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Mapping of Functional Epitopes of Osteopontin by Monoclonal Antibodies Raised Against Defined Internal Sequences

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Abstract Osteopontin (OPN) is a secreted protein that has been implicated in diverse physiological and pathological processes. OPN can bind to integrins, via GRGDS or SVVYGLR amino acid sequences, and to other cell surface receptors, and many of OPN's functions are likely mediated via cell adhesion and subsequent signaling. Here we developed and characterized a series of five monoclonal antibodies, raised to distinct internal peptide sequences of human OPN, and have used these sequence-specific reagents, along with the previously described anti-OPN monoclonal antibody mAb53, to map functional epitopes of OPN that are important to cell adhesion and migration. All antibodies were reactive with native as well as recombinant human OPN. One antibody (2K1) raised against the peptide VDTYDGRGDSVVYGLRS could inhibit RGD-dependent cell binding to OPN, with an efficacy comparable to that of mAb53. Furthermore, 2K1 could inhibit α9 integrin-dependent cell binding to OPN. The epitope recognized by 2K1 was not destroyed by thrombin digestion, whereas mAb53 has been shown to be unable to react with OPN following thrombin cleavage. The two distinct epitopes defined by 2K1 and mAb53 antibodies are closely related to the SVVYGLR cell-binding domain and the GLRSKS containing thrombin cleavage site, respectively, and are involved in cell binding and cell migration. J. Cell. Biochem. 84: 420–432, 2002.

Key words: osteopontin; integrin binding domain; α9 integrin; αv integrin; thrombin cleavage site; RGD tripeptide; structure/function studies

Osteopontin (OPN) is a highly acidic calcium binding glycosylated phosphoprotein. OPN exerts various physiologically and pathologically important functions in vitro and in vivo [Denhardt and Guo, 1993; Rittling and Denhardt, 1997; Uede et al., 1997; Denhardt and Noda, 1998; Chiba et al., 2000; Jono et al., 2000; Shijubo et al., 2000; Sodek et al., 2000; Takemoto et al., 2000]: cell adhesion [Bautista et al., 1994; Helfrich et al., 1994; Hu et al., 1995; Liaw et al., 1995; Katagiri et al., 1996, 1999], migration [Yue et al., 1994; Liaw et al., 1995; Katagiri et al., 1999; Tuck et al., 2000], survival [Lin et al., 2000], tumorigenesis [Chambers et al., 1996], metastasis [Singhal et al., 1997;

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Shijubo et al., 1999; Tuck et al., 1999], immune responses [Kawashima et al., 1999; Ashkar et al., 2000; Wang et al., 2000], host defense [Nau et al., 1999, 2000], and inhibition of complement-mediated cell lysis [Fedarko et al., 2000]. This functional diversity is appeared to be mediated by variety of cell surface receptors. Integrins that recognize Arg-Gly-Asp (RGD) tripeptide sequence including αvβ3, αvβ1, and αvβ5 are major receptors for OPN [Helfrich et al., 1994; Tuck et al., 2000]. The binding of OPN to different integrins via RGD sequence results in distinct functional consequences, which may depend on both the specific integrin and cell type. In vascular smooth muscle cells, αvβ3, αvβ1, and αvβ5 integrins mediate cell adhesion, whereas only αvβ3 supports cell migration in these cells [Liaw et al., 1995]. In contrast, OPN-induced migration of breast cancer cell lines of high versus low malignancy was shown to be αvβ3 versus αvβ5/β1-dependent, respectively [Tuck et al., 1999, 2000]. Integrin α9β1 binds to the SVVYGLR amino acid sequence in amino-terminal fragment of thrombin-cleaved OPN, thus mediating cell adhesion to this OPN fragment [Yokosaki et al., 1999]. It was also shown that OPN could interact with other cell surface receptor(s) than integrins. Weber et al. [1996] demonstrated that CD44 can bind to OPN. We have shown that CD44 variants and β1-containing integrins can cooperate to bind to OPN in RGD-independent manner and promote tumor cell motility and adhesion [Katagiri et al., 1999]. Ashkar et al. [2000] found that interaction of OPN and CD44 on macrophages results in the inhibition of IL-10 secretion, while the interaction of OPN and αvβ3 integrin on macrophages promotes IL-12 secretion.

In order to provide the means to analyze functional epitopes of OPN, we raised five monoclonal antibodies against synthetic peptides corresponding to specific internal sequences of human OPN. The epitope(s) recognized by the resulting monoclonal antibodies are thus predictable. We describe here five monoclonal antibodies that we found to be immunoreactive with recombinant as well as native human OPN. Furthermore, these antibodies could react with OPN after phosphatase treatment. Interestingly, one antibody, (2K1) raised against the synthetic peptide VDTYDGRGDSVVYGLRS, could inhibit the RGD-dependent cell binding to OPN. The efficacy of cell binding inhibition by this antibody is comparable to that shown by the previously described monoclonal antibody mAb 53. It had previously been shown that mAb53 could inhibit RGD-dependent cell binding to OPN, and the epitope recognized by this antibody is destroyed by thrombin treatment, suggesting that the epitope is part of, or is located near, the RGD-cell-binding domain [Bautista et al., 1994]. However, the epitope recognized by 2K1 was not destroyed by thrombin digestion. We found that mAb53 reacted strongly with SVVYGLRSKS and moderately with SVVYGLR, but not with VDTYDGRGDSVVYGLRS or VDTYDGRGD, whereas 2K1 could react with both SVVYGLR and VDTYDGRGDSVVYGLRS, but not with VDTYDGRGD. Importantly, 2K1 could also inhibit α9 integrin-dependent cell binding to OPN. Nevertheless both monoclonal antibodies were specific for human OPN. We thus propose that 2K1 and mAb53 antibodies recognize distinct epitope(s) that are closely related to the SVVYGLR α9β1 integrin-binding domain and the thrombin-cleavable GLRSKS sequence, respectively, and that these sequences are differentially involved in cell binding and migration.

MATERIALS AND METHODS

Cloning, Construction, and Purification of Glutathione S-Transferase–OPN Fusion Proteins and Reagents

The cloning of three human OPN cDNA including OPN-a, carboxy-terminal half OPN-a (C half OPN; K170-N314), and amino-terminal half OPN-a (N half OPN; M1-R168) were reported elsewhere [Kon et al., 2000]. These cDNA 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) as previously described [Kon et al., 2000]. The OPN-a cDNA was also inserted into pcDNA 3.1 (+) vector (Invitrogen, NV Leek, The Netherlands) and transfected into CHO-K1 cells (designated as a CHO/OPN-a cells). Various recombinant GST-OPN fusion proteins prepared in *E. coli* were purified using glutathione-sepharose columns as described previously [Kon et al., 2000]. The GST portion of a previously reported GST fusion protein with recombinant amino-terminal half of OPN (referred to as nOPN) [Yokosaki et al., 1999], was removed by cleaving off with PreScission

protease (Amersham Pharmacia Biotec) and was used in the experiments shown in Figure 7. The glycosylated form of OPN-a was purified from the culture supernatant of CHO/OPN-a cells by using ultrafiltration on a hollow fiber cartridge, a DEAE-sepharose CL-6B column, ULTROGEL AcA44 column, and followed by reverse phase column chromatography on a RESOURCE RPC column as described previously [Kon et al., 2000]. Cloning of rat OPN cDNA was performed as follows. Total RNA was prepared from NRK-52E cells, and first-strand cDNA was generated with reverse transcriptase and random primers. The rat OPN cDNA was then amplified by polymerase chain reaction (PCR) using a rOPN-5′ (5′-CGGGATCCACTACCATGAGACTGGCAGTGGTTTGC-3′) primer containing the rat OPN cDNA sequence at the first seven amino acids (M1–C7) with the signal peptide and the *BamH*I restriction enzyme site, and a rOPN-3′ (5′-CCGCTCGAGTTAATTGACCTCAGAAGATGAACTCTC-3′) primer complementary to the rat OPN cDNA sequence at the carboxy-terminal amino acids (E310–N317) and the stop codon with the *Xho* I restriction enzyme site. The amplified products were digested with *BamH*I and *Xho* I and ligated into pcDNA3.1 Vector (Invitrogen, NV Leek, The Netherlands). The rat OPN cDNA cloned by PCR was confirmed by complete sequencing. The mouse OPN cDNA was obtained as described previously [Katagiri et al., 1996, 1999]. Rat or mouse OPN cDNA were transfected into CHO-K1 with Lipofectamine (Gibco BRL, Rockville, MD) and OPN was purified from the culture supernatants as described above. Various peptides used for immunization and binding studies were purchased from Sigma Genosys Japan, and these were synthesized using Fmoc (*N*-(9-fluoreny)methoxycarbonyl) chemistry on a peptide synthesizer (model 432A, Perkin Elmer Life Sciences), followed by purification with C18 reversed-phase column chromatography.

Production of Monoclonal Antibodies

Synthetic peptides corresponding to the internal sequences of human OPN were prepared (Fig. 1) and used as immunogens. These included IPVKQADSGSSEEKQ (peptide 1; I17–Q31), VDTYDGRGDSVVYGLRS (peptide 5; V153–S169), IDSQELSKVSREFHS (peptide 6; I261–S276), and KHLKFRISHELDSASSEVN (peptide 4; K296–N314). As previously described [Saavedra, 1994; Sorensen et al., 1995; Kon et al., 2000], peptide 1 corresponds to the exon 2-coded region and contains at least three potential phosphorylation sites. Peptide 3 (KSKKFRRPDIQYPDATDE; K170–E187) corresponds to the amino-terminal end of the thrombin-cleaved carboxy-terminal half of OPN and contains one potential phosphorylation site. Peptide 4 corresponds to the carboxy-terminal end of OPN and contains one potential

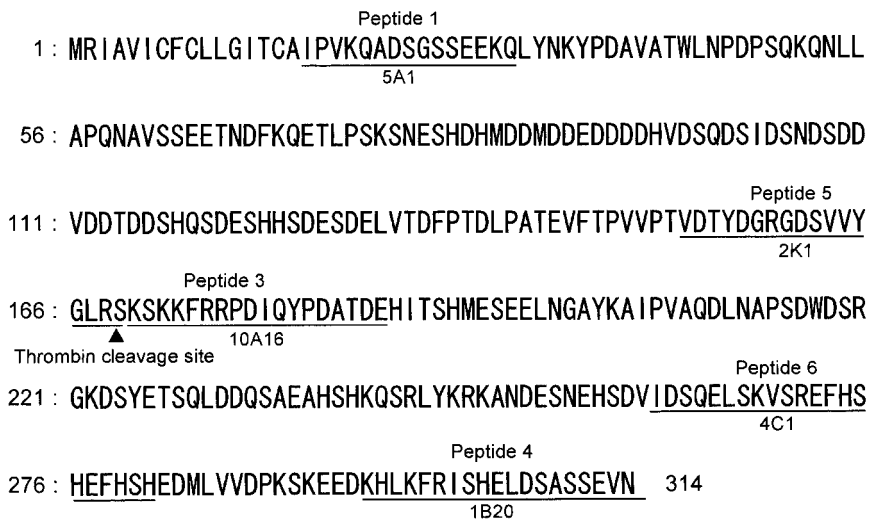

Fig. 1. Amino acid sequence of human OPN-a. The internal sequences corresponding to the synthetic peptides used as immunogens are underlined, and the obtained antibodies are indicated. The site of thrombin cleavage is indicated by a closed triangle, and the cleavage site defines the N and C terminal halves of the OPN molecule.

heparin-binding site. Peptide 5 corresponds to the carboxy-terminal end of thrombin-cleaved amino-terminal half of OPN and may not contain any phosphorylation sites. It should be noted that peptide 5 contains the RGD and SVVYGLR sequences that can be recognized by $\alpha v\beta 3$ and $\alpha 9\beta 1$ integrin receptors, respectively [Denhardt and Guo, 1993; Uede et al., 1997; Denhardt and Noda, 1998; Yokosaki et al., 1999]. Peptide 6 contains at least two potential phosphorylation sites. None of the synthetic peptides contain potential *O*-linked glycosylation sites. These peptides were coupled with thyroglobulin, and were then used to immunize mice. Polyethylene glycol-mediated cell fusions between splenocytes of immunized mice and fusion partner X63-Ag8-653 and the subsequent selection for hybridomas were carried out as described previously [Kinebuchi et al., 1991]. Hybridomas showing positive reactivities for immobilized GST/OPNa and OPN-a derived from CHO cells, but not for GST, were selected. 5A1, 2K1, 4C1, and 1B20 mAb were obtained from mice immunized with peptide 1, 5, 6, and 4, respectively. The human OPN sequence and possible recognition sites for these antibodies are schematically shown in Figure 1. As reported previously, 10A16 mAb was obtained by immunizing mice with peptide 3 [Kon et al., 2000], and mAb53 was obtained by immunization with full-length recombinant human OPN [Bautista et al., 1994]. Anti-human integrin $\alpha 9$ subunit monoclonal antibody, Y9A2 [Wang et al., 1996] was gifted by Dr. Sheppard (Lung Biology Center, UCSF).

Thrombin Cleavage and Phosphatase Treatment

Purified OPN-a was digested by thrombin (Sigma, St. Louis, MO) at 5 μg protein/0.1–2.0 U enzyme and incubated at 37°C for 2 h. Type II potato acid phophatase (Sigma) was used in this study, and 1 μg of purified OPN-a was treated with 3.8 or 7.6 mU of phophatase as described previously [Ashkar et al., 2000].

Western Blot Analysis

Various OPN preparations (100 ng/lane), urine (10 μl), and the supernatant of cell line (10 μl) were loaded onto 12% 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 as described previously [Kon et al., 2000].

Enzyme-Linked Immunoabsorbent Assay (ELISA)

Cysteine residues were introduced at the amino-terminal end of various synthetic peptides and coupled to bovine serum albumin (BSA). In case of SVVYGLR, cysteine residues were introduced at the amino-terminal or carboxy-terminal end and were coupled to BSA (referred to as BSA-SVVYGLR and SVVYGLR-BSA, respectively). These peptides were coated onto 96-well plates at various concentrations (50 μl in volume in 0.1 M carbonate buffer, pH9.5) at 4°C overnight, then blocked with 0.1% BSA in PBS containing 0.05% NaN_3 (referred to as blocking buffer). Purified antibodies were diluted with dilution buffer (0.1% BSA in PBS containing 0.05% Tween 20), added to the plates (50 μl/well), and incubated for 30 min at 37°C. After extensive washing with PBS containing 0.05% Tween 20 (washing buffer), 50 μl of 2 ng/ml horseradish peroxidase-labeled anti-mouse Ig (IBL, Fujioka, Japan) was added to each well and incubated for 30 min at 37°C. After nine washes with washing buffer, 100 μl of *O*-phenylenediamine solution as a substrate was added to each well and incubated for 15 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 490 nm.

Cell Adhesion Inhibition Assay

The 96-well plates were precoated with various concentrations of CHO/OPN-a proteins overnight at 4°C, followed by treatment with 0.5% BSA in PBS for 10 min at 37°C to block nonspecific adhesion. TIG-7 or $\alpha 9$-transfected SW480 cells (see Cells and Cell Culture section, below) were suspended in DMEM containing 0.25% BSA and 200 μl of cell suspension (at a cell density of 5×10^4 cells/well) were applied to 96-well plate precoated with CHO/OPN-a, N half OPN, or nOPN proteins in the presence or absence of various concentrations of monoclonal antibodies or synthetic peptides and incubated for 1 h at 37°C. The medium was removed from the plates, and all wells were washed twice with DMEM containing 0.25% BSA. The adherent cells were fixed and stained by 0.5% crystal

violet in 20% methanol for 30 min. All wells were rinsed three times with water, and adherent cells were then lysed with 20% acetic acid. The resulting supernatants from each well were analyzed by an immunoreader, and the absorbance at 590 (performed in the Sapporo laboratory) or 595 (performed in the Hiroshima laboratory) was measured to determine the relative number of cells adhered to wells. All assays were performed in triplicate and at least three independent experiments were performed. Each value represents the mean of at least three separate experiments.

Cell Migration Assay

The migration of U937 monocyte cell line was measured by using the ChemoTx 101-8 System (Neuroprobe, Gaithersburg, MD) with polyvinylpyrrolidon-free polycarbonate filters (8.0 μm pore size). Cells were suspended to a final concentration of 2×10^6/ml in DMEM containing 0.1% BSA. Cell suspensions (25 μl) were added to the upper surface of the filters, and various concentrations of OPN proteins were added to the lower compartment. ChemoTx plates were incubated at 37°C in a 5% CO_2 atmosphere. After a 4-h incubation, the filters were fixed with methanol, and stained with hematoxylin and eosin (H–E). The cells on the upper surface of the filters were removed by wiping them with a cotton swab. The cells that had migrated to the lower surface were manually counted under a microscope, at a magnification of 400 × (referred to as high power field; HPF). All samples were tested in triplicate, and data were expressed as the mean of the migrating cell number ± SD. A two-tailed Student's *t*-test was used to determine the significance of differences between experimental groups.

Urine Samples

Ten healthy men (mean age = 31 years; range = 24–41 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 −80°C and thawed at room temperature as needed. After thawing, samples were immediately subjected to Western blot analyses.

Cells and Cell Culture

The following human cell lines obtained from Immuno-Biological Laboratories Co., Ltd, Fujioka, Japan were previously described [Kon et al., 2000] and were used in this study. The culture supernatants of NRC-12 (renal cell

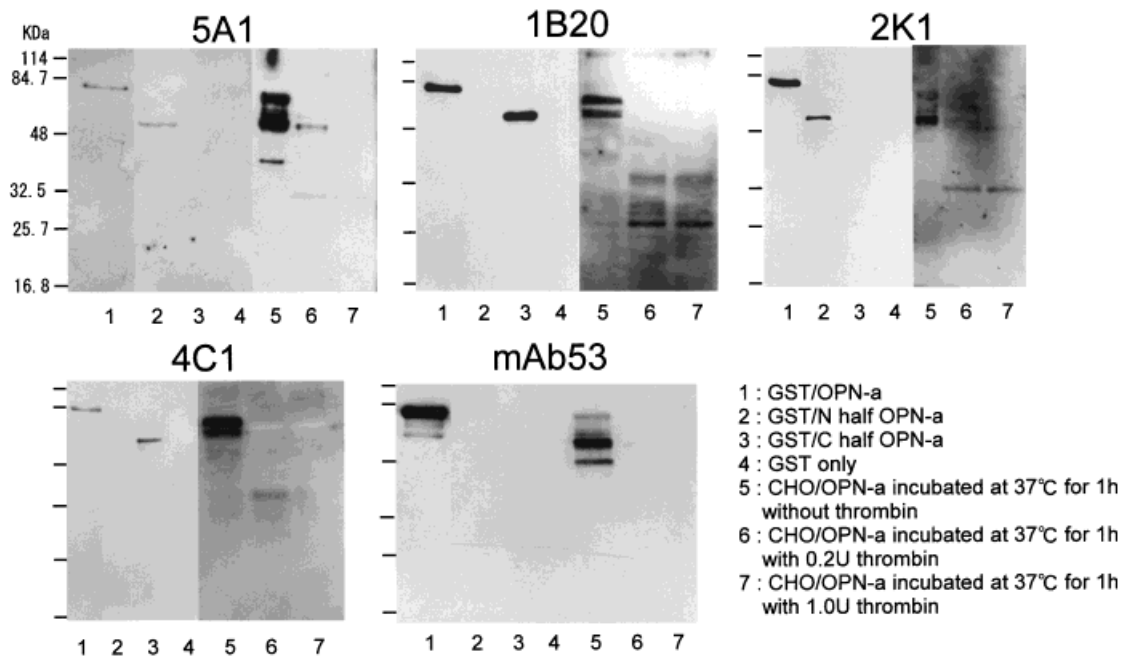

Fig. 2. Western blot analysis of OPN. The various OPN preparations (**lanes 1–7**) were fractionated in SDS–PAGE and detected by 5A1, 1B20, 2K1, 4C1, and mAb53.

carcinoma), HepG2 (hepatocellular carcinoma), PC-9 (lung adenocarcinoma), PC-10 (lung squamous cell carcinoma), and SEKI (malignant melanoma) cells were used for OPN production. To obtain culture supernatant for Western blot analyses, cells were seeded in the TIL Media I (IBL, Fujioka, Japan) containing 10% FCS at a cell density of 2×10^5/ml and cultured for 2 days. TIG-7, a human fibroblast cell was used for cell adhesion study. U937, a human monocytic cell, was used for cell migration assay. α9-transfected human colon cancer cells, SW480 [Yokosaki et al., 1994], were gifted by Dr. Sheppard (Lung Biology Center, UCSF).

RESULTS

Reactivity of Monoclonal Antibodies to Various Forms of Recombinant OPN

We first tested the reactivity of monoclonal antibodies to OPN-a and truncated forms of recombinant OPN derived from *E. coli*. As shown in Figure 2, all monoclonal antibodies including mAb53 tested reacted to human OPN. As expected, 5A1 and 2K1 monoclonal antibodies reacted to the amino-terminal half of OPN, whereas 4C1 and 1B20 reacted to the carboxy-terminal half of OPN. mAb53, however, neither react with the amino-terminal half nor the carboxy-terminal half of OPN. Furthermore, it was found that all antibodies tested reacted not only with non-glycosylated forms of recombinant OPN derived from *E. coli*, but also with glycosylated recombinant OPN-a derived from CHO cells. The epitope recognized by mAb53 was destroyed by thrombin cleavage of OPN, as described previously [Bautista et al., 1994]. In contrast, those recognized by 5A1, 2K1, 4C1, and 1B20 were relatively resistant to thrombin cleavage.

Reactivity of Monoclonal Antibodies to Native Forms of OPN

Urinary OPN was detected by all monoclonal antibodies including mAb53 and found to possess relatively homogenous molecular weight of 70–90 kDa. No small molecular weight OPN was detected in urine of normal individuals (Fig. 3). OPN secreted by tumor cell lines in culture was detected by various monoclonal antibodies (Fig. 4). OPN production by NRC-12, HepG2, and SEKI cells was clearly detected by all antibodies tested, but by PC-9 and PC-10 cells, was little if any detected.

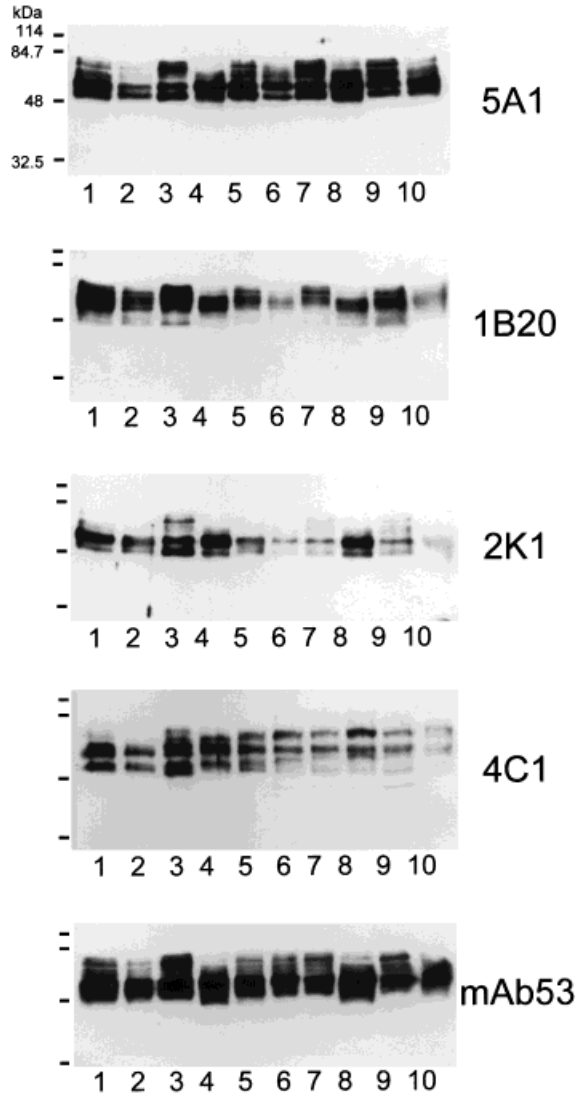

Fig. 3. Western blot analysis of human urinary OPN. Urine obtained from 10 healthy donors (**lanes 1–10**, each lane loaded with 10 μl of urine) were fractionated in SDS–PAGE and were detected by 5A1, 1B20, 2K1, 4C1, and mAb53.

Inhibition of Cell Adhesion to OPN by Monoclonal Antibodies

We examined whether the monoclonal antibodies could inhibit cell adhesion to OPN. Since it was previously shown that mAb53 could inhibit RGD-dependent adhesion of various cells to OPN [Bautista et al., 1994], this antibody was included as a positive control. As shown in Figure 5A, TIG-7 human fibroblast cells were able to adhere well to OPN, and this adhesion was significantly inhibited by GRGDS peptide, but not control peptide, indicating that cell adhesion is RGD-dependent. Only 2K1

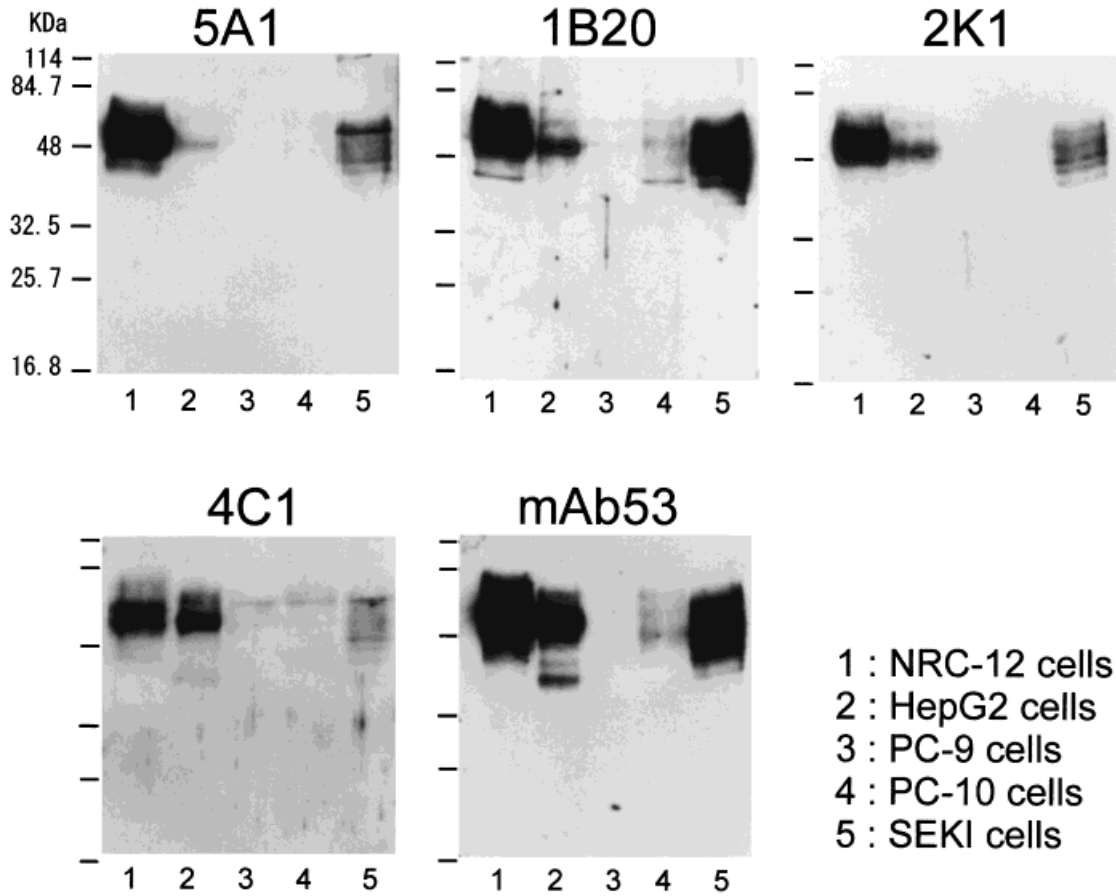

Fig. 4. Western blot analysis of the human OPN secreted by five different tumor cell lines. The equivalent of 5 μg of secreted protein from tumor cells was loaded onto each lane, fractionated in SDS–PAGE, and detected by 5A1, 1B20, 2K1, 4C1, and mAb53.

monoclonal antibody among the five monoclonal antibodies and normal murine IgG as a control antibody (data not shown) tested was able to inhibit cell adhesion to OPN. The efficacy of the inhibition of cell adhesion to OPN by 2K1 was comparable to that shown by mAb53 and was dose-dependent (Fig. 5B,C). 2K1 and mAb53 did not inhibit the cell adhesion of TIG-7 cells to vitronectin (VN) or fibronectin (FN) (data not shown).

Reactivity of Monoclonal Antibodies to Synthetic Peptides and Extracellular Matrix Proteins

mAb53, 2K1, 10A16, 5A1, 4C1, and 1B20 reacted to human OPN, but not to fibronectin (FN) and vironectin (VN) (data not shown). 2K1 antibody was raised against a peptide 5, VDTYDGRGDSVVYGLRS, and this peptide contains two independent cell binding domains, GRGDS and SVVYGLR. Therefore, we tested whether 2K1 could react either with VDTYDGRGD or SVVYGLR. As shown in Figure 6, 2K1 could bind to Peptide 5 and the SVVYGLR peptide, but not to the VDTYDGRGD peptide. It should be noted that 2K1 antibody preferentially bound to SVVYGLR-BSA as compared to BSA-SVVYGLR. In addition, 2K1 antibody did not significantly bind to SVVYGLRSKS, which contains SVVYGLR. mAb53 did not significantly bind to the VDTYDGRGDSVVYGLRS peptide or to the VDTYDGRGD peptide. In contrast to 2K1, mAb53 preferentially bound to BSA-SVVYGLR as compared to SVVYGLR-BSA. More importantly mAb53 strongly bound to SVVYGLRSKS. Nevertheless, 2K1 and mAb53 did not bind to irrelevant Peptide 4 or to the GRGDS peptide. Although the GRGDS sequence is well conserved in various species, SVVYGLR is variable (SLAYGLR in rat and mouse). Therefore, we tested whether 2K1 could react with rat and mouse OPN in Western blot

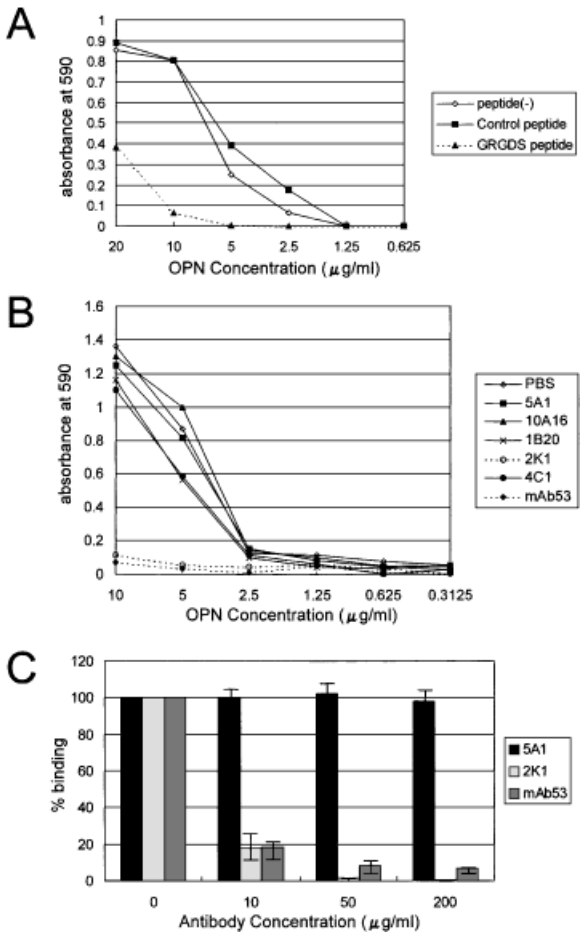

Fig. 5. Inhibition of RGD-dependent cell binding to OPN. TIG-7 cells were allowed to adhere to 96-well plates precoated with OPN-a at various concentrations in the absence or presence of 100 μM GRGDS peptide (**A**), 100 μM peptide 4 as a control (A), or 50 μg/ml monoclonal antibodies against OPN (**B**). Data are presented as a mean of triplicate experiments. In **C**, cells were allowed to adhere to 96-well plates precoated with 5 μg/ml OPN in the presence of various concentrations of monoclonal antibodies against OPN. Data are presented as a mean ± SD.

analysis and found that 2K1 is specific for human OPN, and does not recognize rat or murine OPN (data not shown). Since it was recently shown that the SVVYGLR sequence is recognized by the α9β1 integrin receptor, we next tested whether 2K1 can inhibit integrin α9β1 integrin-mediated cell adhesion to OPN. As shown in Figure 7, the binding of α9-transfected SW480 cells to the RGD-containing extracellular matrix protein, VN was totally RGD-dependent. In contrast, the same α9-transfected cells adhered to the nOPN in both RGD-dependent and -independent manners. The RGD-independent adhesion to nOPN was mediated by α9β1, since Y9A2 completely blocked residual adhesion of GRGDSP-treated cells. This multiple receptors-mediated adhesion of α9-transfected SW480 cells to nOPN was completely blocked by 2K1 but not by 5A1, while the adhesion to VN was not blocked either by 2K1 or 5A1.

Inhibition of OPN-Induced Monocyte Migration by Monoclonal Antibodies

OPN induced the migration of human monocytic cell line, U937, in a dose-dependent manner (Fig. 8). Full-length OPN, thrombin-cleaved OPN, and N half OPN similarly induced cell migration. 2K1 antibody significantly inhibited cell migration induced by full-length, thrombin-cleaved, and N half OPN. In contrast, mAb53 could only inhibit cell migration induced by full-length OPN.

Reactivity of Monoclonal Antibodies to OPN After Phosphatase Treatment

Recently, it has been reported that phosporylated and non-phosporylated OPN have differential functions [Jono et al., 2000]. To examine whether those monoclonal antibodies can detect both forms, glycosylated recombinant human OPN derived from CHO cells was treated with acid phosphatase. As shown in Figure 9, all monoclonal antibodies tested could detect OPN before and after phosphatase treatment. It should be noted that the molecular weight of OPN was significantly reduced after phosphatase treatment.

DISCUSSION

We describe here five monoclonal antibodies that specifically recognize OPN in addition to previously described mAb53. This approach permits that 1) monoclonal antibodies against targeted epitope(s) can be obtained and 2) monoclonal antibodies against the protein backbone can be obtained rather than those reacting to post-translationally modified epitopes that might have antigenicity similar to other unrelated proteins. Those five and mAb53 antibodies recognize *E. coli*-derived non-glycosylated form of OPN and CHO-derived glycosylated form of OPN (Fig. 2) and could react similarly with OPN before and after phosphatase treatment (Fig. 9). Furthermore, those six antibodies could react with native human OPN derived from urine (Fig. 3) and tumor cell lines (Fig. 4). Taken

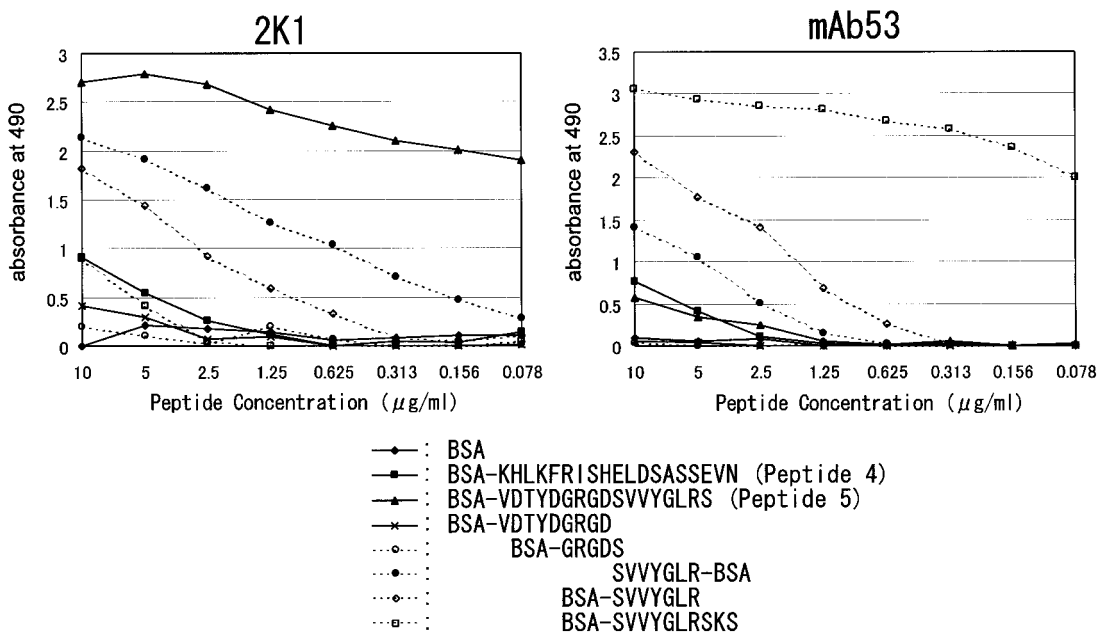

Fig. 6. The reactivity of 2K1 and mAb53 to synthetic peptides corresponding to internal sequences of human OPN. The synthetic peptides were coated onto 96 wells at various concentrations. 2K1 or mAb53 at a concentration of 5 μg/ml was added to the plates and the bound antibody was quantified as described in Materials and Methods. Data were expressed as a mean of triplicate experiments.

together, epitopes recognized by these monoclonal antibodies are in regions of the protein backbone unaltered by post-translational modifications.

The multiple bands of urinary OPN in Figure 3 and the two bands of CHO-derived OPN in Figure 9 may be due to the multiple isoforms [Saitoh et al., 1995] and heterogeneity of post-translational modification [Singth et al., 1990]. In addition, full-size OPN produced by different tumor cells gave bands of different intensities in Figure 4. The amounts of OPN produced by each tumor cell line can be different as previously reported [Kon et al., 2000]. The degree of OPN glycosylation can be different among tumor cell lines. Both above factors may influence the intensity of OPN band in Western blot analysis.

We found that the RGD-dependent cell adhesion to human OPN was blocked by mAb53 and 2K1 in a dose-dependent manner, thus indicating that the epitope recognized by these two

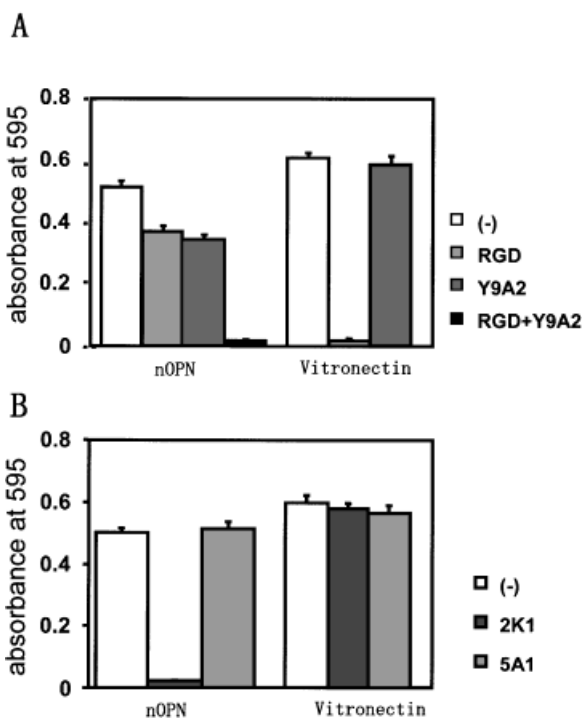

Fig. 7. The inhibition of RGD-independent cell adhesion of α9-transfected SW480 cells to nOPN by 2K1. In this experiment, the amino-terminal half OPN was cleaved off from GST by PreScission protease (nOPN). **A**: α9-Transfected SW480 cells bound to nOPN (3 μg/ml) in both RGD-dependent and -independent manners and RGD-dependent manner to VN (1 μg/ml). The adhesion is tested in the presence of GRGDSP peptide (200 μM), anti-α9 monoclonal antibody Y9A2, or in combination of these two reagents. **B**: The effects of 2K1 and 5A1 on the adhesion of α9-transfected SW480 cells to the nOPN and VN. The antibodies at a final concentration of 200 μg/ml were added into the well after blocking with BSA. Adhesion is expressed as absorvance at 595 nm. Each bar represents the mean ± SD of tripricated well.

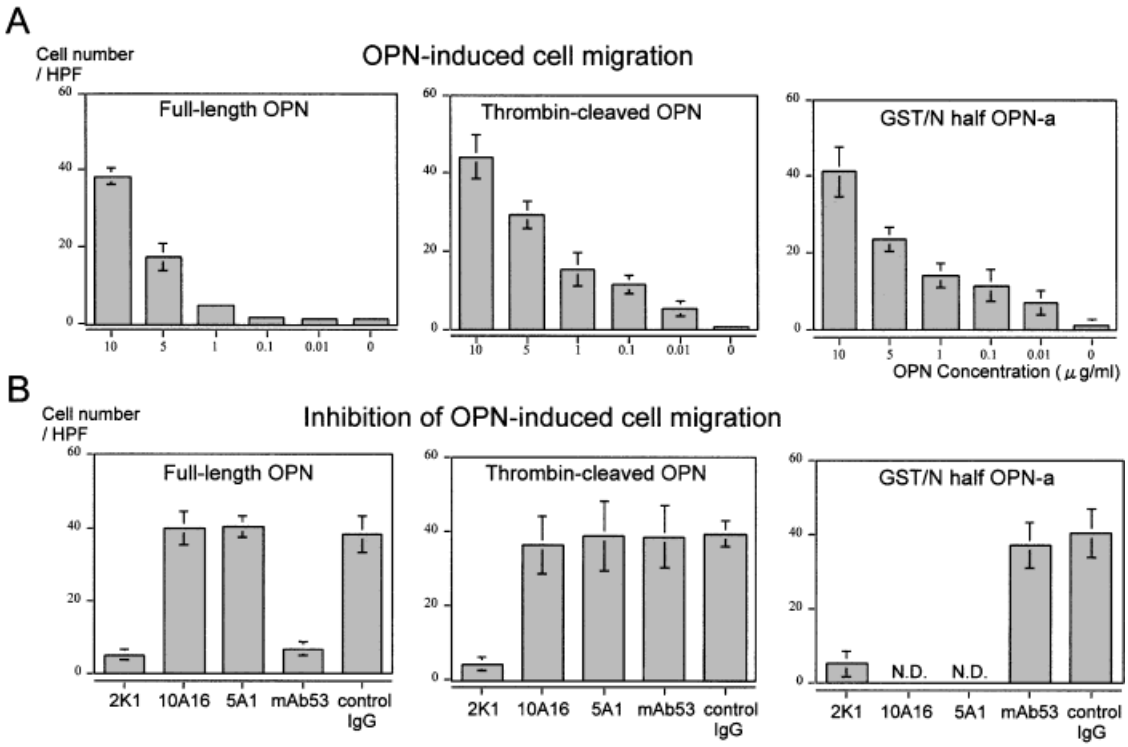

Fig. 8. The OPN-induced cell migration. **A**: The cell migration of U937 cells against indicated concentrations of full-length, thrombin-cleaved, and N half OPN. **B**: The inhibition of OPN-induced cell migration by monoclonal antibodies against OPN. The cell migration against 10 μg/ml OPN was examined in the presence of 50 μg/ml of affinity purified 2K1, mAb53, 5A1, 10A16, or control murine IgG. Migrated cell number was counted under a microscope at a magnification of 400× and expressed as the mean of cell number per HPF ± SD.

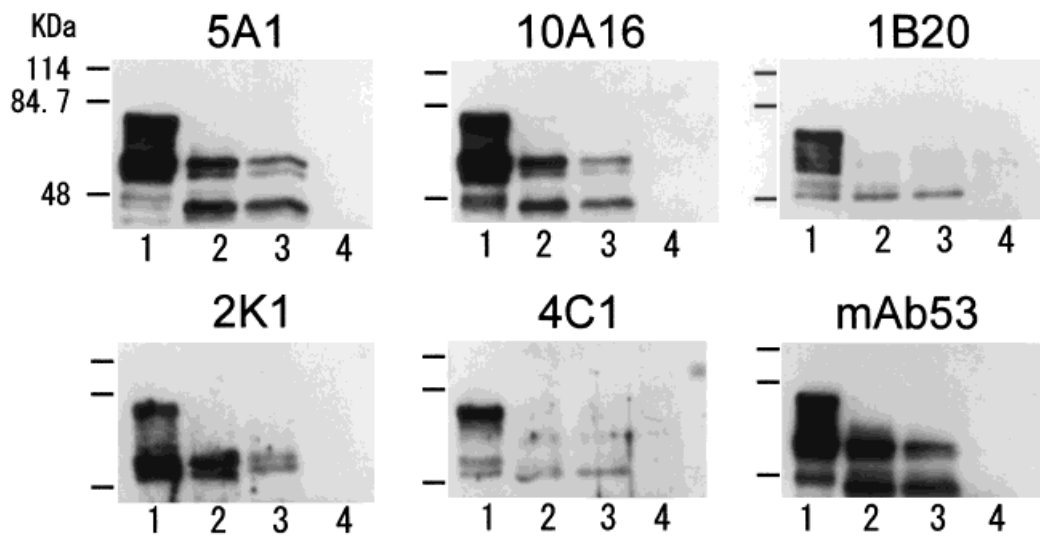

Fig. 9. The reactivity of various antibodies with phosphatase-treated OPN. One microgram of OPN treated with or without potato phosphatase was fractionated in SDS–PAGE and blotted by 5A1, 10A16, 1B20, 2K1, 4C1, and mAb53. **Lane 1**: OPN. **Lane 2**: OPN treated with 3.8 mM phosphatase. **Lane 3**: OPN treated with 7.6 mM phosphatase. **Lane 4**: 7.6 mM phosphatase.

antibodies is near the GRGDS sequence or functionally related to this sequence. It was previously shown that the epitope recognized by mAb53 is thrombin-labile, suggesting that its epitope either includes the RGD/thrombin cleavage region or is conformationally dependent on the intact molecule [Bautista et al., 1994]. Whereas, 2K1 could react with both thrombin-cleaved and non-cleaved OPN (Fig. 2). 2K1 was raised against VDTYDRGDSVVYGLRS, and this peptide contains two cell binding domains, GRGDS and SVVYGLR. 2K1 did not bind to GRGDS. Interestingly, 2K1 preferentially bound to the SVVYGLR peptide when a cysteine residue was introduced at the carboxy-terminal end (SVVYGLR-BSA) as compared to BSA-SVVYGLR. This may indicate that the critical epitope for 2K1 binding is located at the amino-terminal side of SVVYGLR. This speculation is supported by the fact that 2K1 antibody does not react with rat and mouse OPN, and the rat, mouse, and human OPN sequences differ in this region (VV in human; LA in rat and mouse). In contrast to 2K1, mAb53 preferentially bound to the SVVYGLR peptide when the cysteine residue was introduced at the amino-terminal end (BSA-SVVYGLR), indicating that the epitope is located at carboxy-terminal side of SVVYGLR. mAb53 failed to react with the full VDTYDGRGDSVVYGLRS peptide which includes the SVVYGLR sequence, supporting the notion that the epitope recognized by mAb53 may be influenced by the conformation of the molecule. More importantly, we found that mAb53 bound more strongly to the SVVYGLRSKS peptide, which includes the thrombin-cleavage site, than to SVVYGLR. Since it was recently shown that the SVVYGLR sequence can be recognized by the $\alpha 9\beta 1$ integrin receptor, we examined whether 2K1 would inhibit integrin $\alpha 9\beta 1$-mediated cell adhesion to the amino-terminal half of OPN. $\alpha 9$-transfected SW480 cells bound to the amino-terminal half of OPN both in RGD-dependent and -independent fashions. The RGD-independent adhesion to the amino-terminal half of OPN was mediated by $\alpha 9\beta 1$ integrin. We found that 2K1 could inhibit both RGD-dependent and -independent adhesion of $\alpha 9$-transfected SW480 cells to the amino-terminal half of OPN, and thus 2K1 completely blocks adhesion of $\alpha 9$-transfected SW480 cells to the amino-terminal half of OPN.

We also found that the binding of TIG-7 cells to OPN was RGD-dependent, and this binding was significantly inhibited by 2K1 and mAb53. In addition, both antibodies inhibited OPN-induced cell migration. In contrast, 10A16 and 5A1 that recognize the peptides within the carboxy-terminal and amino-terminal half of OPN, respectively, could not inhibit cell migration. Taken together, our findings suggest that the epitopes recognized by mAb53 and 2K1 are functionally important for both cell binding and migration. Overall, this study demonstrates that different regions of the OPN molecule are functionally distinct, and the precisely defined reagents which we have developed are providing clues as to the roles these different regions play in the various functions of OPN. Especially since 2K1 possesses a neutralizing ability, it can contribute in addressing the in vivo role of the thrombin-cleaved fragment of OPN, which have recently attracted much attention in inflammation, tissue remodeling, and tumor metastasis.

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