by using OPN-a cDNA as a template using two steps of PCR. In the first PCR step, two sets of PCR reactions were conducted with an OPN-5 primer and an OPNct-3 (5′-ACACAGCATTCTTTTCCTCAGAACTTCCAGAATCAGC-3′) primer complementary to the human cDNA sequence of nine amino acids (A_{22}–K_{30}) and 10 nucleotides following N59, and an OPN-3 primer and an OPNct-5 (5′-TGAGGAAAAGAATGCTGTGTCCTCTGAAGAAACC-3′) primer containing human cDNA sequence of 10 nucleotides prior to K_{30} and eight amino acids following N_{59} (N_{59}–T_{66}). Then, in the second PCR step, products of the first PCR were mixed, annealed after heat denaturation, and amplified by PCR using OPN-5 and OPN-3 primers. The amplified products were digested with *Bam* HI and *Xho* I and ligated into pBlueScript-KS vector.

Carboxy-terminal half OPN-a (C half OPN; K_{170}–N_{314}) was constructed from human OPN-a as a template. Briefly, in the first PCR step, two sets of PCR reactions were conducted with an OPN-5 primer and an OPNch-3 (5′-TCTTAGATTTGGCACAGGTGATGCCTAGGAG-3′) primer complementary to human cDNA sequence of the seven amino acids (L_{10}–A_{16}) and 10 nucleotides following K_{170} and an OPN-3 and an OPNch-5 (5′-CACCTGTGCCAAATCTAAGAAGTTTCGCAGA-3′) primer containing human cDNA sequence of 10 nucleotides prior to A_{16}, and seven amino acids following K_{170} (K_{170}–R_{176}). Then, in the second PCR step, products from the first PCR were amplified by using OPN-5 and OPN-3 primers.

Amino-terminal half OPN-a (N half OPN; M_1–R_{168}) was constructed using PCR with an OPN-5 and an OPNnh-3 (5′-GCCTCGAGTTACCTCAGTCCATAAACCACACT-3′) primer complementary to human cDNA sequence of seven amino acids (S_{162}–R_{168}) and stop codon with *Xho* I restriction site. The amplified products were digested with *Bam* HI and *Xho* I and ligated into pBlueScript-KS vector.

Construction of the GST-OPN Fusion Plasmid

The five OPN cDNAs were inserted into pGEX-4T vector (Amersham Pharmacia Biotech, Tokyo, Japan) in the same reading frame as the carrier gene (GST; glutathione S-transferase, EC 2.5.1.18) and transformed in *Escherichia coli* JM109 cells. Thus, five human GST/OPN were produced: GST fused to mature human OPN-a, OPN-b, and OPN-c, an amino-terminal half of human OPN-a (M_1–R_{168}), and a carboxy-terminal half of human OPN-a that lacks $R_{159}G_{160}D_{161}$ (K_{170}–N_{314}) (Fig. 1). The OPN-a

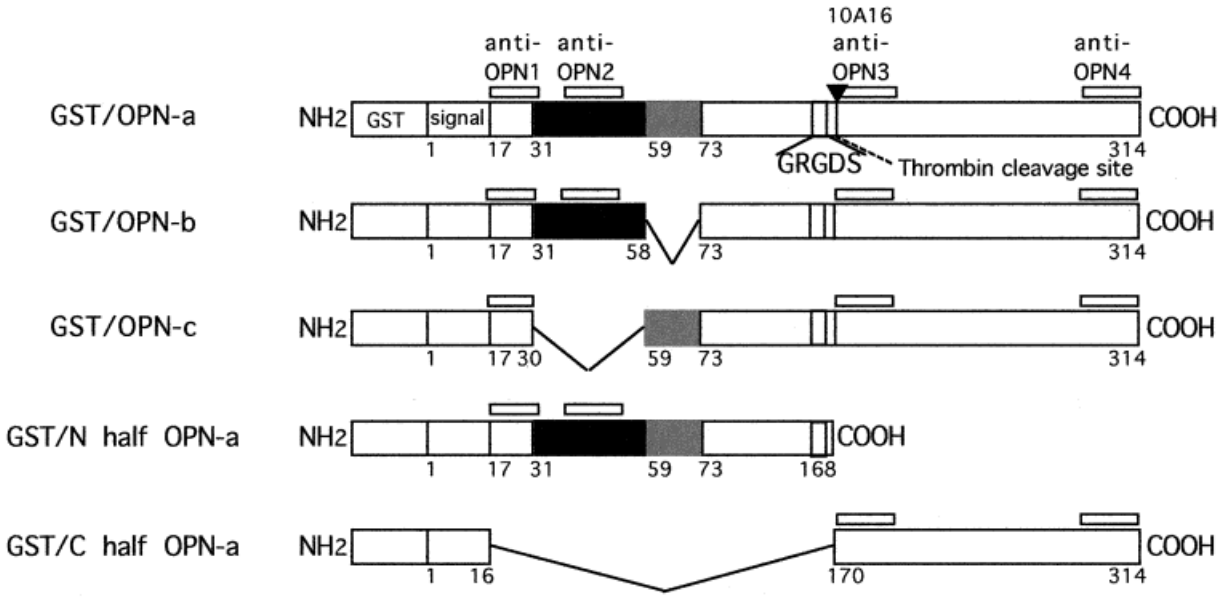

Fig. 1. Structure of osteopontin (OPN) preparations and possible sites of antibody recognition. Black box indicates the domain lacking in OPN-c isoform. Gray shading indicates the domain lacking in OPN-b isoform. A closed triangle indicates the position of thrombin cleavage site. GST, glutathione-S-transferase.

cDNA was also inserted into pcDNA 3.1 (+) vector (Invitrogen, NV Leek, The Netherlands) and transfected to CHO-K1 cells.

Protein Purification

The various recombinant GST/OPN fusion proteins were prepared in *E. coli* as described previously [Xuan et al., 1994]. The GST fusion proteins were purified on glutathione-sepharose columns as described [Smith and Johnson, 1988]. CHO-K1 transfectants with OPN-a cDNA were cultured in TIL I medium (IBL, Fugioka, Japan) to a cell density of 4×10^7 cells/liter for four days. Culture supernatant was collected, concentrated to 1/10 volume by ultrafiltration on a hollow fiber cartridge (HIP10-20, Amicon, Beverly, MA), urea was added up to final concentration of 6 M, and applied to a DEAE-sepharose CL-6B column equilibrated with 50 mM Tris HCl buffer, pH 7.2 containing 6 M urea (referred to as standard buffer). After washing with 0.14 M NaCl in standard buffer, proteins were eluted with 0.7 M NaCl in standard buffer. The eluted proteins were pooled and then reapplied to a DEAE-sepharose CL-6B column as described above. The eluted pool was concentrated with polyethylene glycol, subjected to gel filtration chromatography on an ULTROGEL AcA44 column (BioSepra SA, Cergy, France) equilibrated with standard buffer and eluted with the same buffer at a flow rate of 6 ml/h. The protein of interest was pooled and dialyzed against trifluoroacetic acid (TFA)/water (0.1:100) and then applied to reverse phase column chromatography on a RESOURCE RPC column (Amersham Pharmacia Biotech, Tokyo, Japan) with the same buffer. The protein was eluted with a 150-ml linear gradient of acetonitrile to H_2O (each containing 0.1% TFA) from 0 to 80% using an HPLC system (Waters, Milford, MA). The main peak of OPN was lyophilized, reconstituted with phosphate-buffered saline (PBS), and applied to affinity chromatography on formyl-cellulofine column (Seikagaku Kogyo, Tokyo, Japan) conjugated with pooled IgG of rabbits immunized with number 4 synthetic peptides as described below and eluted with 0.2 M glycine-HCl, pH 2.5. The eluted OPN was immediately neutralized with 1 M tris buffer, pH8.6. Quantitation of protein concentration of OPN utilizing the principle of protein-Coomassie Blue binding yield anomalously low values. Therefore an extinction coefficient for OPN was obtained by amino acid analysis using ninhydrin method. Thus, $E^{1\ mg/ml}_{280\ nm} = 0.75$ was used for determining OPN concentration.

Thrombin Cleavage

Purified OPN-a protein was digested by thrombin (Sigma) at 5μg protein/0.1–2.0 U enzyme, incubated at 37°C for 2 h.

Production and Purification of Monospecific Polyclonal and Monoclonal Antibodies Against OPN

Synthetic peptides used for immunization were as follows. Number 1 = IPVKQADSGSSEEKQ, number 2 = NKYPDAVATWLNPDPSQ, number 3 = KSKKFRRPDIQYPDATDE, and number 4 = KHLKFRISHELDSASSEVN, which correspond to the I_{17}–Q_{31}, N_{34}–Q_{50}, K_{170}–E_{187}, and K_{296}–N_{314} internal amino acid sequences of human OPN, respectively. Number 1 peptide corresponded to the exon 2-coded region and contained at least three potential phosphorylation sites (S_{24}, S_{26}, and S_{27}). Number 2 peptide corresponded to the exon 3-coded region and was deleted in OPN-c isoform and may not contain any phosphorylation sites. Number 3 peptide corresponded to the carboxy-terminal end of the thrombin-cleaved amino-terminal half of OPN and contained one potential phosphorylation site at T_{185}. Number 4 peptide corresponded to the amino-terminal end of OPN and contained one potential heparin-binding site (K296HLKFRI302) and three potential phosphorylation sites at S_{303}, S_{308}, and S_{310}. None of the synthetic peptides contained potential glycosylation sites. This information was based on previous publications [Saavedra, 1994; Sorensen et al., 1995]. These peptides were coupled with thyroglobulin, which was then used to immunize rabbits or mice.

The immunoglobulin (Ig) G fraction was obtained using protein G from the serum of the immunized rabbits. The resulting IgG was applied to a sepharose column coupled with the synthetic peptide used as the immunogen. Eluted IgG was used as anti-OPN1, anti-OPN2, anti-OPN3, or anti-OPN4 antibody, respectively. Polyethylene glycol-mediated cell fusions between splenocytes of immunized mice with peptide number 3 and fusion partner X63-Ag8-653, and the subsequent selection for hybridomas was carried out as described previously [Kinebuchi et al., 1991]. First, candidate hybridomas were screened for production of antibody specifically reactive to immobilized synthetic peptides used for the immunization, but not to immobilized irrelevant synthetic peptides. As a secondary screen, hybridomas having the following reactivities were selected: positive for immobilized GST/OPN-a and OPN-a derived from CHO cells, but negative for GST, and these were subcloned by a limiting dilution method. One hybridoma was chosen for this study on the basis of reactivity to OPN and designated as 10A16 (IgG1). The structure of various forms of OPN and possible sites of antibody recognition are schematically shown in Fig. 1.

Western Blot Analysis

Various OPN preparations (100 ng/lane) were loaded onto 4–20% polyacrylamide gels, fractionated by sodium dodecyl sulfate (SDS)-polyacryamide gel electrophoresis and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes. The membranes were immunoblotted (anti-OPN antibody dilution of 5 μg/ml) by the enhanced chemiluminescence system.

Establishment of ELISA System

Microtiter plates with 96 wells were coated with purified anti-OPN1, anti-OPN2, or anti-OPN4 antibody (20 μg/ml, 100 μl in volume in 0.1 M carbonate buffer, pH 9.5) at 4°C overnight, then blocked with 1% bovine serum albumin (BSA) in PBS containing 0.05% NaN_3 (referred to as blocking buffer). Samples and purified OPN-a derived from CHO cells as a standard OPN were diluted with dilution buffer (1% BSA in PBS containing 0.05% Tween 20), added to the plates (100 μl/well) and incubated for 1 h at 37°C. After extensive washing with PBS containing 0.05% Tween 20 (washing buffer), 100 μl of 2 ng/ml horseradish peroxidase-labeled anti-OPN1 antibody, anti-OPN3 antibody or 10A16 monoclonal antibody was added to each well and incubated for 30 min at 37°C. After nine washes with washing buffer, 100 μl of tetramethyl benzidine buffer as a substrate was added to each well and incubated for 30 min at room temperature in the dark. Color development was stopped by addition of 100 μl of stop solution (1 N H_2SO_4). A BioRad plate reader was used to quantify the signal at 450 nm.

Cells and Cell Culture

The following human cell lines were obtained from Immuno-Biology Laboratories, Fujioka,

Japan: lung carcinoma cells, PC-9 (well differentiated adenocarcinoma) and PC-10 (moderately differentiated squamous carcinoma) [Kinjo et al., 1979]; malignant melanoma cells, SEKI [Mori et al., 1991]; and renal cell carcinoma cells, NRC-12 [Komatsubara, 1978]. Human hepatocellular carcinoma cells, HepG2 [Imai et al., 1999], were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) plus penicillin and streptomycin. Human promyelocytic leukemia cells, HL-60 [Sekiya et al., 1994], and human histiocytic lymphoma cells, U937 [Okada et al., 1995], were cultured in RPMI containing 10% FCS plus penicillin and streptomycin in the presence or absence of 0.1 nM phorbol 12-myristate 13-acetate (PMA). To obtain culture supernatant for OPN analysis, cells were seeded in the above medium at a cell density of 2×10^5/ml and cultured for 2 days.

Urine Samples

Nine healthy men (mean age = 30.44 years; range = 24–42 years) were recruited into an experimental group. Midstream first morning urine samples were collected before any exercise or work and tested free of bacterial contamination. All samples were immediately frozen and stored at −20°C and thawed at room temperature as needed. After thawing, samples were immediately subjected to ELISA analyses. Because the urinary OPN concentration has been shown to vary inversely with urinary volume [Min et al., 1998], we measured urinary creatinine level and adjusted the OPN concentration by dividing the ELISA OPN value by the creatinine value for each sample.

RESULTS

Purification of OPN

After the purification steps described in Materials and Methods, the purity of the various OPN preparations was evaluated by SDS-polyacrylamide gel electrophoresis and visualization by Coomassie Blue staining. As shown in Fig. 2, the various GST/OPN fusion proteins produced in *E. coli* and OPN-a derived from a CHO cell culture supernatant were purified. In addition, human OPN-a derived from CHO cells was cleaved by thrombin digestion.

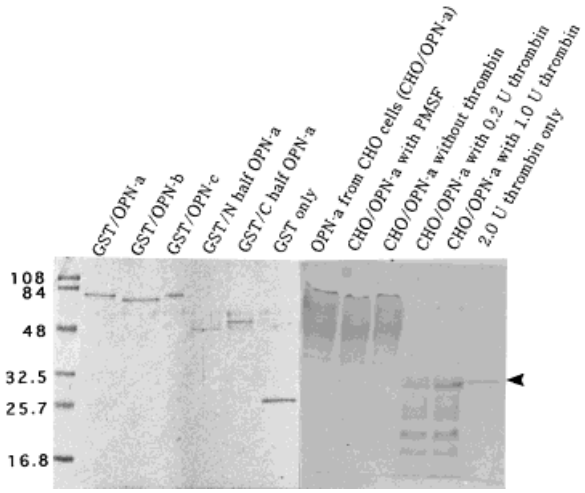

Fig. 2. Protein purification and thrombin cleavage. Various purified osteopontin (OPN) preparations (5 μg/lane) were electrophoresed on SDS-PAGE (4–20% gel) under reducing conditions. The gels were stained with Coomassie Brilliant Blue R-250. An arrow indicates the position of thrombin. GST, glutathione-S-transferase; PMSF, phenylmethylsulfonyl fluoride; CHO, Chinese Hamster Ovary.

Specificity of Antibodies Raised Against Synthetic Peptides

The specificities of purified rabbit IgG raised against synthetic peptides were examined by immunoblot analysis as shown in Fig. 3. GST/human OPN-a and OPN-b with approximate molecular weights of 80 kDa were clearly detected by the four polyclonal antibodies tested. GST/human OPN-c was detected by anti-OPN-1, 3, and 4 antibodies. However, anti-OPN2 antibody failed to detect GST/OPN-c, as expected, because the immunoreactive epitope is lacking in OPN-c. The 47-kDa GST/N half OPN fragment was detected by anti-OPN-1 and anti-OPN-2 antibodies, whereas the 50-kDa GST/C half OPN was detected by anti-OPN-3 and anti-OPN-4 antibodies. None of the four polyclonal antibodies reacted with GST. Anti-OPN-3 and anti-OPN-4 antibodies clearly detected both thrombin-cleaved and non-cleaved forms of OPN-a derived from a CHO cell supernatant, whereas the reactivities of anti-OPN-1 and anti-OPN-2 to the thrombin-cleaved form of OPN-a were positive but faint. The 10A16 monoclonal antibody recognized GST/OPN-a, GST/OPN-b, GST/OPN-c, and GST/C half OPN, but not GST/N half OPN. This monoclonal antibody also recognized the thrombin-cleaved form of OPN-a derived from CHO cell supernatant.

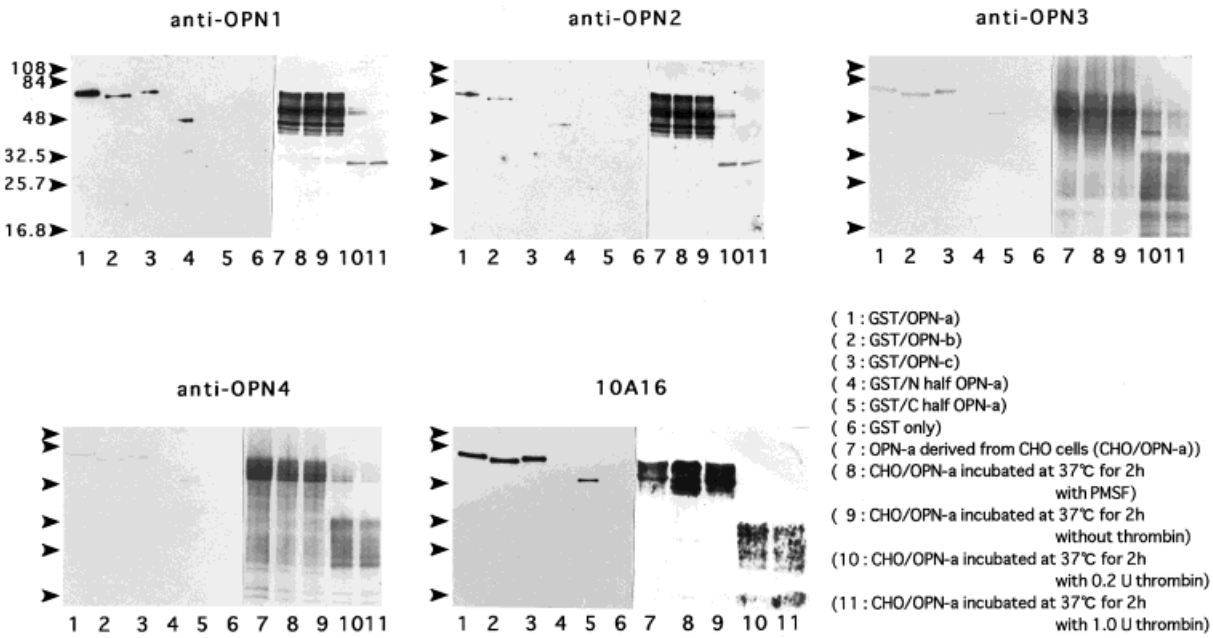

Fig. 3. Specific recognition of osteopontin (OPN) by polyclonal and monoclonal antibodies. Western blot analysis of the various OPN preparations fractionated in 4–20% SDS-PAGE as shown in Fig. 2 and detected with anti-OPN1 immunoglobulin G (IgG), anti-OPN2 IgG, anti-OPN3 IgG, anti-OPN4 IgG, or 10A16 monoclonal antibody. Lanes 1–11 are the same as described in Fig. 2. Arrowheads indicate the position of molecular weight markers. GST, glutathione-S-transferase; PMSF, phenylmethylsulfonyl fluoride; CHO, Chinese Hamster Ovary.

Establishment and Characterization of ELISA Systems for Human OPN

We prepared six different ELISA systems as described in Materials and Methods. Briefly, plates were coated with purified anti-OPN1, anti-OPN2, or anti-OPN4 IgG. After sample application, captured OPN was detected by peroxidase-conjugated anti-OPN1 IgG, anti-OPN3 IgG, or 10A16 monoclonal antibody. Thus, the combinations of antibodies used were as follows: 1-3 system (anti-OPN1 and 3), 2-3 system (anti-OPN 2 and 3), 4-3 system (anti-OPN4 and 3), 4-1 system (anti-OPN 4 and 1), 2-1 system (anti-OPN2 and 1), and 1-Mono system (anti-OPN1 and 10A16). By using purified recombinant human OPN-a derived from CHO cells as a standard sample, standard curves were established (data not shown). The data demonstrated that all ELISA systems exhibited comparable avidity for the glycosylated form of CHO-derived OPN-a except a 1-Mono system, which demonstrated an avidity equivalent to one-tenth of the other systems. Various recombinant nonglycosylated forms of GST/OPN fusion proteins were also measured using the six ELISA systems. It should be noted that all ELISA systems exhibited similar avidity to glycosylated and nonglycosylated forms of OPN. All ELISA systems exhibited a linear log/log plot over the range of 4 to 100 ng/ml. The 2-1 ELISA system failed to detect the OPN-c isoform as expected. GST/N-half OPN fragments were detected only by the 2-1 ELISA system, whereas the 4-3 system was able to detect GST/C-half OPN fragments. C-half OPN and OPN-c were nonspecifically detected by the 1-3 and the 2-3 systems, respectively. However, values were at most $<7\%$ compared to the specifically recognized osteopontin. Thus, a value <7 ng/ml OPN concentration should be considered as a nonspecific detection in both 1-3 and 2-3 systems.

Detection of Native OPN Secreted by Tumor Cell Lines in Culture

The various ELISA systems were further tested to determine their ability to recognize native OPN secreted in vitro by seven tumor cell lines (Fig. 4). Osteopontin secretion was not detected in U937 and HL60 cells by any of the six distinct ELISA; however, upon PMA stimulation, production of OPN was detected in both cell lines. Osteopontin production by the hepatocellular carcinoma cell line HepG2 was

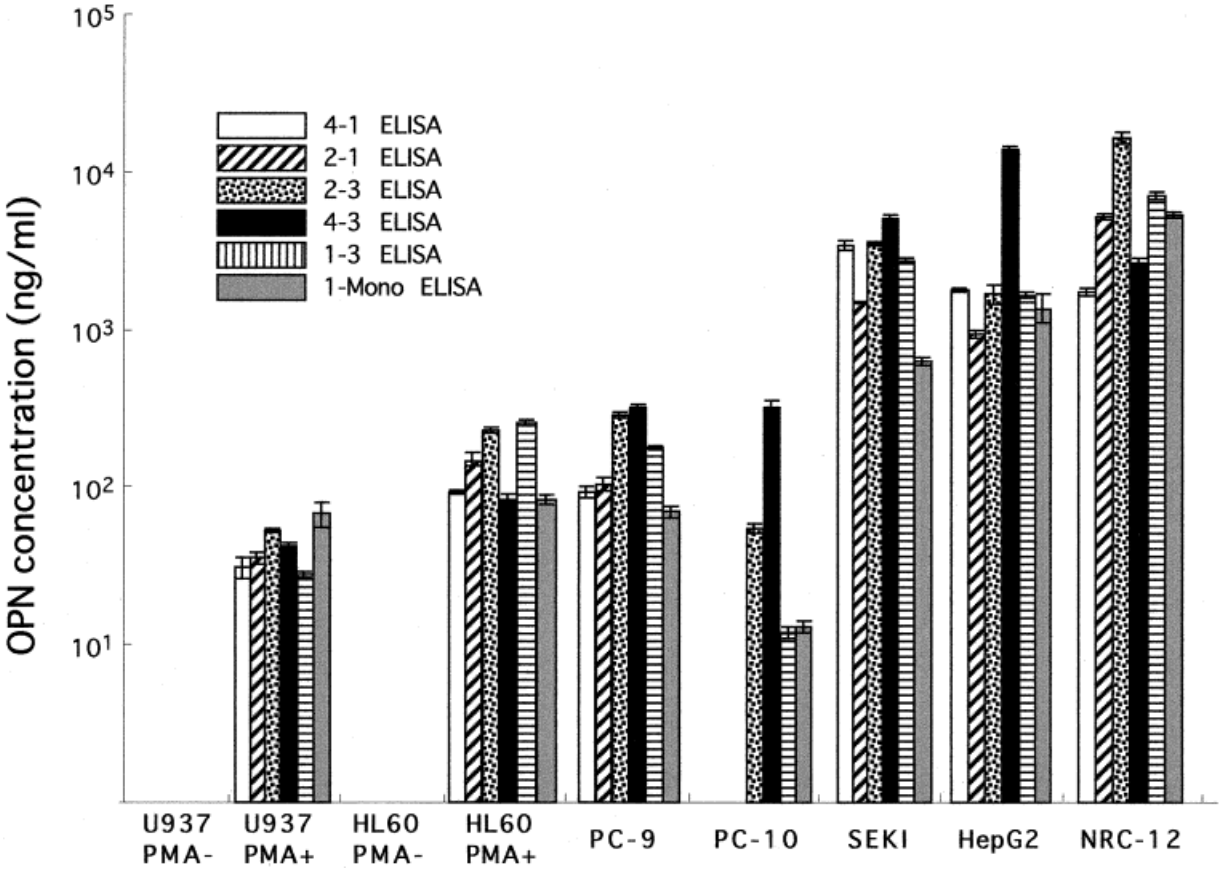

Fig. 4. Secretion of osteopontin (OPN) by various human tumor cell lines. Various tumor cells were cultured for 2 days at a cell density of 2×10^5/ml in the presence or absence of 0.5 nM phorbol 12-myristate 13-acetate (PMA). Culture supernatants were applied to ELISA systems and OPN concentration (ng/ml) in samples was determined as described in Materials and Methods. All samples were tested in triplicate and the data indicate the mean ± SD.

preferentially detected by the 4-3 system, whereas other systems detected considerably lower amounts of OPN, with the 2-1 system providing the lowest value. In contrast, the renal cell carcinoma cell line NRC-12 produced OPN that was preferentially detected by the 2-3 system, with the 4-1 system yielding the lowest OPN value.

Heterogeneity of OPN production by tumor cells was also demonstrated by western blot analysis (Fig. 5). All polyclonal antibodies and the monoclonal antibody could detect native OPN purified from CHO cells (Lane 11). Specific bands that ranged in size from 40 to 70 kDa were detected. It is likely that the differences in size are due to post-translational modifications including glycosylation, and possibly alternative splicing or proteolytic cleavage. OPN produced by NRC-12, HepG2, and SEKI cells detected by all the antibodies tested. In contrast, OPN produced by the lung carcinoma cells, PC-9, and PC-10 was detected only by anti-OPN3 and anti-OPN4. OPN produced by U937 cells was detected by anti-OPN4 only after PMA stimulation, whereas OPN produced by HL60 cells was detected by anti-OPN3 and anti-OPN4 only after PMA stimulation.

Detection of Urinary OPN

Urinary OPN was first examined by western blot analysis. In contrast to the results obtained from tumor-derived OPN, the majority of antibodies tested could detect urinary OPN with molecular weights in the 40–70 kDa range (Fig. 6). In addition, there were no small molecular weight forms of OPN detected by any antibody tested, indicating that there are very few, if any, cleaved forms of OPN in urine. Next, urinary OPN was measured by using the six distinct ELISA systems. Values were normalized by dividing the amount of OPN (μg/ml)

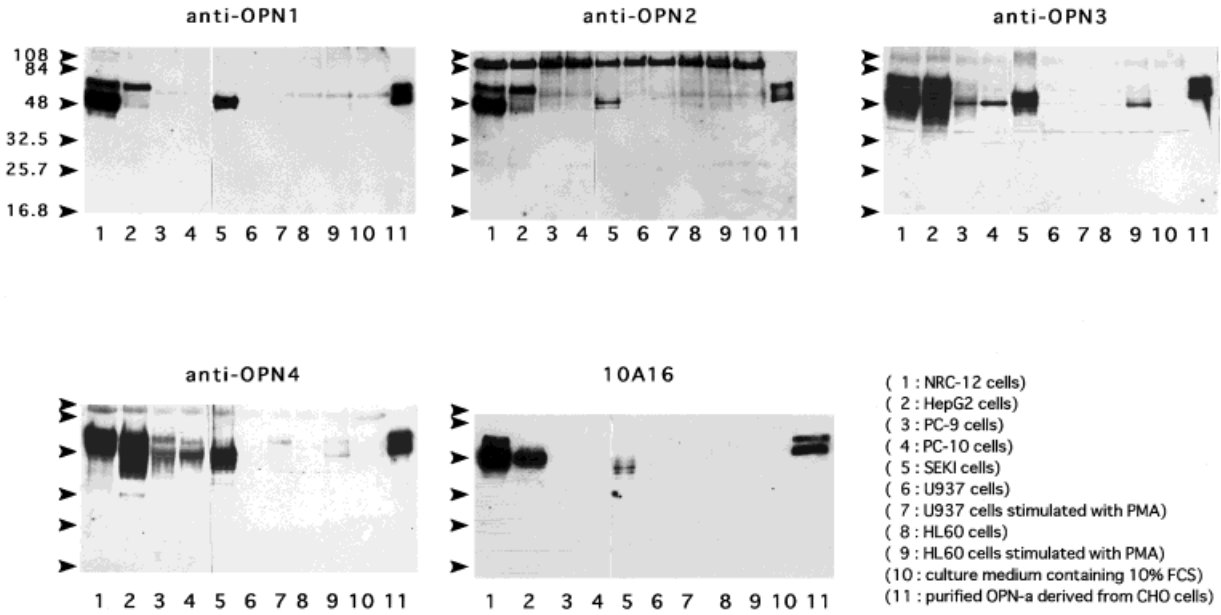

Fig. 5. Western blot analysis of human osteopontin (OPN) secreted by different tumor cell lines. Western blot analysis of secreted OPN fractionated in SDS-PAGE and detected with anti-OPN1, anti-OPN2, anti-OPN3, anti-OPN4, or 10A16. The equivalent of 5 μg of total secreted protein was loaded for each lane. For comparison, 1 μg of purified OPN-a from CHO cells was also analyzed. Culture conditions are as shown in Fig. 4. PMA, phorbol 12-myristate 13-acetate; FCS, fetal calf serum; CHO, Chinese Hamster Ovary.

by creatinine value (mg/dl). The amount of urinary OPN detected depended on the ELISA system used, and was 27.5 ± 5.2, 24.6 ± 5.6, 19.1 ± 3.8, 16.1 ± 3.4, 16.1 ± 3.9, and 7.7 ± 1.9 by 2-1, 1-Mono, 1-3, 4-1, 2-3, and 4-3 system, respectively (Fig. 7). Thus, urinary OPN was detected by all ELISA systems tested, which was in contrast to the western blot analysis in which 10A16 monoclonal failed to detect urinary OPN in case 8.

DISCUSSION

Osteopontin Heterogeneity

Osteopontin has recently received considerable attention for its potential role in several disease processes. It is known to exist in various forms due to post-translational modifications such as glycosylation, sulfation, and phosphorylation, which in some cases possess functional significance [Denhardt and Guo, 1993; Saavedra, 1994; Sorensen et al., 1995].

There are at least three differentially spliced isoforms of human OPN, such as OPN-a, OPN-b, and OPN-c [Saitoh et al., 1995]. OPN-a is a complete transcript of the OPN gene. As shown in Fig. 1, OPN-b lacks 14 amino acids encoded by exon 4 of OPN gene and OPN-c lacks 28 amino acids encoded by exon 3 of OPN gene. In addition, OPN proteins are susceptible to thrombin cleavage, and cleaved forms of OPN may express cryptic cell binding domains [Patarca et al., 1993; Smith et al., 1996]. However, the potential functional differences among those isoforms are largely not known and the differential expression of OPN fragments in various disease states is not well characterized. Thus, it is essential that reagents and assays able to detect these forms be developed to clarify the physiologic and pathologic functions of OPN. With these points in mind, we generated monoclonal and polyclonal antibodies raised against specific internal sequences of human OPN.

Nature of Antibodies

Min et al. [1998] generated polyclonal anti-osteopontin antibodies by immunizing rabbits with human urinary uropontin, a glycosylated form of osteopontin. Bautista et al. [1994] generated two monoclonal antibodies against *E. coli*-derived recombinant OPN-a, a nonglycosylated form of human OPN. One clone, mAb53, does not recognize thrombin-cleaved OPN, whereas the other clone, mAb87-B, does recognize thrombin-cleaved OPN. However, it is not known whether those antibodies can rec-

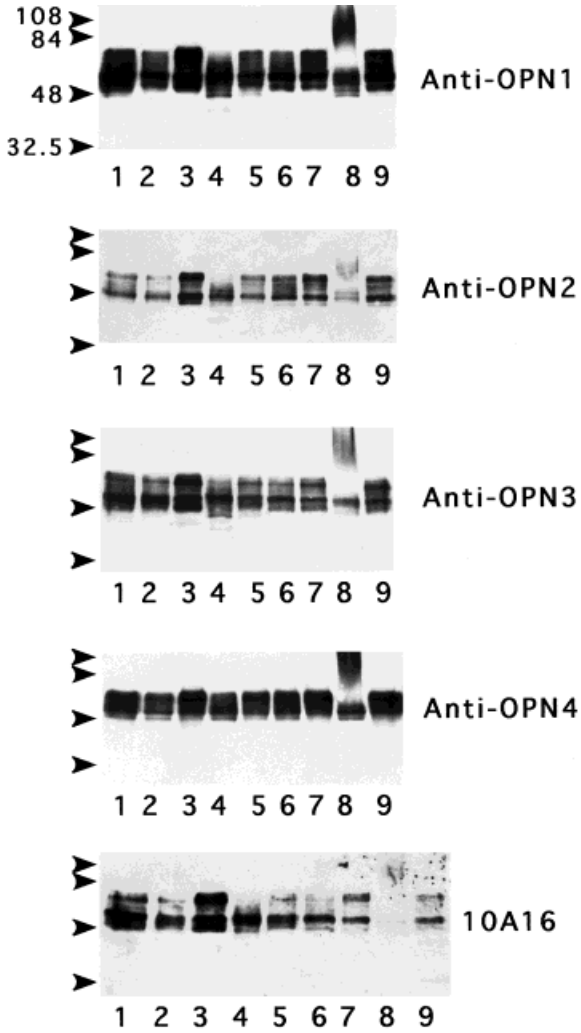

Fig. 6. Western blot analysis of human urinary osteopontin (OPN). Western blot analysis of urinary OPN fractionated in SDS-PAGE and detected with anti-OPN1, anti-OPN2, anti-OPN3, anti-OPN4, or 10A16. Urine (10 μl) was loaded for each lane. Lanes 1–9 represent urine samples obtained from Cases 1 to 9, respectively (see Fig. 7).

ognize human OPN-b and OPN-c. We immunized rabbits and mice with four different synthetic peptides corresponding to specific internal OPN sequences. The rationale for using four distinct peptides as immunogens and developing six distinct ELISA systems is as follows. The quantitation of secreted proteins and cytokines by ELISA can be dependent on the nature of the antibody used [White et al., 1996; Krakauer, 1998] and OPN is susceptible to proteolytic fragmentation by thrombin and this process may have physiologic importance [Xuan et al, 1994; Senger et al, 1994]. Therefore a system to detect thrombin-cleaved forms of OPN is needed. Anti-OPN 2 antibody does not recognize OPN-c because the target epitope is lacking in OPN-c. OPN-a and OPN-b were detected by all antibodies tested. The N-half OPN was detected by the 2-1 system, whereas the C-half OPN was detected by the 4-3 system. These antibodies could detect both the nonglycosylated form of OPN derived from *E. coli* and the glycosylated form of OPN such as CHO cell-derived and urinary OPN as shown in Figs. 3–5. Although the data are not shown, our antibodies could also react with the highly phosphorylated form of OPN derived from human milk. Importantly, all polyclonal and monoclonal antibodies used in this study could detect thrombin-cleaved forms of OPN in western blot analysis. It should be noted that our antibodies described here could measure the thrombin-cleaved forms of both CHO-derived and *E. coli*-derived OPN in ELISA systems (data not shown).

Tumor-Derived Osteopontin

It has been shown that elevated plasma OPN levels in metastatic breast cancer are associated with increased tumor burden and decreased survival [Singhal et al., 1997]. Furthermore, it has been shown that OPN immunopositivity in breast and lung tumors is associated with poor patient survival [Chambers et al., 1996; Tuck et al., 1997; Tuck et al., 1998]. However, it should be noted that some of the protease-cleaved forms of OPN and isoforms of OPN would escape detection because the antibody used in these studies does not recognize thrombin-cleaved OPN and the nature of epitope was not fully characterized [Bautista et al., 1994; Min et al., 1998]. There are widely differing efficiencies of detection of OPN by the different ELISA systems described, and these differences do not always correlate with differences observed by western blot analysis. It is possible, but unlikely, that differences in detection are due to differences in glycosylation of samples, because none of the antibodies were raised against internal sequences of OPN where post-transcriptional modification of glycosylation occur. All synthetic peptides except the number 2 peptide corresponded to internal sequences of OPN that can be phosphorylated. Therefore, differences in phosphorylation may explain differences in detection of OPN from tumor cells. Tumor cells may produce differentially spliced

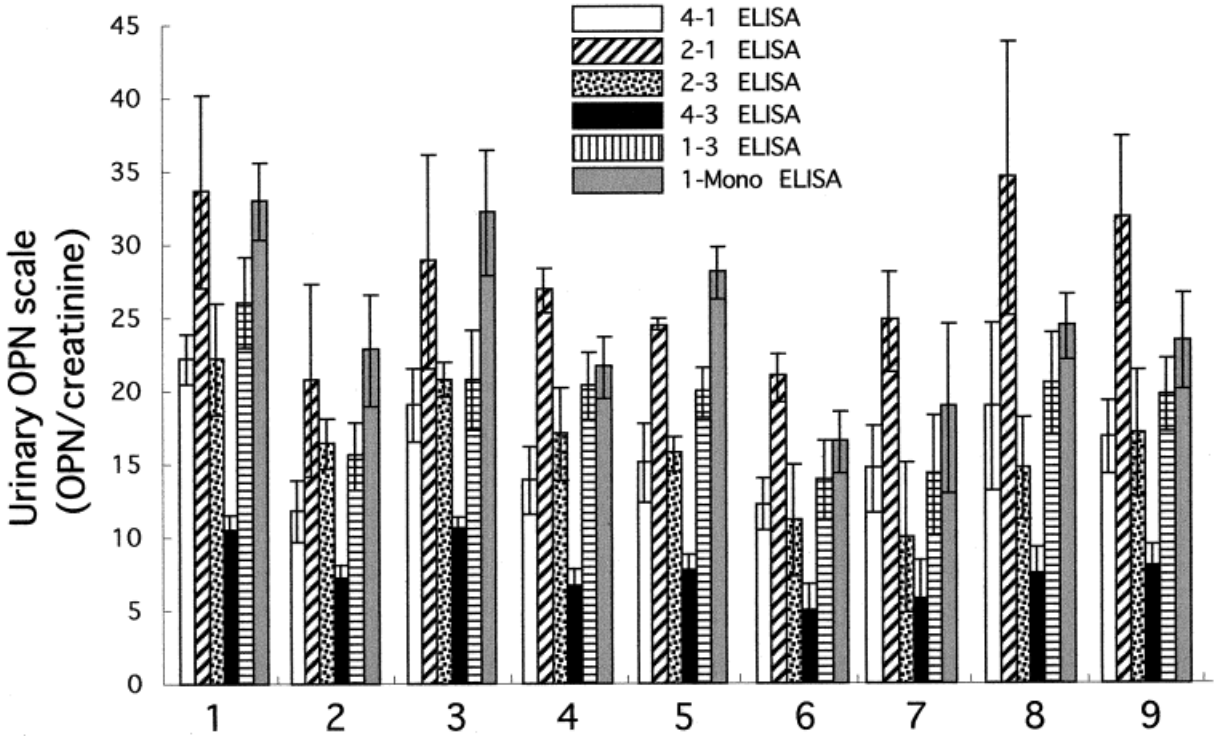

Fig. 7. Detection of male urinary osteopontin (OPN). Midstream urine was obtained and diluted urine samples were applied to ELISA systems. OPN concentration (μg/ml) in samples was determined as described in Materials and Methods. Data represent the normalized value with the amount of OPN (μg/ml) being divided by creatinine (mg/dl). Experiment was repeated three times with similar results. The data indicate the mean ± SD.

forms of OPN. It has been shown that the OPN-a and OPN-b isoforms can be produced by a variety of cells, whereas the OPN-c isoform is produced by certain cells such as malignant glioma cells [Kiefer et al., 1989; Young et al., 1990; Saitoh et al., 1995]. Our unpublished data showed that both NRC-12 and HepG2 cells expressed a complete OPN transcript, OPN-a and a differentially spliced variant OPN-b, but not OPN-c. However, OPN produced by the two cell lines was differentially detected. Osteopontin produced by NRC-12 was detected by all antibodies tested, whereas OPN produced by HepG2 cells was detected by antibodies reacting to the C half of OPN, thus indicating that OPN can be differentially degraded during or after protein synthesis and secretion. Lung carcinoma cell lines (PC-9 and PC-10) secreted low amounts of OPN as compared to HepG2, SEKI, and NRC-12 cells. Note that both HL-60 and U937 cells produced detectable amounts of OPN only after PMA stimulation. These data are in good agreement with a previous report that HL-60 cells do not express OPN mRNA, and that PMA treatment, which induces differentiation of HL-60 cells into monocytes, augmented expression of OPN mRNA [Atkins et al., 1998].

Urinary Osteopontin

The mean urinary OPN concentration detected among normal Caucasian individuals was 1.9 μg/ml [Min et al., 1998]. Bautista et al. [1996] developed an antigen-capture ELISA to quantify human OPN in plasma and urine using a combination of a mouse monoclonal antibody, mAb53, and rabbit polyclonal antibodies reacting to human OPN. By using this ELISA system, it was demonstrated that urinary OPN levels ranged from 0.01 to 2.7 μg/ml. Osteopontin/creatinine ratios detected by the 2-1, 1-3, and 4-1 systems were in the order of 27.5, 19.1, and 16.1, respectively. This may indicate that truncated forms of OPN protein exist in urine. The 2-1 system, which can detect the amino-terminal portion of OPN, exhibited the highest value of 27.5, whereas the 4-3 system, which can detect OPN having the carboxy-terminal half, exhibited the lowest value of 7.7. This may simply indicate that mature OPN is

susceptible to enzymatic cleavage and that the carboxy-terminal half of OPN is rather unstable. Another possibility is that proteolytic cleavage of OPN may significantly alter the conformation of the OPN molecule. In this regard, we recently demonstrated that cleavage of OPN by thrombin resulted in a significant reduction of the reactivity to antibody recognizing the epitopes located in the carboxy-terminal half, but not in the amino-terminal half [Hotta et al., 1999]. However, western blot analysis of urine did not show any fragmentation of OPN and the molecular weight of urinary OPN was rather homogenous. Another point to be discussed here is why OPN detected by the 4-3 system (OPN/creatinine ratio of 7.7) was significantly less than that detected by the 4-1 system (16.1). One possibility is that the two epitopes recognized by the anti-OPN3 and anti-OPN4 antibodies are located closely so that binding of urinary OPN by one antibody may sterically inhibit the binding of the second antibody. However, recombinant OPN detected by the 4-3 system was not lower than that detected by the 4-1 system (data not shown), thus arguing against this hypothesis. It is also possible that urinary and recombinant OPN differ in their conformation.

Here we have documented that OPN can be differentially detected depending on the reagents used. The antibodies and ELISA systems described here will contribute to an understanding of the physiologic significance and functional roles of various forms of OPN.

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