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Characterization of antibodies for osteopontin fragments in rheumatoid arthritis

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CHARACTERIZATION OF ANTIBODIES FOR OSTEOPONTIN
FRAGMENTS IN RHEUMATOID ARTHRITIS

A Thesis

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San Jose State University

In Partial Fulfillment

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Masters of Science

by

Shadi Sharif

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ABSTRACT

CHARACTERIZATION OF ANTIBODIES FOR OSTEOPONTIN
FRAGMENTS IN RHEUMATOID ARTHRITIS

by Shadi Sharif

The pro-inflammatory cytokine osteopontin (OPN) is present in rheumatoid arthritis (RA) synovial fluid. Osteopontin can be cleaved by thrombin (OPN-R) exposing a cryptic $\alpha_4\beta_1$ / $\alpha_9\beta_1$ integrin-binding motif (SVVYGLR). The C-terminal arginine in thrombin-cleaved OPN can be further cleaved by plasma carboxypeptidase B (CPB) abrogating thrombin-cleaved OPN's binding to the integrins (OPN-L). Specificity of antibodies against OPN-R and OPN-L were determined by Western blot and peptide blocking studies. Epitope mapping of antibodies revealed Anti-OPN-R and Anti-OPN-L recognize the bold amino acids from SVV**YGLR** and S**VV**YGL respectively. Anti-OPN-R and Anti-OPN-L ELISA were developed with sensitivity of 0.6 ng/ml. Significantly elevated OPN-R and OPN-L levels were detected from rheumatoid arthritis synovial fluids compared to osteoarthritis (OA) and psoriatic arthritis (PsA). Furthermore, preferential adherence of fibroblast-like synoviocytes from joints to OPN-R rather than OPN-L, suggests thrombin-cleaved OPN may play a role in pathogenesis of RA which in turn is being regulated by CPB.

ACKNOWLEDGMENT

this research project. I should also note the antibodies used in this work were initially generated at Berlex Laboratories.

I would also like to acknowledge the support of Dr. William Robinson and Dr. Jason Song, fine rheumatologists and researchers, who provided the synovial fluid samples and their knowledge of RA.

Last but not least, I am grateful to my family and friends for their constant support and understanding.

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Introduction

Osteopontin is a highly glycosylated serine/threonine phosphoprotein found in plasma and in extracellular matrix. It functions as a pro-inflammatory cytokine at sites of inflammation and is also implicated in bone remodeling (1, 2). OPN is expressed by many different cell types including macrophages, natural killer cells, and activated T lymphocytes (3, 4, 5, 6). Recently several groups have shown that there is local production of OPN in human rheumatoid arthritic joints (7, 8, 9, 10).

Rheumatoid arthritis (RA) is a chronic, debilitating autoimmune inflammatory joint disease with complex pathophysiology. RA is characterized by the infiltration of inflammatory cells into the joint space leading to invasive growth of synovial tissue (pannus) and destruction of cartilage and bone (11). Recently, several independent groups have suggested that osteopontin plays an essential role in RA (7, 12, 8).

Osteopontin contains an Arg-Gly-Asp (RGD) sequence (Fig. 1) that mediates its binding to multiple integrins including $\alpha_v\beta_3$ (the vitronectin receptor) on the surface of osteoclasts. It also contains a CD44-binding domain for bone matrix interactions important in bone remodeling (13). OPN is an inflammatory cytokine that has been shown to induce cell attachment (14) and chemotaxis (15) through RGD-dependent interactions with different integrins including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_8\beta_1$ (1, 2).
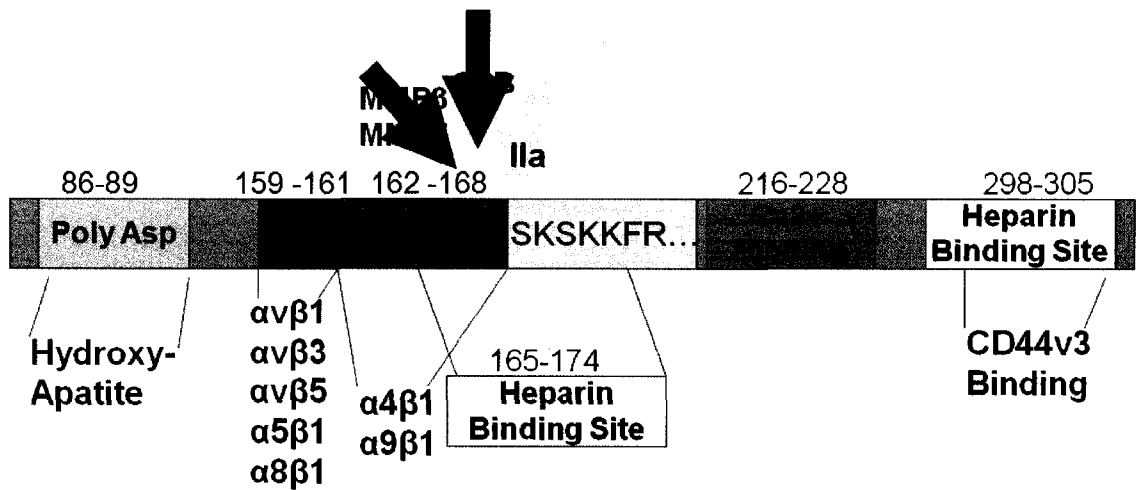
FIGURE 1. Important features of human osteopontin.
Some features of human osteopontin, integrin binding motifs and receptor binding sites are depicted. Thrombin cleavage site and the sequential plasma CPB cleavage sites are indicated by arrows.

OPN also has an integrin-binding motif that is only exposed after its cleavage by thrombin (Fig. 1 & Fig. 2). This cryptic motif is the short peptide sequence SVVYGLR (in human OPN) and SLAYGLR (in mouse OPN) which interacts with $\alpha_4\beta_1$ (16, 17, 18) and $\alpha_9\beta_1$ integrins (19, 20). $\alpha_4\beta_1$ otherwise known as VLA-4 or CD49d / CD29 is expressed on the surface of lymphocytes and smooth muscle cells (16, 17, 18) while $\alpha_9\beta_1$ is expressed on neutrophils, epithelial cells and smooth muscle cells (19, 20, 21). According to both Bayless (17) and Green (18) the interactions of SVVYGLR (located on the C-terminal end of the N-terminal thrombin-cleaved OPN fragment) with $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins are independent of the RGD motif.
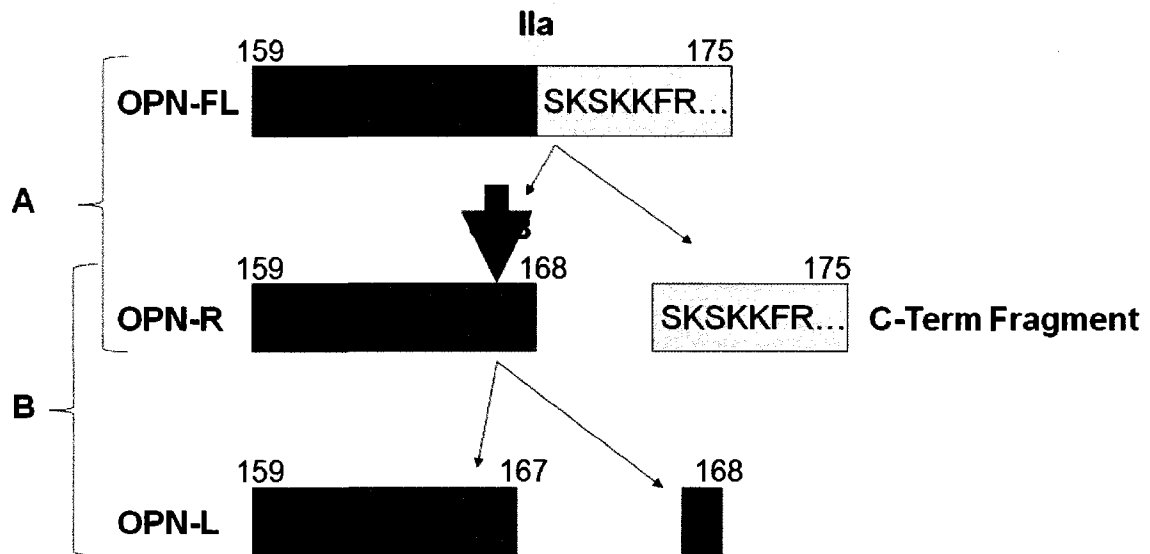
FIGURE 2. Generation of OPN-R and OPN-L by sequential cleavage by thrombin and plasma carboxypeptidase B.
The sequence of full-length human osteopontin near its thrombin cleavage site is depicted. *A*, Treatment of OPN with thrombin (Factor IIa) results in generation of OPN-R and C-terminal fragment. *B*) Plasma carboxypeptidase B (CPB) removes C-terminal arginin from OPN-R to generate OPN-L.

Recently Yamamoto et al. demonstrated that the cryptic epitope of OPN (SLAYGLR) has an essential role in a mouse model of rheumatoid arthritis (12). They successfully demonstrated that a blocking antibody against the exposed cryptic epitope of OPN in RA inhibited pannus proliferation and infiltration of inflammatory cells, in addition to reducing bone erosions *in vivo*. This finding is direct evidence that in the murine model of RA the thrombin-cleaved OPN plays an essential role in progression of disease. The thrombin cleavage of OPN is depicted in Figure 2. The existence of thrombin-cleaved OPN has also been detected in human rheumatoid arthritis synovial fluids by Ohshima et al. using an indirect ELISA method (10). Ohshima et al. compared

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the binding of an antibody that recognizes intact and cleaved OPN to one that recognizes full-length OPN only. Clearly, findings by both Yamamoto et al. and Ohshima et al. suggest that thrombin-cleaved form of OPN is important in both human and murine model of RA.

Plasma carboxypeptidase B (CPB), also called thrombin-activatable fibrinolysis inhibitor or TAFI, can inactivate thrombin-cleaved OPN adhesion to Jurkat T cells by removing the C-terminal arginine from the exposed SVVYGLR epitope (14). Cleavage by CPB converts the thrombin-cleaved OPN (OPN-R) into the inactive des-arg form (OPN-L) (Fig. 2). Both thrombin and CPB are generated locally within the joint space in RA (22). Therefore, one may hypothesize that CPB regulates the inflammatory role of thrombin-cleaved OPN in RA.

In order to investigate the specific inflammatory role of thrombin-cleaved OPN (OPN-R) in RA and its regulation by CPB, I have characterized two antibodies and determined their sensitivity and specificity for their respective antigens, OPN-R and OPN-L. I have also developed specific sandwich ELISA for direct detection of OPN-R and OPN-L from biological fluids, and investigated the levels of OPN-R and OPN-L in rheumatoid arthritis in comparison to osteoarthritis (OA) and psoriatic arthritis (PsA). Furthermore, to explore the functional role of OPN-R in RA, I have demonstrated that fibroblast-like synoviocytes lining the inflamed joints of RA patients, which express α_4 integrin, bind preferentially to OPN-R.

Materials and Methods

Reagents

Human OPN Quantikine ELISA kit was purchased from R&D Systems (Minneapolis, MN). Goat anti-rabbit HRP-labeled antibody conjugate was from Jackson Immunological (West Grove, PA). Heteroblock was purchased from Omega Biologicals (Bozeman, MT). Human thrombin was obtained from Haematologic Technologies (Essex Junction, VA), while CPB was from ACTICHROME CPB activity kit purchased from American Diagnostica (Stamford, CT). The peptides SLAYGL, SVVYGL and SLAYGLR were synthesized by the peptide synthesis facility at Beckman Center, Stanford University School of Medicine. For epitope mapping of antibodies, a series of peptides were designed based on the immunogen sequences with introduction of alanine in place of each amino acid (Table I). The peptides for Anti-OPN-R (CA1999) and anti-OPN-L (CA1998) were synthesized at Elim Biopharmaceuticals Inc (Hayward, CA).

Table I. *List of peptides used to map the epitope for Anti-OPN-R and Anti-OPN-L*

Anti-OPN-R Peptides	Anti-OPN-L Peptides
RLGYALS (random peptide)	RLGYALS (random peptide)
SLAYGLR (Anti-OPN-R immunogen)	SVVYGL (Anti-OPN-L immunogen)
SAAYGLR	SAVYGL
SLAYGLR	SVAYGL
SLAAGLR	SVVAGL
SLAYALR	SVVYAL
SLAYGAR	SVVYGA
SLAYGLA	SVVYGL-**NH2**
SLAYGLR-**NH2**	

To investigate significance of each amino acid within the immunogen sequence, alanine was introduced in place of each amino acid (shown in blod), and affinities of the antibodies for the mutated peptides were determined as discussed in text.

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Antibodies

The antibodies were generated at Berelex Laboratories Incorporated, and were kindly donated to Dr. Lawrence Leung's Laboratory at Stanford School of Medicine, where this research was conducted. Rabbits were immunized with short peptides from the carboxyl termini of human VDTYDRGDSVVYGLR (CA2001) for detection of OPN-FL; human SVVYGLR (CA1997) and mouse SLAYGLR (CA1999) for detection of OPN-R, and carboxyl termini of human SVVYGL (CA1998), mouse SLAYGL (CA2000) for detection of OPN-L (Table II).

Table II. *List of antibodies and their respective immunogens*

Antibody Name	Immunogen	Antigen
Anti-OPN (CA2001)	VDTYDRGDSVVYGLR	Human OPN-FL, OPN-R, OPN-L
Anti-OPN-R (CA1999)	SLAYGLR (Mouse)	Human & Mouse OPN-R
Anti-OPN-L (CA1998)	SVVYGL (Human)	Human OPN-L
CA1997	SVVYGLR (Human)	Failed to recognize OPN-R Specifically

Synovial fluid samples

Archived synovial fluid samples were kindly provided by Dr. Jason J. Song and Dr. William H. Robinson. 26 rheumatoid arthritis, 18 osteoarthritis and 10 psoriatic arthritis samples were analyzed. Samples were stored at -80°C until use.

Human osteopontin purification from milk

OPN was purified from human milk (Mothers Milk Bank, San Jose, CA) according to previously published procedure with minor modifications (17). One liter of human milk pooled from several donors was incubated on ice to allow for separation of the whey from the curd. The whey was then clarified from the curd by centrifuging at 23000 x g for 60 min at 4°C, and filtered through a 0.2μM filter unit. 1mM benzamidine and 2mM DTT were added and the clarified whey was batch adsorbed to Q-sepharose resin (GE Healthcare, Pittsburgh, PA) for 2 h at 4°C. Following batch-adsorption, the resin was washed with 10mM NaPhosphate pH 7.4, 0.2M NaCl, 2mM DTT, 1mM benzamidine and the protein was eluted using 10mM Na Phosphate pH 7.4, 1M NaCl, 2mM DTT, 1mM benzamidine. The eluant was dialyzed in 10mM Na Phosphate pH 7.4, 4M NaCl, 2mM DTT, 1mM benzamidine overnight at 4°C, and loaded onto a 5ml HiTrap Phenyl Sepharose HP column (GE Healthcare, Pittsburgh, PA) at 1ml/min. Then, the column was washed until OD280 plateaued to a baseline level and a linear gradient from 4M to 2M NaCl at 1 ml/min was used to elute the proteins. 1ml fractions were collected and analyzed by SDS-page. Pure fractions were pooled and dialyzed against PBS pH 7.4. Impure fractions were dialyzed against 4M salt and re-applied to the phenyl sepharose column until purified. The purified protein was sent out to Stanford University Beckman Center for mass spectroscopy analysis and was confirmed as osteopontin (Data not shown).

Thrombin-cleaved OPN and thrombin / CPB-cleaved OPN preparation

To generate OPN-R, 10μg of purified milk osteopontin was digested with 100nM of thrombin in presence of 20mM HEPES, 1mM $CaCl_2$, 150mM NaCl, 0.1% PEG_{8000}, pH 7.6 at 37°C. After 2, 5, 10, 20, 30, 40, 50, 60, 75 minutes of incubation with thrombin, the reaction was stopped using 200nM of the thrombin inhibitor, PPACK (D-Phe-Pro-Arg-chloromethyl ketone). 500ng of OPN from each time point was analyzed by SDS-PAGE. 60 minutes was determined to be the ideal time for complete conversion of OPN to OPN-R. To make OPN-L, the thrombin-cleaved OPN-R was treated with 100nM CPB for 1 h at room temperature. Due to thermal instability of CPB, further treatment was not necessary for stopping the reaction. OPN-R and OPN-L were analyzed by SDS-PAGE and Western blot to ensure complete cleavage of milk OPN and the proteins were aliquoted and stored at -80°C for use as a standard in ELISA.

Specificity determination of antibodies to OPN-FL, OPN-R and OPN-L by Western blot

2μg of each of the three forms of osteopontin (prepared as described above), were incubated with 1mM DTT and sample loading buffer (Invitrogen, Carlsbad, CA) at 90°C for 15 min and were vortexed. Three identical 10% Nuvex polyacrylamide gel were prepared. Per gel, 3 identical sets of protein ladder, 100ng of OPN-Fl, OPN-R and OPN-L were run using MOPS running buffer. The proteins were later transferred onto PVDF membranes (BioRad, Hercules, CA). Stained Precision Plus Protein Marker (BioRad, Hercules, CA) was used to ensure complete transfer of proteins to PVDF membrane. The

blots were incubated in blocking buffer consistent of 50mM Tris-HCL, 2mM $CaCL_2$, 80mm NaCl, 5% w/v non-fat milk and 0.2% Niodent P40, pH 8.0. Each membrane was cut into three pieces, so that each piece contained a stained protein ladder, 100ng of OPN-Fl, OPN-R, and OPN-L. Membranes were probed with 1μg/ml of each of the antibodies, Anti-OPN, Anti-OPN-R, and Anti-OPN-L (Table II) respectively for 1 h. Goat anti-rabbit horse radish preoxidase-linked IgG (Jackson Immunological, West Grove, PA) was used as secondary antibody at 1/15000 in blocking buffer for 40 min, and developed using ECL reagents (GE Healthcare, Pittsburgh, PA).

In order to determine the specificity of Anti-OPN-R and Anti-OPN-L antibodirs, after incubation with blocking buffer the two blots were each cut into three identical pieces, each piece containing a protein ladder, 100ng of OPN-Fl, OPN-R and OPN-L. Antibodies were pre-incubated with their cognate and non-cognate peptides (SVVYGL and SLAYGLR) at a molar ratio of 1:50 for 2 h at room temperature before probing the membranes. The first three blots from the same gel were incubated for 1 h with Anti-OPN-R, 1:50 molar ratio of Anti-OPN-R to SLAYGLR (congnate peptide), and 1:50 molar ratio of Anti-OPN-R to SVVYGL (non-cognate peptide) respectively. The second set of blots from the same gel were incubated for 1 h with Anti-OPN-L, 1:50 molar ratio of Anti-OPN-L to SVVYGL (congnate peptide), and 1:50 molar ratio of Anti-OPN-L to SLAYGLR (non-cognate peptide) respectively. After incubation with the primary antibodies blots were treated as described above.

Identical 10% Nuvex polyacrylamide gel with 1μg of each of the three forms of OPN was run along with the Western blot gels and was stained with Bio-Safe Coomassie

G250 Stain (BioRad, Hercules, CA) to not only prove purity of the purified proteins in each lane, but also as positive control for the electrophoresis.

Development of sandwich ELISA for detection of human OPN

To detect OPN-FL from biological samples, a commercial ELISA kit (R&D Systems OPN Quantikine ELISA Kit) was used according to the manufacturer's instructions. For detection of OPN-R and OPN-L, the commercial R&D Systems OPN antibody was used to capture, and Anti-OPN-R and Anti-OPN-L antibodies were used for detection respectively. Various concentrations of the three forms of OPN ranging from 0.625ng/ml to 50ng/ml were incubated in the antibody-coated wells for 2 h at room temperature. Wells were washed three times with wash buffer (PBS, 0.05% Tween-20, pH 7.4) and were incubated with 0.5 μg/ml of the corresponding antibody for 1 h at room temperature. After three washes, goat anti-rabbit HRP-linked antibody was incubated in the wells at 1/15000 dilution for 40 min. Then, wells were washed three times and 100 μl of substrate (TMB; Alpha Diagnostic, San Antonio, TX) was added to the wells for 15 min. The reaction was stopped with 100 μl of stop solution (Alpha Diagnostic, San Antonio, TX) and read at 450 nm.

Epitope mapping of Anti-OPN-R and Anti-OPN-L antibodies

To map the epitopes for Anti-OPN-R and Anti-OPN-L antibodies, the commercial R&D Systems OPN antibody was used as the capture antibody. 10 ng/ml of milk OPN-R and OPN-L were incubated in the antibody-coated wells. Peptides <u>A</u>LAYGLR,

SAAYGLR, SLGYGLR, SLAAGLR, SLAYALR, SLAYGAR, SLAYGLA, SLAYGLR-NH2 , RLGYALS were used to epitope-map Anti-OPN-R antibodies, and SAVYGL, SVAYGL, SVVAGL, SVVYAL, SVVYGA, SVVYGL-NH2, RLGYALS were used for Anti-OPN-L antibodies (Table I). Various molar excesses of peptides to antibodies (ranging from 1:1 to 50:1, peptide to antibody ratio) were pre-incubated for 2 h at room temperature, then were added to the wells and incubated for an additional hour. The plates were then washed three times with wash buffer and 1/15000 dilution of goat anti-rabbit HRP-linked antibody was incubated in the wells for 40 min. Following three washes the plates were developed as described in the previous section.

Detection of OPN-FL, OPN-R and OPN-L from RA, OA, and PsA synovial fluids by ELISA

Synovial joint fluids were thawed on ice and were incubated with 3 µg/ml of HeteroBlock (Omega Biologicals, Bozeman, MT) to reduce the chance of cross-linking between capture and detection antibodies by rheumatoid factor. Following a 10 min centrifugation at 400 x g at 4°C (to clarify the samples), samples were diluted in R&D Systems ELISA kit RD1-6 assay diluent. OPN-FL in synovial fluids was detected using the R&D Systems commercial assay. OPN-R and OPN-L proteins were measured using the sandwich ELISA systems described above. Due to the wide range of OPN levels detected (ranging from 1-2 ng/ml to 50 µg/ml), median OPN levels are reported. Statistical analysis were performed using the Wilcoxon Rank-Sum (Mann-Whitney) test with p values less than 0.05 being considered significant.

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Adhesion assay on preferential binding of fibroblast-like synoviocytes to OPN-R

Primary cultured fibroblast-like synoviocytes from RA joints, kindly provided by Dr. Robinson, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS. Recombinant wild type and RGD-mutated human OPN-FL, OPN-R and OPN-L (Table III), kindly provided by Dr. Leung, were coated onto 96-well micro black fluorescent plates at 500 ng/ml in 0.1M NaHCO$_3$, pH 8.5, and incubated for 2 h at room temperature. Wells were washed with PBS and blocked using 2% BSA, 0.05% Tween 20, PBS pH 7.4 for 1 h. Fibroblast-like synoviocytes were labeled with CFDA cell tracer dye (Invitrogen, Carlsbad, Ca) for 30 min at 37°C and washed twice with PBS. The OPN-coated wells were washed with PBS and 5000 cells were added per well and incubated for 1 h at 37°C. The wells were washed three times with PBS at room temperature, and the plate was read at excitation wavelength of 488 nm and emission of 538 nm using Fluoroskan Ascent (Thermo Scientific, Waltham, MA). Data were recorded as relative fluorescence unit (RFU).

Table III. *List of recombinant osteopontin proteins*

Recombinant proteins	Sequence near thrombin cleavage site
OPN-FL Wild Type	...153 VDTYDG**RGD**SVVYGLRSKSKKFR 175 ...
OPN-R Wild Type	...153 VDTYDG**RGD**SVVYGLR 168
OPN-L Wild Type	...153 VDTYDG**RGD**SVVYGL 167
RAA-OPN-FL	...153 VDTYDG**RAA**SVVYGLRSKSKKFR 175 ...
RAA-OPN-R	...153 VDTYDG**RAA**SVVYGLR 168
RAA-OPN-L	...153 VDTYDG**RAA**SVVYGL 167

Sequence near the thrombin cleavage site is depicted. RGD has been mutated to RAA in some of the proteins as indicated. Vector construction, expression and purification of the proteins were performed by Dr. Timothy Myles in Dr. Leung's Laboratory and have been extensively described in reference 14.

Adhesion assay on preferential binding of fibroblast-like synoviocytes to RGDS and SVVYGLR peptides

Primary cultured fibroblast-like synoviocytes were maintained as described in the previous section. 0, 0.1, 1, 10, 100, 1000 µM RGDS, GRGES, SVVYGLR, and SVVYGL peptides were coated onto 96-well micro black fluorescent plates in 0.1 M NaHCO$_3$, pH 8.5, and incubated for 2 h at RT. Wells were washed with PBS and blocked using PBS, 2% BSA, pH 7.4 for 1 h. Fibroblast-like synoviocytes were labeled with CFDA cell tracer dye (Invitrogen, Carlsbad, Ca) for 30 min at 37°C, and washed twice with PBS. 5000 cells were added per well and incubated for 1h at 37°C. The wells were washed three times with PBS at RT and the plates were read at excitation wavelength of 488 nm and emission of 538 nm.

Results

Thrombin cleavage of milk osteopontin

To determine efficiency of thrombin cleavage of milk osteopontin, a time study was conducted. 10μg of OPN was treated with 100nM of thrombin. At various time points reaction was stopped by 200nM PPACK, and 500ng aliquots of OPN were analyzed by SDS-PAGE (Fig. 3). Analysis of the SDS-PAGE revealed disappearance of the full-length milk OPN (55KD) and appearance of OPN-R (30KD band) and the 25KD C-terminal fragment with increasing time. It is interesting to note that after only 5 minutes of incubation with 100nM thrombin the cleaved products are already visible. The reaction seemed to have reached completion at 50 minutes. Therefore, to generate OPN-R for ELISA calibration curves 1 h was chosen as the ideal incubation time with 100nM of thrombin.

FIGURE 3. Thrombin time-course of osteopontin cleavage
Purified milk osteopontin (10μg) was incubated with 100nM of thrombin at 37°C and at various time points the reaction was stopped by PPACK and 500ng of osteopontin (per lane) was analyzed by SDS-PAGE.
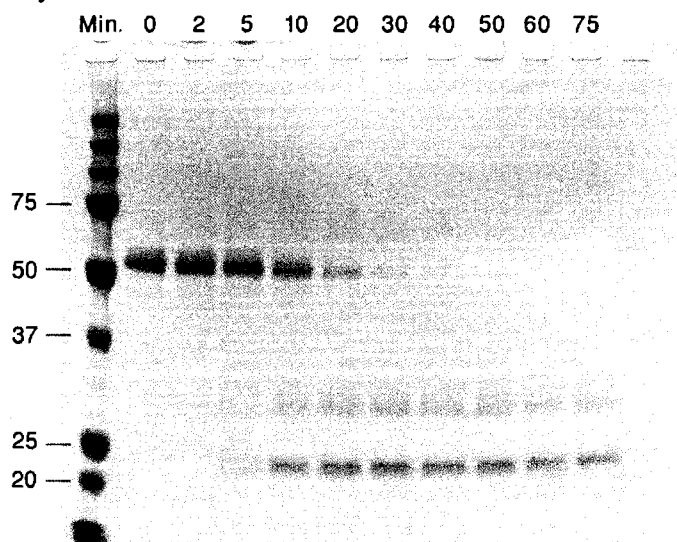
Specificity determination of antibodies Anti-OPN-R and Anti-OPN-L for OPN-R and OPN-L respectively by Western blot

Specificity of antibodies, Anti-OPN-R (CA1999) and Anti-OPN-L (CA1998) were tested by Western blot analysis. Anti-OPN (CA2001) recognized all forms of OPN (Fig. 4*A*). Anti-OPN-L (CA1998) recognized human OPN-L with high degree of specificity (Fig. 4*A right panel*). The antibody raised against the mouse epitope SLAYGLR for detection of OPN-R (CA1999 or Anti-OPN-R) was found to detect both the human and mouse version of OPN-R specifically (Table II). The antibody raised against the human version of the peptide SVVYGLR although recognized OPN-R was not as reactive as the mouse antibody. Therefore, CA1999 was used for detection of human OPN-R and CA1998 was used for detection of OPN-L for the remainder of the study. The specificity of the two antibodies were further confirmed by using the cognate and non-cognate peptides to block the antibody-antigen interactions. The peptide SLAYGLR pre-incubated with Anti-OPN-R prior to probing the membrane, blocked recognition of OPN-R by Anti-OPN-R (Fig. 4*B*). However, pre-incubation of the Anti-OPN-R with the non-cognate peptide at the same molar ratio did not block recognition of OPN-R by the antibody. Similarly, the peptide SVVYGL pre-incubated with Anti-OPN-L blocked recognition of OPN-L by Anti-OPN-L, but pre-incubation of the antibody with the non-cognate SLAYGLR peptide had no effect on Anti-OPN-L recognition of OPN-L (Fig. 4*C*). In this study the focus was on CA1998 (Anti-OPN-L) for detection of human OPN-L and CA1999 (Anti-OPN-R) for detection of human OPN-R.

FIGURE 4. Specificity of antibodies against OPN determined by Western blot analysis.
A, Left panel: SDS-PAGE on different forms of OPN. Milk OPN was treated with nothing (-), thrombin (IIa) or thrombin/CPB (IIa/CPB) to generate OPN-FL (60kD), OPN-R (30KD) and OPN-L (30KD) respectively. *A,Right Panel* from left to right: Western blot analysis of different forms of OPN with CA2001 (for recognition of all forms of OPN), CA1999 (Anti-OPN-R), and CA1998 (Anti-OPN-L). *B*, Specificity of Anti-OPN-R. From left to right: blot incubated with Anti-OPN-R only, 1:50 molar ratio of Anti-OPN-R to cognate peptide, 1:50 molar ratio of Anti-OPN-R to non-cognate peptide. *C*, Specificity of Anti-OPN-L. From left to right: blot incubated with Anti-OPN-R, 1:50 molar ratio of Anti-OPN-L to cognate peptide and 1:50 molar ratio of Anti-OPN-L to non-cognate peptide.

Detection of human OPN by specific sandwich ELISA

The two antibodies, Anti-OPN-R and Anti-OPN-L, were used to develop ELISA for detection of human OPN-R and OPN-L from biological fluids. The different forms of milk OPN (OPN-FL, OPN-R, OPN-L) were used to construct calibration curves. CA2001 antibody although detected the DTT, heat-denatured OPN in Western blot, it was unable to recognize the native form of full-length OPN in the ELISA system. Therefore, R&D Systems ELISA kit was used to measure the OPN-FL levels according to manufacturer instructions. The R&D Systems OPN ELISA kit capture antibody was also used for capture of OPN-R and OPN-L. The kit was very sensitive for detection of OPN-FL, but not for OPN-R and OPN-L (Fig. *5A*). Anti-OPN-R and Anti-OPN-L were much more sensitive and specific for detection of the cleaved forms of OPN. Anti-OPN-R antibodies detected OPN-R, but not OPN-FL or OPN-L (Fig. *5B*). Similarly, Anti-OPN-L antibodies reacted specifically with OPN-L, but not OPN-FL or OPN-R (Fig. *5C*). The ELISA detected as low as 0.6 ng/ml of OPN-R and OPN-L using the antibodies. To ensure that presence of high level of full-length OPN does not affect the detection efficiency of OPN-R and OPN-L, a 20-fold molar excess of intact full-length OPN was incubated with either OPN-R or OPN-L in the wells (Fig. *6A&B*). Such excess did not affect the read-out of the ELISA, signifying that the OPN-R and OPN-L assays are appropriate for measuring the cleaved forms of OPN from blood and other biological fluids in presence of high levels of intact OPN.

FIGURE 5. Development of specific sandwich ELISA for OPN-R and OPN-L
A, R&D Systems OPN kit is specific for OPN-FL, but not OPN-R and OPN-L. *B,* Development of sandwich ELISA specific for OPN-R. *C,* Development of sandwich ELISA specific for OPN-L.
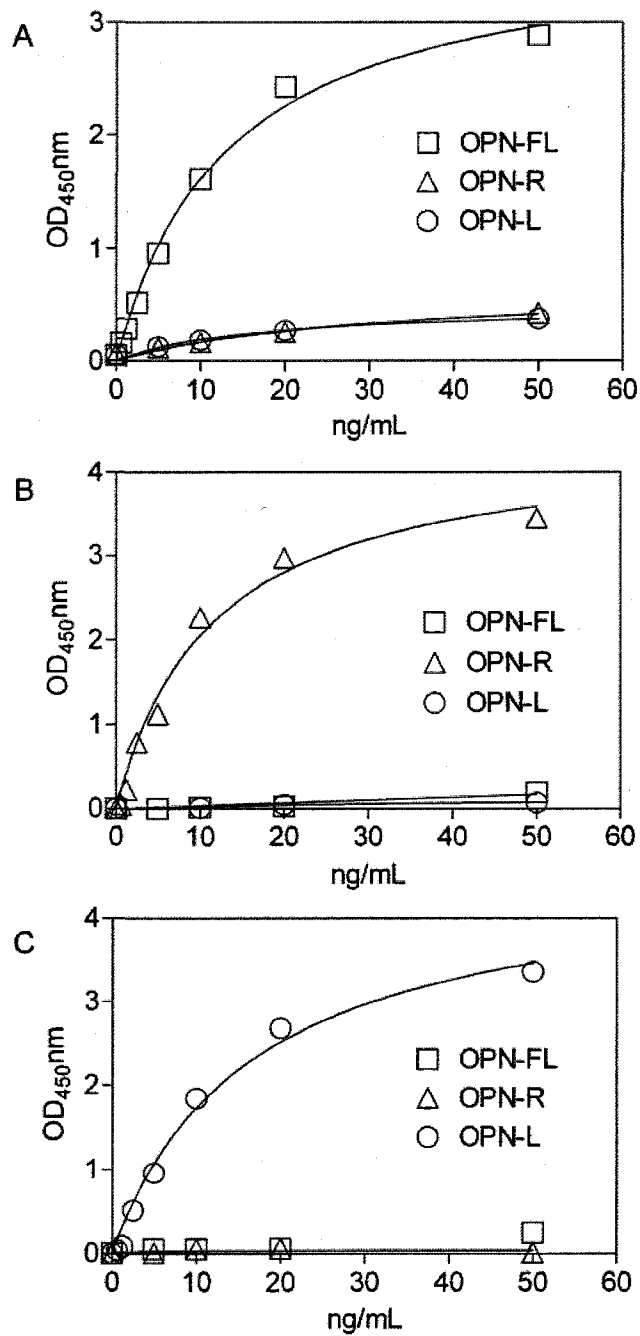
FIGURE 6. OPN-FL does not interfere with OPN-R and OPN-L in sandwich ELISA
A, OPN-FL does not interfere with OPN-R ELISA. 20 fold molar excess of OPN-FL incubated with OPN-R at different concentrations were compared with OPN-R only wells, at 20 fold molar excess. *B*, OPN-FL does not interfere with OPN-L ELISA. 20:1 ratio of OPN-FL to OPN-L vs. OPN-L only wells, showed non-interference of OPN-FL
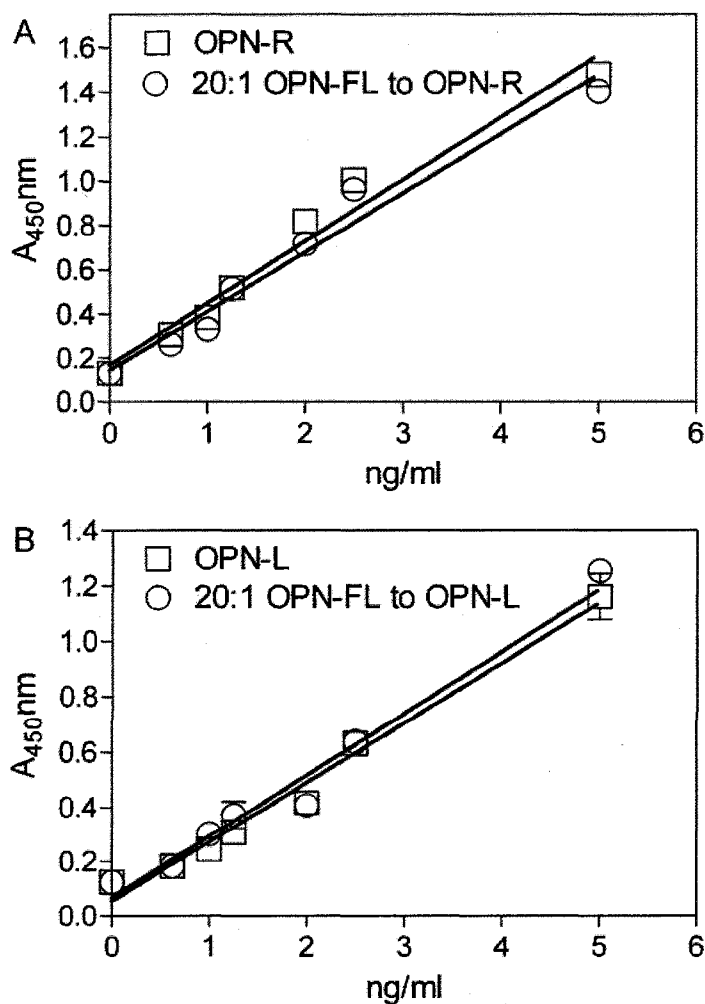
Epitope mapping of Anti-OPN-R and Anti-OPN-L antibodies

To identify the exact epitopes of OPN-R and OPN-L recognized by Anti-OPN-R and Anti-OPN-L antibodies respectively, a series of peptides were synthesized based on the sequence of peptides used as immunogens for generation of the antibodies (Table II).

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The cognate peptide for Anti-OPN-R was the mouse sequence SLAYGLR, and the peptide used as the immunogen for generation of Anti-OPN-L was SVVYGL. In order to identify which amino acids from the peptides are important for interaction with the respective antibodies, alanine was introduced in place of each of the amino acids in the synthesized peptides (Table I). The peptides were used as competitive inhibitors of the antibodies binding to OPN-R and OPN-L in the sandwich ELISA systems. The competitive inhibition assay with all peptides were done with increasing molar ratio of peptides to antibodies, but the dose dependent-curves are only depicted for the cognate and the non-cognate peptides (Fig. 7A&C). The rest of the peptide blocking experiments are only shown at 50:1 molar ratio of peptides to antibodies for convenience (Fig. 7B&D).

For Anti-OPN-R antibodies, pre-incubation of the antibodies with increasing molar excess of the peptides SLAYGLR and SVVYGLR inhibited the binding of the antibodies to OPN-R in a dose-dependent manner (Fig. 7A). However, the peptides SLAYGL and SVVYGL did not interfere with binding of Anti-OPN-R antibodies to OPN-R coated plate, confirming the C-terminal arginine in the thrombin-cleaved OPN is essential for recognition of OPN-R by Anti-OPN-R. The peptides ALAYGLR, SAAYGLR, SLGYGLR effectively inhibited the binding of Anti-OPN-R, indicating that serine, leucine, and alanine residues of SLAYGLR are not essential for Anti-OPN-R recognition (Fig. 7B). On the other hand, substitution of alanine for tyrosine, second leucine, arginine, and glycine rendered the peptides SLAAGLR, SLAYGAR, SLAYGLA, and to some degree SLAYALR ineffective in inhibiting the binding of Anti-

OPN-R antibodies to OPN-R. This means tyrosine, second leucine, arginine, and to some degree glycine in SLA**YGLR** are involved in defining the epitope for Anti-OPN-R. The epitope YGLR is shared by both human and mouse version of thrombin-cleaved OPN, which explains why Anti-OPN-R (raised against the mouse epitope SLAYGLR, and not the human epitope SVVYGLR), effectively recognizes both human and mouse OPN-R. In addition, substituting the C-terminal carboxyl group of arginine by an amide group diminished inhibitive effect of the peptide, indicating that the carboxyl group is also essential for recognition by Anti-OPN-R.

For Anti-OPN-L antibodies, the cognate peptide SVVYGL effectively blocked antibody binding in a dose-dependent manner while the peptide sequence SVVYGLR was completely ineffective (Fig. 7*C*). Furthermore, the blocking studies indicated tyrosine, glycine, leucine, the C-terminal carboxyl group, and to a lesser extend the first valine in the peptide sequence S**V**V**YGL** are essential in defining the epitope for Anti-OPN-L (Fig. 7*D*). The blocking studies showed Anti-OPN-R and Anti-OPN-L essentially recognize three to four residues, with the C-terminal residues arginine or leucine and their carboxylates being most important for recognition by the specific antibodies.

FIGURE 7. Epitope mapping of Anti-OPN-R and Anti-OPN-L antibodies by competitive ELISA

A & B, Epitope mapping of Anti-OPN-R antibodies by various peptides. *A*, Anti-OPN-R antibodies recongnition of OPN-R were blocked by pre-incubation of antibodies with increasing concentrations of cognate peptides (SLAYGLR and SVVYGLR). *B*, Anti-OPN-R inhibition by various peptides at 50:1 molar excess of peptides to antibodies signifies the bold underlined epitopes in SLA**YGLR** are important for recognition of OPN-R by Anti-OPN-R. *C*, Anti-OPN-L antibodies' recongnition of OPN-L were blocked by pre-incubation of antibodies with increasing concentration of cognate peptide (SVVYGL). *D*, Anti-OPN-L inhibition by various peptides at 50:1 molar excess of peptides to antibodies signifies the bold underlined epitopes in S**VVYGL** are important for recognition of OPN-L by Anti-OPN-L.
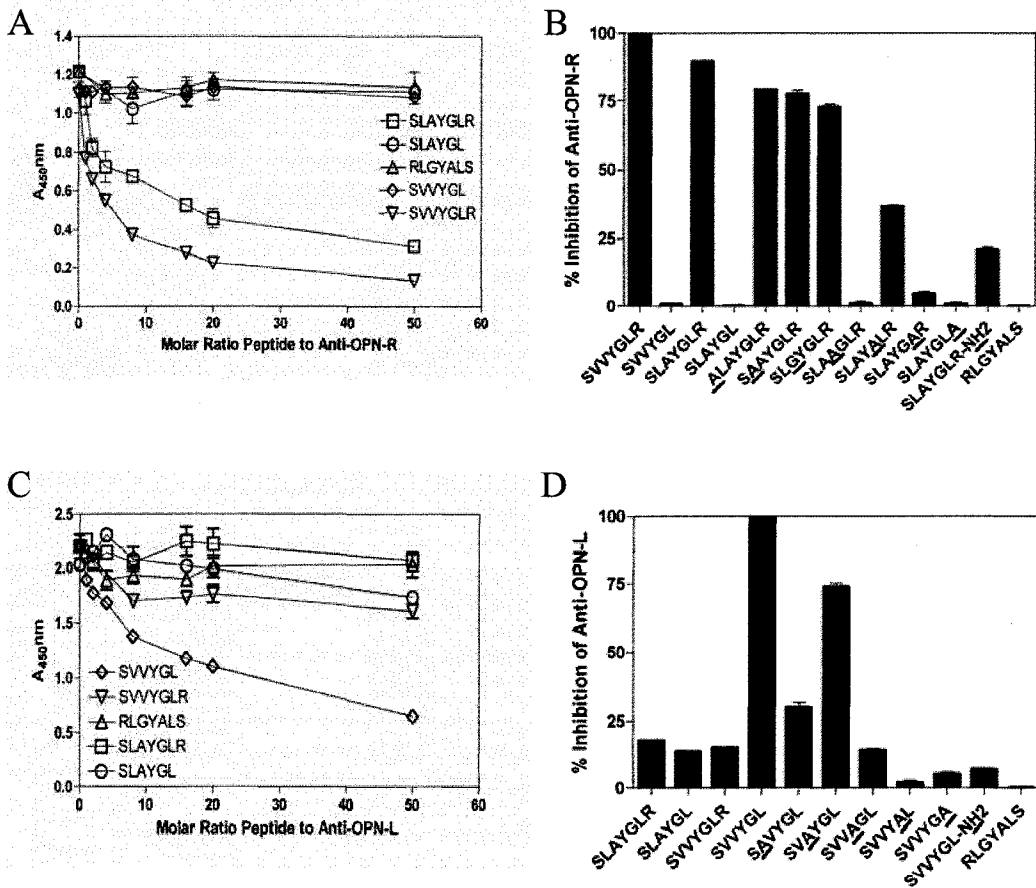
Determination of OPN-Fl, OPN-R and OPN-L levels from synovial fluids of RA,

OA, and PsA patients by ELISA

Previous studies have reported increased OPN levels in synovial fluids from RA

patients (8, 10). To test whether cleaved forms of OPN are also detectable in the synovial

fluid samples of RA patients, ELISA systems were developed for detection of cleaved

forms of OPN. The commercial R&D Systems ELISA kit was used for detection of

OPN-FL. For comparison of RA synovial fluid OPN levels, synovial fluid samples from

OA and PsA were used since synovial fluid from healthy joints cannot be obtained. The

Wilcoxon rank sum test was used to compare the median values of each OPN form

between the three patient groups. In rheumatoid arthritis patients synovial fluids OPN-

FL (352.5 ng/ml) was significantly elevated compared to their normal plasma level (50

ng/ml), however, this level did not reach significance when compared to the OPN-FL

level from OA (157.9 ng/ml, p-value 0.142) and PsA (143.4 ng/ml, *p* value 0.074)

synovial fluids (Fig. 8*A*). On the other hand, OPN-R and OPN-L levels were

significantly higher in RA synovial fluids when compared to OA and PsA. The median

RA OPN-R level was 138.6 ng/ml compared to a 10.6 ng/ml for OA and 2.2 ng/ml for

PsA (*p* value < 0.001), and the median OPN-L level of RA was 205.3 ng/ml compared to

25.9 ng/ml for OA and undetectable for PsA (*p* value < 0.006) (Fig. 8*A*). There was a

wide range of full-length and cleaved OPN levels in the synovial fluids of RA patients

compared to OA and PsA, ranging from undetectable to more than 50 µg/ml. Such

heterogeneity in OPN levels may be due to differences in stage of the disease, the

particular treatment in individual patients, or other factors. The median ratio of cleaved

OPN to total OPN in rheumatoid arthritis synovial fluid was 0.5, indicating that a

significant amount of OPN exists in its cleaved forms within the rheumatic joint space

compared to the OA and PsA (Fig. 8*B*).

FIGURE 8. Comparison of OPN-FL, OPN-R and OPN-L from OA, PsA, and RA synovial fluids by ELISA.
A, Comparison of OPN levels in Osteoarthritis (OA) n=18, psoriatic arthritis (PsA) n=10, rheumatoid arthritis (RA) n=26. Bars indicate median values of each disease group. *p value < 0.006 by Wilcoxon Ranked Sum test. *B*, Significantly higher cleaved OPNs detected in RA patients' synovial fluids. The ratio of cleaved OPNs (OPN-R plus OPN-L) to total OPN (sum of the three different OPN forms) for OA, PsA, and RA are depicted. * p value < 0.003.
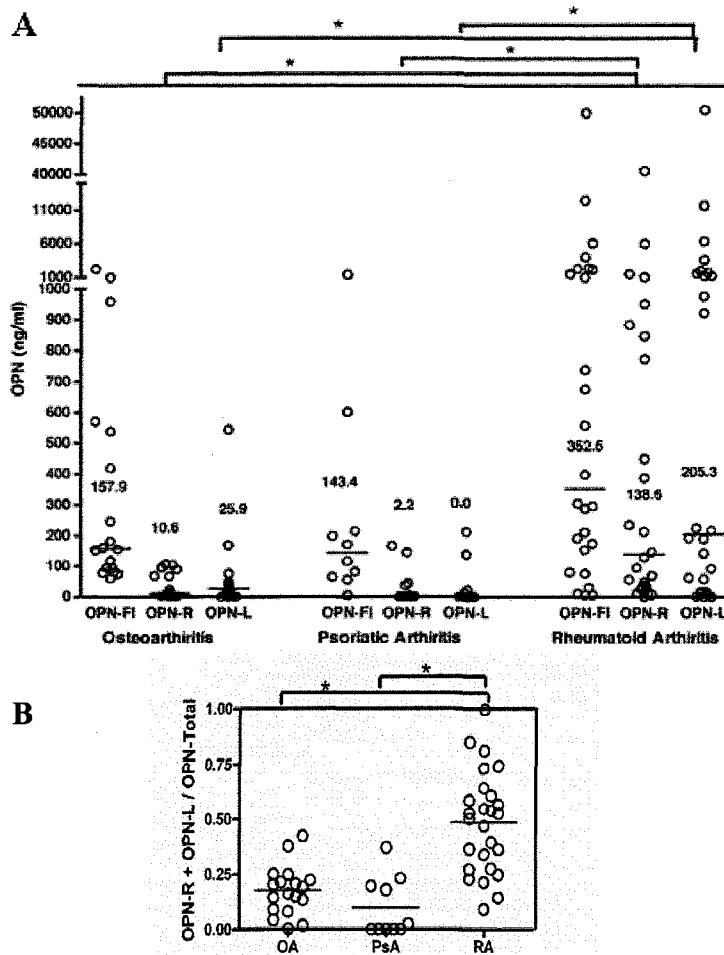
Determination of preferential binding of fibroblast-like synoviocytes to OPN-R

To investigate the role of thrombin-cleaved OPN in RA, adhesion of arthritic fibroblast-like synoviocytes to different forms of osteopontin was studied. Previously described wild type and RGD-mutated recombinant OPN-FL, OPN-R, and OPN-L (14) were coated onto a 96-well plate. In the RGD-mutated forms of OPN, the RGD site was mutated into RAA in order to distinguish RGD-dependent adhesions (through integrins such as $\alpha_v\beta_3$) from RGD-independent adhesions. A significant two-fold increase in FLS adhesion was seen with wild type OPN-R compared to wild type OPN-FL and OPN-L, suggesting the exposure of the cryptic integrin-binding motif SVVYGLR in OPN-R to be the reason for the enhanced binding (Fig. 9A). However, in the case of both OPN-Fl (where the SVVYGLR is sterically-hindered) and OPN-L (where the C-terminal arginine has been cleaved off by CPB) the enhanced binding of FLS to OPN is significantly diminished. Lack of specific binding to RGD-mutated (RAA) versions of the three forms of OPN in comparison with the wild type OPNs suggests that RGD is essential for the initial binding of FLS to OPNs. In the absence of the RGD sequence, SVVYGLR alone was unable to support binding to $\alpha4$ integrin subunits (RAA-OPN-R), perhaps due to lack of conformational integrity. The magnitude of cell binding was clearly enhanced when both RGD and SVVYGLR were present.

To further investigate whether RGD is essential for adhesion of FLS, various amounts of RGDS peptide (ranging from 0.1 to 1000 µM) were coated onto 96-well plates and FLS adhesion to RGDS peptide-coated wells were compared to adhesion to GRGES peptide (Fig. 9B). As expected, FLS adhered to RGDS peptide in a dose-

dependent manner. Similarly, when SVVYGLR and SVVYGL peptides were coated onto the wells, FLS adhered to SVVYGLR peptide, but not SVVYGL in a dose-dependent manner (Fig. 9C).

FIGURE 9. Preferential adhesion of fibroblast-Like synoviocytes (FLS) to thrombin-cleaved OPN (OPN-R)

A, Fluorescently labeled fibroblast-like synoviocytes adhesion to WT OPN-Fl, OPN-R, OPN-L, RGD mutated OPNs (indicated as RAA-FL, RAA-R, and RAA-L), and BSA coated wells. Data representative of four experiments with n = 8. *B,* Fluorescently labeled FLS adhesion to RGDS ($\alpha_v\beta_3$ integrin binding site) coated wells in a dose-dependent manner. *C,* FLS adhesion to SVVYGLR ($\alpha_4\beta_1$ integrin binding site) coated wells in a dose-dependent manner. *B & C,* Data representative of two experiments with n=6.

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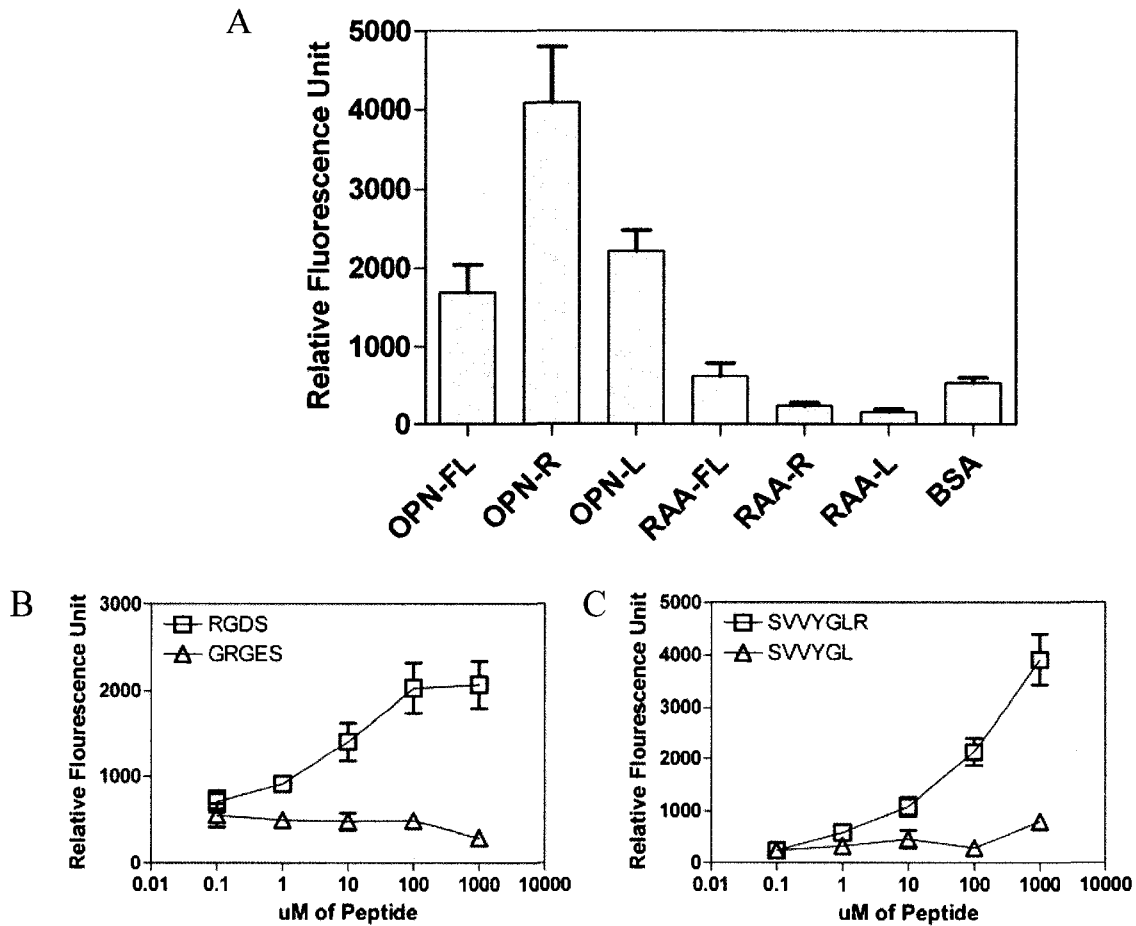
Discussion

The hypothesis that prompted this study was the premise that thrombin cleavage of OPN plays a role in the pathophysiology of RA, and that cleavage of OPN-R by CPB may down regulate the role OPN-R plays in rheumatoid arthritis. Recent studies have reported increased OPN levels in the RA synovial fluids (9, 10). Ohshima et al. also reported presence of cleaved forms of OPN in RA synovial fluids using an indirect ELISA method (10). They compared binding of an antibody that recognizes both intact and cleaved forms of OPN to one which recognizes intact OPN only, in order to deduce the level of thrombin-cleaved OPN is high in RA compared to osteoarthritis. On the contrary, the OPN-R and OPN-L ELISA described in this study directly detect the thrombin-cleaved and the thrombin / CPB-cleaved osteopontin in biological fluids and can distinguish these forms from the MMP3 and MMP7-cleaved products.

Extensive characterization of the two antibodies, Anti-OPN-R and Anti-OPN-L, by Western blot (Fig. 4) and ELISA (Fig. 5 and Fig. 6) is indicative of the specificity of the antibodies for their respective antigens. Epitope mapping of OPN-R antibodies revealed that Anti-OPN-R, which was raised against SLA<u>YGLR</u> peptide, specifically binds to the four amino acids YGLR. On the other hand, Anti-OPN-L raised against SVVYGL recognizes the first valine and the YGL sequence. The fact that the C-terminal residues N-terminal to the thrombin / CPB cleavage site, namely leucine (in the case of Anti-OPN-L) and arginine (in the case of Anti-OPN-R) and their carboxyl groups are essential for antibody recognition (Fig. 7), confirm that the antibodies would not recognize MMP3 and MMP9-cleaved OPN. Since the two metalloproteinases are known

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to cleave after glycine within the SVVYGLR sequence, cleavage with these metalloproteinases essentially destroys the Anti-OPN-R and Anti-OPN-L recognition sites. It should be noted, even though Anti-OPN-R and Anti-OPN-L specifically recognize the thrombin-cleaved and thrombin / CPB-cleaved OPN, they cannot distinguish whether these cleaved forms of OPN have been further cleaved by other proteases.

Once specificity of the antibodies were determined, they were used to investigate whether OPN-R and OPN-L could be detected from the RA synovial fluids. Synovial fluid OPN has not been characterized, but it is known that the protein is highly modified post-translationally. Therefore, it could be speculated that the glycosylation and phosphorylation patterns of OPN found in synovial fluid would be different from milk OPN and could possibly contribute to differential affinities of the antibodies for the two forms. Therefore, the initial attempt was to analyze the synovial fluid OPN by Western blot. However, due to the presence of high concentrations of albumin, immunoglobulins and rheumatoid factor in the synovial fluid samples which non-specifically reacted with the antibodies, Western blot analysis of synovial fluid OPN failed. Therefore, sandwich ELISA was used as the method of choice, to minimize non-specific interactions. In this way only osteopontin would bind to the Anti-OPN capture antibody on the plate and all else would be washed away, therefore, the chance of cross-reactivity with antibodies were minimized. However, in a Western blot setting where whole synovial fluid is run on a gel and all proteins are transferred onto a blot the chance of non-specific interactions are considerably higher.

Using the specific OPN-R and OPN-L ELISA, levels of both OPN-R and OPN-L were found to be significantly elevated within the rheumatic joints compared to their levels in OA and PsA joints, supporting the notion that thrombin cleavage of OPN and the subsequent cleavage of OPN-R into OPN-L by CPB occur within the rheumatic joints.

There was a wide range of full-length and cleaved OPN levels in the synovial fluids of RA patients compared to OA and PsA, ranging from undetectable to more than 50 μg/ml (Fig. 8*A*). Such wide distribution of OPN level may be partly attributed to stage of disease, its severity, and the particular treatment regimen at time of synovial fluid collection. Therefore, further studies of the inflammatory state of the patients are necessary to understand the heterogeneity of OPN levels found in RA.

Contrary to previous published results (10), in this study the median OPN-FL level of RA synovial fluids was not significantly higher compared to OA and PsA (Fig. 8*A*). This finding may be partly explained by the small sample size, or by variations in the inflammatory stage of disease and treatment regimens of individual patients at time of fluid collection.

The ratio of cleaved OPNs (OPN-R and OPN-L) to OPN-FL in RA synovial fluid samples had a median value of 0.5, suggesting that almost half of the OPN within the rheumatic joint space has been cleaved into OPN-R and OPN-L (Fig. 8*B*). On the contrary, the ratio of cleaved OPNs to OPN-FL in OA and PsA was significantly lower. This finding is suggestive that the cleaved forms of OPN may play important roles in the pathogenesis of RA, but not OA and PsA.

Once it was established that cleaved OPNs are present at high levels in at least a subset of RA synovial fluids, the biological relevance of this finding was explored using cells from RA joints. The primary fibroblast-like synoviocytes (FLS) within the joint space express $\alpha4$ (CD49d) on their surface (23). On the other hand, thrombin-exposed C-terminal sequence SVVYGLR of OPN-R binds to the $\alpha4$ subunit of $\alpha4\beta1$ (18). Furthermore, data on integrin profile of fibroblast-like synoviocytes showed there is a 3-4 fold increased expression of $\alpha4$ in rheumatoid arthritic synovium compared to normal synovium (23). This information along with the finding that FLS binds OPN-R preferentially when compared to OPN-FL and the doubly-cleaved OPN-L (Fig. 9*A*), suggest thrombin-cleaved form of osteopontin may be biologically active in rheumatoid arthritis. While OPN-R was able to support substantial cell binding, adhesion to OPN-L was reduced to levels seen with OPN-FL, suggesting that *in vivo* CPB conversion of OPN-R into OPN-L, may possibly serve as a regulator of integrin-mediated cell adhesion to OPN-R. Although the present study is suggestive that OPN-R has a possible role in the pathogenesis of RA, further studies are needed to investigate the specific nature of the interaction of OPN-R with the fibroblast-like synoviocytes within the joint space and the role OPN-R may play in the progression of rheumatoid arthritis. In addition, the cleaved OPN ELISA systems developed for this study may serve as an effective tool for studying thrombin-cleaved OPN and thrombin / CPB-cleaved OPN levels in different inflammatory conditions.

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