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Detection of Various Epitopes of Murine Osteopontin by Monoclonal Antibodies

Hiroshi Hotta,*,† Shigeyuki Kon,* Yohko U. Katagiri,* Noriko Tosa,* Taiji Tsukamoto,† Ann F. Chambers,‡ and Toshimitsu Uede*,[1]

**Section of Immunopathogenesis, Institute of Immunological Science, Hokkaido University, Kita-15, Niishi-7, Kita-ku, Sapporo, Japan; †Department of Urology, Sapporo Medical University School of Medicine, Sapporo, Japan; and ‡The London Regional Cancer Centre, London, Ontario, Canada N6A 4L6*

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We immunized rats with recombinant murine osteopontin protein and obtained four monoclonal antibodies recognizing distinct epitopes of murine osteopontin. OPN1.2 recognized the amino-terminal half of OPN, while OPN2.2, OPN2.3, and OPN3.1 recognized the carboxy-terminal half of OPN. The epitope recognized by OPN2.2 was destroyed by further cleavage of the carboxy half of OPN. The epitope recognized by OPN2.3 was located in the amino-terminal end of the carboxy half of OPN, whereas that recognized by OPN3.1 was located in the carboxy-terminal end of the carboxy half of OPN. OPN1.2 and OPN2.2 recognized thrombin-cleaved osteopontin, whereas thrombin-cleaved osteopontin was not recognized by OPN2.3 and OPN3.1. Thus, these monoclonal antibodies will be useful in structure/function studies of the role of osteopontin in murine models of disease.

Osteopontin (OPN) is an acidic calcium-binding phosphoprotein secreted by a variety of cell types including macrophages, endothelial cells, kidney tubules, tumor cells, and activated T cells (1). OPN has now received considerable attention for its potential role in several disease processes (1-7). To better understand the role of OPN in various disease processes, one obvious approach is to establish murine models. Monoclonal antibodies (mAb) that detect human (8) and rat (9) OPN have been generated, and polyclonal antibodies that can detect rat and mouse OPN have been used (10-15). However, mAb specific for mouse OPN, and especially those able to detect defined epitope regions of the murine molecule, have not been available. These reagents will be particularly important in clarifying the function of OPN in murine models of disease, and in determining the contribution of different regions of the OPN molecule to OPN function. It has been shown that the Arg-Gly-Asp (RGD) tripeptide sequence within the OPN molecule provides a cell binding domain which interacts with cell surface αv integrin receptors (12,16,17). We and others demonstrated that in addition to RGD, OPN contains multiple cell binding domains which interact at least with CD44 and $\alpha 9\beta 1$ (10,18-20). Furthermore, various forms of OPN have been detected, possibly due to differential RNA splicing, glycosylation, and proteolytic fragmentation. Therefore, it is important to clarify the structure of OPN expressed in vivo to better understand the role of OPN in various pathological and physiological processes. We thus have raised mAb against recombinant GST-murine OPN fusion protein and obtained four distinct mAb specifically reacting to distinct epitopes of murine OPN.

MATERIALS AND METHODS

Construction of the GST-OPN fusion plasmid. Three murine GST-OPN fusion plasmids; GST fused to full length murine OPN(WT OPN; L_1-N_{278}), the N-terminal half of mOPN lacking $R_{128}G_{129}D_{130}$ (N-half OPN; L_1-G_{127}) and the C-terminal half of mOPN lacking $R_{128}G_{129}D_{130}$ (C-half OPN; L_{132}-N_{278}) were constructed as described previously (10). Oligonucleotides used in this study were synthesized based on the published murine OPN cDNA sequence (11); Primer 1, (5′AAAGGATCCGCTTGGCTTATGGACTGAGG3′) contains a *Bam* HI restriction site and encodes the sequence of the 6 amino acids starting from L_{132}. Primer 2, (5′TTTCCCGGGTTAGTGACCTCAGAAGATGA3′) is complementary to the cDNA sequence of the last 6 amino acids of mature OPN and contains a stop codon with a *Sma* I restriction site. Primer 3, [5′TTTCCCGGGGATTCTGTGTGTTTCCAGACT3′] is complementary to the cDNA sequence encoding the 6 amino acids prior to L_{209} and contains a stop codon with a*Sma* I restriction site. Primer 4, [5′AAAGGATCCTTGAGCATTCCAAAGAGAGC3′] contains a *Bam* HI site followed by cDNA encoding 7

[1] To whom correspondence should be addressed. Fax: 81-11-736-9836. E-mail: toshi@imm.hokudai.ac.jp.

Abbreviations used: OPN, osteopontin; GST, glutathione-S-transferase; PCR, polymerase chain reaction; RGD, arginine-glycine-asparatic acid; AAA, alanine-alanine-alanine; RGE, arginine-glycine-glutamic acid; ΔRGD, RGD deleted; N, amino; C, carboxy; mAb, monoclonal antibody; WT, wild type; kDa, kilodalton.

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amino acids sequence starting from L_{209}. PCR reactions were conducted using full length murine OPN cDNA as a template using an appropriate combination of the above two primers. Primers 1 and 3 were used for 3/4 C-half OPN (3/4 C; L_{132}-R_{208}) and primers 2 and 4 for 4/4 C-half OPN (4/4 C; L_{209}-N_{278}). PCR products were purified and cloned into pCRII vector. The OPN cDNAs cloned by PCR were completely sequenced and inserted into the pGEX-3X vector in the same reading frame as the carrier gene (glutathione S-transferase, EC 2.5.1.18) and transformed in *E. coli* DH5α cells. In order to make mutated OPN in which the RGD sequence is replaced with AAA (designated as AAA-OPN), we further synthesized primer 5 which is complementary to the cDNA sequence of the 14 amino acids (T_{121}VDVPNGAAASLAY$_{134}$) and which was engineered to contain a *Sal*I restriction enzyme site: 5′ACAGTCGAC$^{\#}$GTCCCCAACGGC-GCAGCTGCTAGCTTGGCATAT3′ [the original T was substituted by C$^{\#}$ to create a *Sal* I site since no amino acid substitution occurred as a result of this change]. A PCR reaction was conducted using the full length mouse OPN cDNA as a template and was directed by primers 2 and 5. The PCR product was sequenced and digested with *Sal* I and *Sma* I enzymes. The cDNA of mutated OPN (D_{123}-N_{278}) thus obtained was ligated with the N-half OPN/pGEX-3X plasmid cleaved with *Sal* I/*Sma* I restriction enzymes. Two additional mutated GST-OPN fusion plasmids [mutation of RGD to RGE (Arg-Gly-Glu) by a single base change, GAT (Asp) to GAG (Glu), designated as RGE-OPN, and deletion of the RGD sequence, designated as ΔRGD, were constructed as described previously (16). A fusion protein consisting of GST and full length human OPN, designated as r-hOPN was prepared as described previously (21).

Protein purification. The various recombinant GST-OPN fusion proteins and GST proteins were purified on glutathione-Sepharose columns. The purity of the proteins was analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB) (Fig. 1A).

Production of monoclonal antibodies against murine OPN. Conventional polyethylene glycol-mediated cell fusions were made between lymph node cells of rats immunized with GST-murine OPN fusion protein and fusion partner X63-Ag8-653. First, candidate hybridomas were screened for production of antibody specifically reactive to immobilized GST-murine OPN, but not to GST. As a secondary screening, hybridomas having following reactivities were selected: positive for either immobilized GST-OPN, GST-C half OPN, or GST-N half OPN, but negative for GST, fibronectin and vitronectin. Several hybridomas were chosen for this study and designated as OPN1.2 (IgG1; κ), OPN2.2 (IgG2a; κ), OPN2.3 (IgG2a; κ), and OPN3.1 (IgG2a;κ).

Urine samples. Urine was recovered from mice by pressuring the lower abdominal wall and stored at −80°C until use.

Thrombin cleavage. GST-OPN fusion proteins were digested by thrombin (Sigma) at 10 μg protein/0.1 U enzyme, incubated at 37°C for 2 hours.

SDS-PAGE and western blot analysis. Various OPN preparations were loaded onto 12.5% polyacrylamide gels, fractionated by SDS-PAGE and transferred electrophoretically onto PVDF membranes. The membranes were immunoblotted by the enhanced chemiluminescence system. The monospecific polyclonal antibody raised against an internal sequence of mouse OPN (10) was used as a positive control. The polyclonal antibody recognizing GST was purchased from Amersham Pharmacia Biotech Japan, Tokyo, Japan.

Immunohistochemistry. Tissue speciments were fixed with 10% buffered formalin and embedded in paraffin. For antigen retrieval, the deparafinized sections were pretreated in 10mM citrate buffer, pH6.0 in a microwave oven for 10 min. The sections were stained with a monoclonal antibody followed by biotin-conjugated rabbit anti-rat immunoglobulin antibody, and then avidin-biotin-peroxidase complex as described previously (22). The enzyme reaction was done as described previously (22).

RESULTS

Specificity of monoclonal antibodies raised against GST-murine OPN. The specificity of mAb OPN1.2, OPN2.2, OPN2.3, and OPN3.1 was established by immunoblotting as shown in Fig. 1. Fig. 1A shows a Coomassie Blue stained 12.5% gel depicting the electrophoretic pattern of various forms of OPN, with immunoblot equivalents in Fig. 1C (OPN1.2), 1D (OPN2.2), 1E (OPN2.3), and 1F (OPN3.1). OPN 1.2 recognized N-terminal, but not C-terminal halves of OPN. The major staining band in the N-half OPN lane (Fig. 1A), which migrated with an apparent molecular weight of 45 kDa, was detected by OPN 1.2 (Fig. 1C). Several smaller bands (43, 40, 35, and 32 kDa) that were stained less intensely in Fig. 1A (presumed to be truncated N-half OPN translational products because they co-purified by repeated affinity chromatography) were not immunoreactive with OPN 1.2. Therefore, OPN1.2 recognized an epitope very close to the RGD tripeptide sequence. OPN 2.2, OPN 2.3 and OPN 3.1 recognized carboxy-half of OPN, but not N-terminal half of OPN (Fig. 1D–F). OPN 2.3 recognized an epitope in C3/4, but not in C4/4 of OPN, while OPN 3.1 recognized C4/4, but not C3/4 of OPN. The epitope recognized by OPN 2.2 is found in C-half OPN, but not in C3/4 and C4/4 of OPN, indicating that the epitope very close to the junction between C3/4 and C4/4, or OPN 2.2 recognized a conformational epitope. All the mAb tested here could immunostain recombinant human OPN. It was shown that several bands in Fig. 1A were truncated translational GST-OPN fusion proteins because they were all stained by anti-GST antibody (Fig. 1B).

Modulation of antibody reactivity by mutation and deletion within RGD tripeptide sequence. The critical involvement of the RGD sequence within the OPN molecule for cell binding has been repeatedly shown. Therefore, we prepared wild type full length GST-OPN (WT), RGE-OPN, AAA-OPN, and ΔRGD. After several rounds of affinity chromatography, several bands were detected (Fig. 2A). These bands were all stained by anti-GST antibody (Fig. 2B), indicating that these bands contained mature as well as truncated translational GST-OPN fusion proteins. We then tested their reactivity to mAb (Fig. 2C–F). WT, RGE, AAA, and ΔRGD-OPN are equally detected by OPN 2.2, OPN 2.3 and OPN 3.1. In contrast, the reactivity of OPN1.2 to WT and RGE is much stronger than that to AAA and ΔRGD.

Modulation of antibody reactivity by thrombin digestion of OPN. As shown in Fig. 2A, purified OPN consited of mature and several truncated translational fusion proteins (Fig. 3). After thrombin digestion of full length wild type OPN, 23, 43 and 48 kDa OPN fragments were detected (Fig. 3). From the site of thrombin cleavage and size of fragments, it seems that 23 kDa

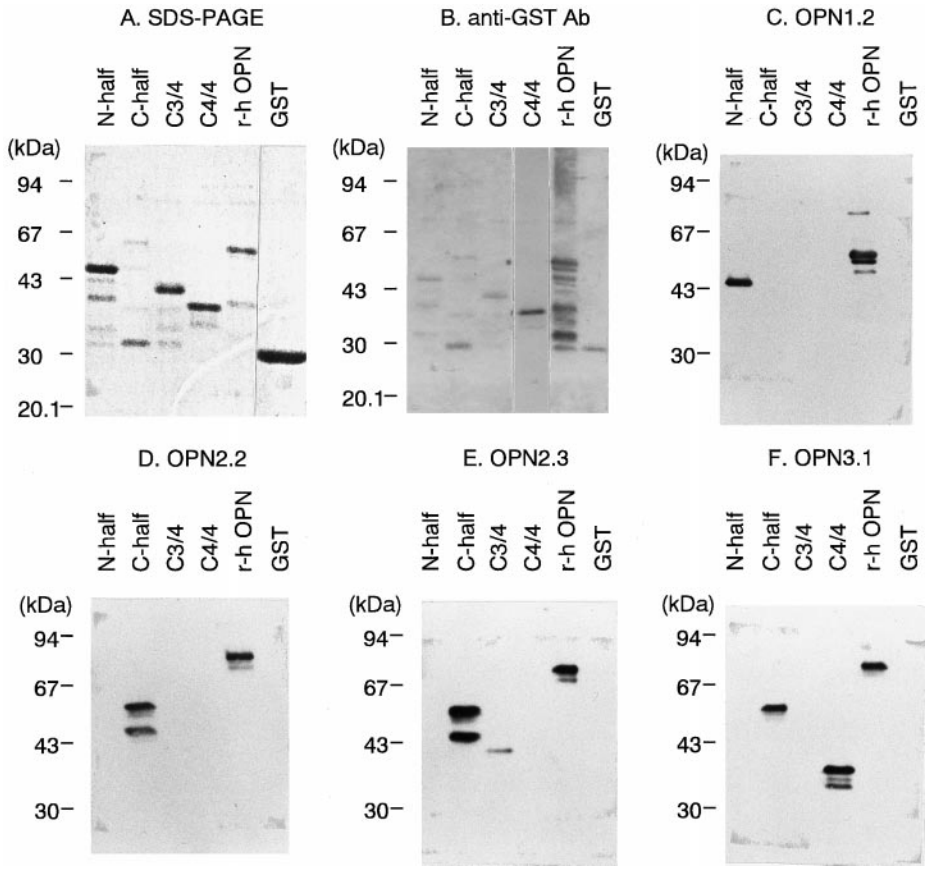

FIG. 1. Reactivity of various monoclonal antibodies with distinct fragments of recombinant OPN. Ten (A and B) or one (C–F) μg of affinity purified recombinant OPN was applied to each lane of gels followed by SDS-PAGE and electrotransfered to PVDF membranes. The membranes were then stained with CBB (A), anti-GST antibody (B), and various mAb (C–F).

fragments represent the C-terminal portion of OPN, while the 43 and 48 kDa bands are likely from the N-terminal portion. OPN 1.2 immunostained the thrombin-cleaved 43 and 48 kDa fragments of OPN. However, only thrombin-cleaved 23 kDa fragments are faintly recognized by OPN 2.2. OPN 2.3 and OPN 3.1 very faintly detected 23 kDa fragments only after longer exposure.

Monoclonal antibodies recognize native murine OPN. mAb were further characterized to determine their ability to recognize native OPN in urine and tissues (Fig. 4). Since it was shown that the expression of OPN was detected in calcified tissues and cardiac myocytes (1,6,23), those tissues obtained from BALB/C mice were immunostained with various monoclonal antibodies. As shown in Fig. 4A, the expression of OPN was detected by all monoclonal antibodies tested in the calcified tissues. Among them, OPN2.2 immunostained calcified tissues most effectively. Furthermore, cardiac myoctes were positively immunostained by OPN1.2 and 2.3, but not by OPN2.2 and OPN3.1. The monospecific polyclonal antibody, used as a positive control, was shown to immunostain 70 and 41 kDa bands in urine sample. OPN2.2 stained 70 and 41 kDa bands in urine sample strongly, while OPN2.3 and OPN3.1 stained, but less strongly. However OPN1.2 failed to detect any band (Fig. 4B).

DISCUSSION

OPN has received considerable attention for its potential role in wound healing, bone mineralization, vascular remodeling, tumor metastasis, glomerulonephritis, and granulomatous inflammation (2-6,22,24). It has been known that multiple species of OPN exist due to post-translational modification such as phosphorylation and glycosylation, cleavage by proteases, and alternative splicing of mRNA (1). In addition, recent reports have shown that the OPN molecule contains multiple cell binding sites in addition to the classical RGD sequence (10,18-20). These cell binding sites within the OPN molecule differentially interact with various cellular receptors and the consequence of those interactions may result in distinct cellular functions (6,20). To detect the expression of murine OPN protein, polyclonal antibodies have been raised against murine

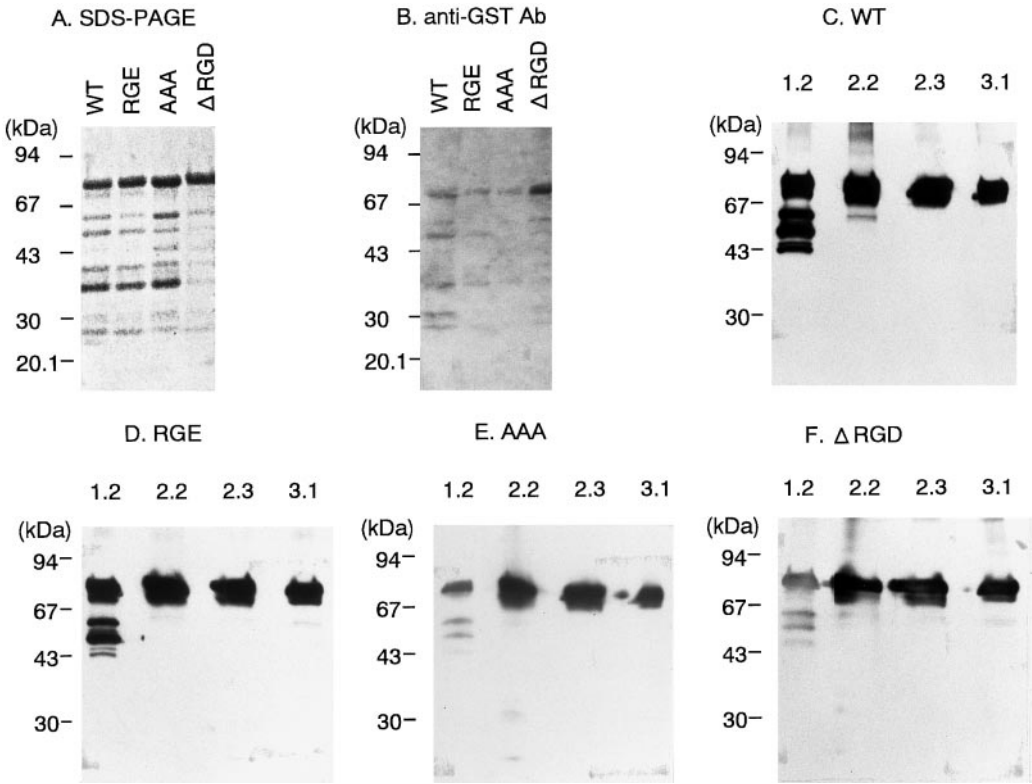

FIG. 2. Reactivity of monoclonal antibodies with mutated forms of OPN. Ten (A and B) or one (C–F) μg of affinity purified OPN was applied to each lane of gels followed by SDS-PAGE and electrotransfered to PVDF membranes. The membranes were then stained with CBB (A), anti-GST antibody (B), and various mAb (C–F).

OPN and a murine mAb and a goat antibody were raised against rat OPN which crossreacted with murine OPN (10-15). Together with detection of mRNA expression by in situ hybridization, such antibodies have been quite useful for analyzing the expression and distribution of OPN proteins in various cells and tissues. However, the epitopes recognized by those antibodies are rather limited or unknown. Thus, it is

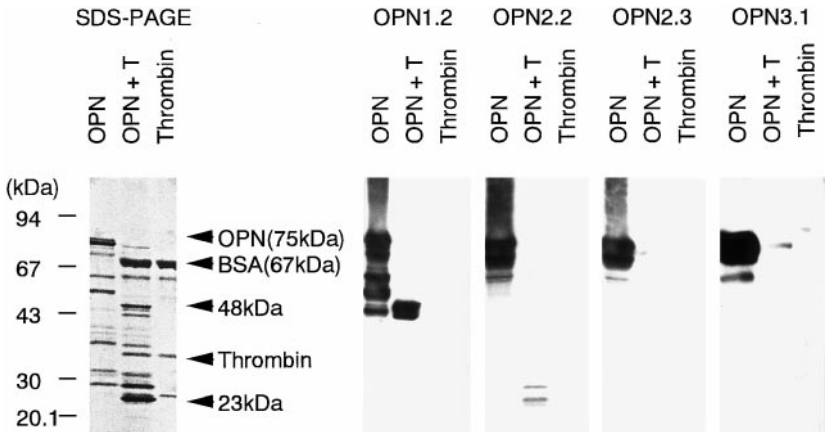

FIG. 3. Reactivity of OPN with monoclonal antibodies after thrombin digestion. Analysis of recombinant full length murine OPN in 12.5% SDS-PAGE and visualization by CBB staining, at left. OPN + T; 10 μg OPN treated with 0.2 U thrombin. Ten μg of affinity purified recombinant OPN was applied to each lane of gels. Arrows indicate the positions of GST-murine OPN fusion protein (GST-OPN), bovine serum albumin (BSA), thrombin cleaved products of GST-OPN with 48 kDa and 23 kDa, and 37 kDa thrombin (Thrombin). Western blot analysis of the recombinant GST-OPN fractionated in 12.5% SDS-PAGE and detected with the mAb, as indicated on the figure. OPN; 5 μg/lane. OPN + T; 5 μg of GST-OPN treated with 0.2 U thrombin.

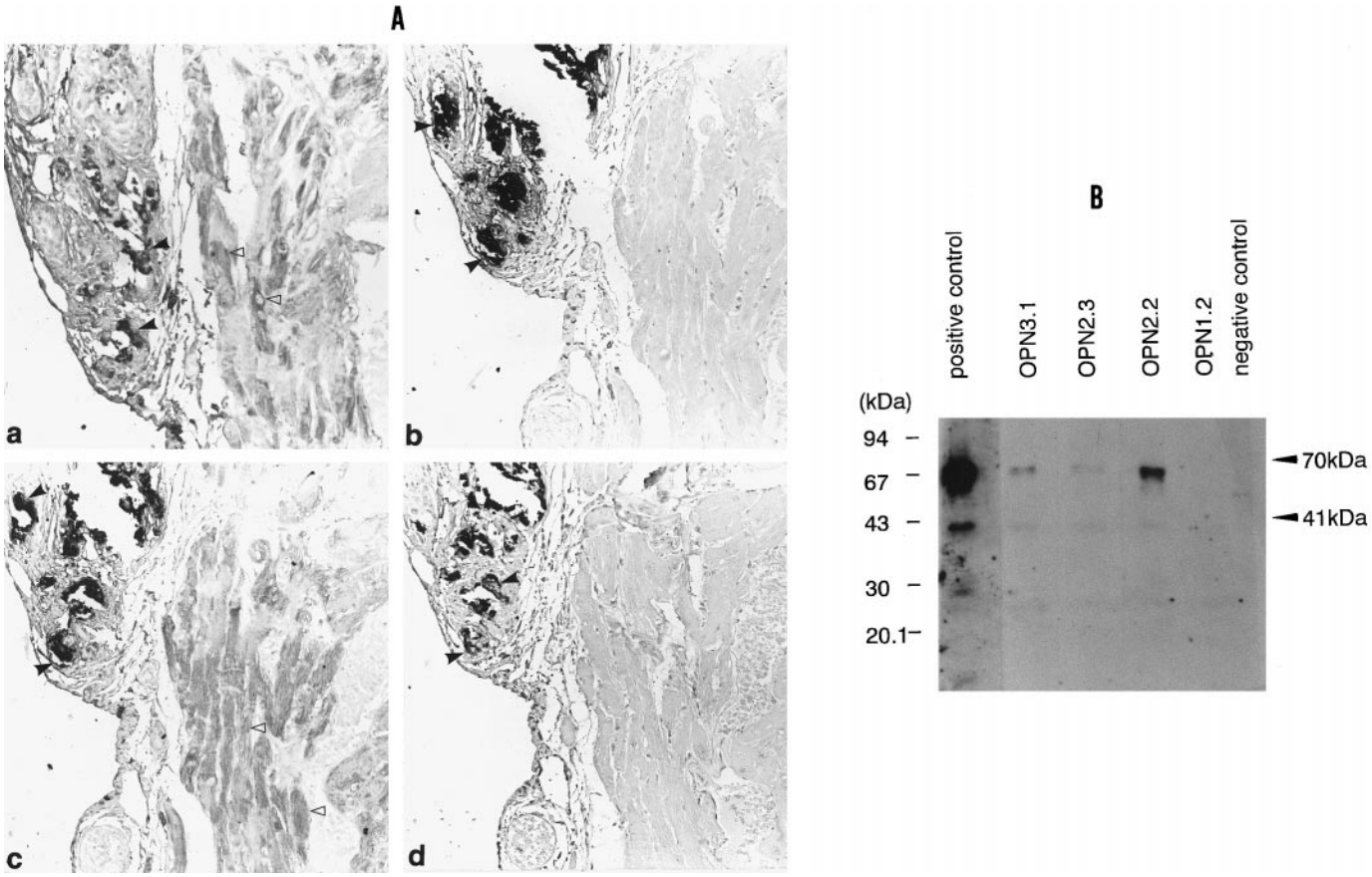

FIG. 4. Detection of native osteopontin by monoclonal antibodies. (A) Murine hearts with ectopic calcification were immunostained with monoclonal antiodies. a: OPN1.2. b: OPN2.2. c: OPN2.3. d: OPN3.1. Closed arrow heads indicate the positive staining in calcified tissues. Open arrow heads indicate the positive staining in cardiac myocytes. (B) Western blot analysis of urinary OPN in 12.5% SDS-PAGE and detected with polyclonal and mAb. All urinary samples were 10 μl/lane. Monospecific polyclonal antibody against mouse OPN was used as a positive control. Negative control indicates first antibody was omitted, followed by peroxidase-labeled anti-rat immunoglobulin antibody.

important to generate mAb to the distinct epitopes of OPN. Therefore, we used a purified bacterially produced GST-murine OPN fusion protein for immunization and recombinant truncated forms of OPN for screening. This approach enabled us to generate a set of mAb, directed against the distinct portions of the protein backbone of recombinant as well as native OPN. We describe here four rat mAb recognizing four distinct epitopes of murine OPN; OPN1.2 recognized the N-terminal half of OPN, while OPN2.2, OPN 2.3 and OPN3.1 recognized the C-terminal half of OPN.

It has been repeatedly shown that the RGD cell binding site and the adjacent thrombin cleavage site of OPN are important for the function of OPN (12-15,17,21,22,24-26). Thus, we tested whether a mutation or a deletion in the RGD region affect its reactivity to antibody. Three mAb, OPN 2.2, OPN2.3 and OPN3.1 recognizing the C-terminal half of OPN reacted with WT, RGE, AAA and ΔRGD-OPN. Thus, mutations and a deletion in the region of RGD have little impact if any on the immunoreactive epitopes located in the C-terminal half of OPN. OPN1.2 reacted equally with WT and RGE. However, replacement of RGD by AAA or deletion of RGD significantly reduce the immunoreactivity to OPN1.2. These results suggested the possibility that mutations and a deletion of RGD domain significantly alter the conformation of N-terminal half of OPN. Since OPN1.2 recognized an epitope that is sensitive to the integrity of RGD domain, we examined whether OPN1.2 can modulate the RGD-dependent cell binding of OPN. However, OPN1.2 failed to affect cell binding to OPN (data not shown). In contrast to the RGD mutational studies, thrombin-cleavage of wild type OPN resulted in significant reduction of the reactivity to those mAb recognizing the epitopes located in the C-terminal half of OPN (OPN2.2, OPN2.3 and OPN3.1). OPN1.2 which recognize epitopes located in the N-terminal half of OPN, reacted with thrombin-cleaved 43 kDa fragments. Although the thrombin-cleavage site in OPN is very close to the RGD cell binding domain, thrombin-cleavage and mutation of the RGD region appear to differentially alter the conformation of the OPN molecules. Finally, we demonstrated that these monoclonal antibodies were differ-

entially reactive with native OPN in urine and tissues. A better understanding of function/structure relationships will help in clarifying the role of OPN in various physiological and pathlogical conditions.

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