Journal of Cellular Biochemistry 73:20–30 (1999)

CD44 Is Not an Adhesive Receptor for Osteopontin

Laura L. Smith,[1] Brad W. Greenfield,[2] Alejandro Aruffo,[2] and Cecilia M. Giachelli[1]*

[1]Department of Pathology, University of Washington, Seattle, Washington 98195
[2]Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543

Abstract Osteopontin is a secreted glycoprotein with adhesive and migratory functions. Cellular interactions with osteopontin are mediated through integrin receptors which recognize the RGD domain. Recently, CD44, a non-integrin, multifunctional adhesion molecule was identified as an osteopontin receptor. CD44 is a ubiquitous surface molecule that exists as a number of different isoforms, generated by alternative splicing. To analyze which forms of CD44 mediate binding to osteopontin, we used the standard form of CD44 as CD44-human immunoglobulin fusion proteins and several splice variants in enzyme-linked immunosorbant assays. Multiple preparations of osteopontin were used including native osteopontin derived from smooth muscle cells, human urinary osteopontin, full-length recombinant osteopontin, and two recombinant osteopontin fragments expected to be formed following thrombin cleavage. Our data show that although the CD44-hIg fusion proteins could interact with hyaluronic acid as expected, there was no interaction between CD44H, CD44E, CD44v3,v8-v10, or CD44v3 with osteopontin. These studies suggest that CD44-osteopontin interactions may not be common *in vivo* and may be limited to a specific CD44 isoform(s), and/or a particular modified form of osteopontin. J. Cell. Biochem. 73:20–30, 1999.

Key words: adhesion; integrin; RGD; CD44 splice variants; CD44-hIg

Osteopontin (OPN) is a multifunctional secreted glycoprotein implicated in a number of diseases [Denhardt and Guo, 1993; Giachelli et al., 1993, 1994, 1995b, 1997; Murry et al., 1994; O'Brien et al., 1995; Oates et al., 1997]. Some of the potential functions of osteopontin include regulation of nitric oxide, bone mineralization, and dystrophic calcification. Osteopontin also may act as a cytokine and is thought to play a role in malignancy, tissue injury, wound healing, and bacterial infections [Butler et al., 1996; Weber, 1996; Denhardt and Chambers, 1994; Denhardt et al., 1995; Giachelli et al., 1995b; Hwang et al., 1994; Liaw et al., 1998; Oates et al., 1997; Patarca et al., 1993]. In vitro, osteopontin has been shown to be an adhesive protein and a migratory stimulus for a variety of different cell types [D'Errico et al., 1995; Flores et al., 1996; Hu et al., 1995; Smith et al., 1996; Xuan et al., 1995] including smooth muscle cells (SMC) and endothelial cells [Liaw et al., 1994, 1995b; Yue et al., 1994]. Liaw et al. has shown that the interaction of these cell types with OPN is mediated through the $\alpha_v\beta_3$, $\alpha_v\beta_1$, and $\alpha_v\beta_5$ integrin receptors in an RGD-dependent manner [Liaw et al., 1995b]. In addition to α_v-containing integrins, $\alpha_4\beta_1$ was shown to mediate adhesion of a macrophage cell line [Nasu et al., 1995], and the $\alpha_9\beta_1$ integrin was shown to interact with the N-terminal fragment of osteopontin following thrombin cleavage [Smith et al., 1996].

More recently, Weber et al. identified osteopontin as a ligand for CD44, a non-integrin, cell surface glycoprotein [Weber et al., 1996]. CD44 is expressed on a broad range of normal and malignant tissues [Hofmann et al., 1991; Matsumura and Tarin, 1992]. It plays a role in cell adhesion, cell migration, lymphocyte activation, lymphocyte homing, and cancer metastasis [Aruffo et al., 1990; Gunthert et al., 1991; Jalkanen et al., 1986; Lesley et al., 1993; Taher et al., 1996]. The primary ligand for CD44 is hyaluronic acid (HA) [Aruffo et al., 1990; Culty et al., 1990; Miyake et al., 1990], however it has also been shown to interact with fibronectin, collagen, and heparin-binding growth factors [Bennett et al., 1995a; Jalkanen and Jalkanen, 1992; Tyrrell et al., 1993]. The multifunctional

Contract grant sponsor: NIH; Contract grant numbers: HL-18645 and 5T32 GM07270–20; Contract grant sponsor: NSF; Contract grant number: UWEB ERC EEC9529161.

*Correspondence to: Cecilia Giachelli, Ph.D., Pathology Department, University of Washington, Vascular Biology, Box 357335, Seattle WA 98195. E-mail: ceci@u.washington.edu

Received 4 May 1998; Accepted 2 October 1998

property of CD44 is probably due to the many different variant isoforms that exist. Human CD44 contains 20 exons and at least 12 exons can be alternatively spliced [Screaton et al., 1992]. The most abundant isoform is the hematopoietic variant (CD44H or CD44s) which is widely distributed [Stamenkovic et al., 1989]. This 85–95 kD variant lacks all the variable exons. Larger variant forms are generated by alternative splicing (CD44v), and some of these variants seem to correlate with invasive and metastatic capacity of tumor cells in vivo [Gunthert, 1996; Gunthert et al., 1991; Koopman et al., 1993; Matsumura and Tarin, 1992; Tanabe et al., 1993; Terpe et al., 1994]. In addition to alternative splicing, gagosylation can also lead to variants containing chondroitin sulphate and heparin sulfate [Cooper and Dougherty, 1995; Freed et al., 1989; Jackson et al., 1995; Labarriere et al., 1994].

CD44-osteopontin interactions could potentially be very important in a number of systems including inflammation, tumorogenesis, and tissue injury. For example, both osteopontin and certain CD44 variants seem to be correlated with metastases [Gunthert, 1996; Oates et al., 1997]. The secretion and binding of OPN to CD44 receptors could trigger signals for invasiveness or promote migration of CD44 positive tumor cells.

We are particularly interested in the potential role of OPN interactions with CD44 in vascular disease because both molecules are expressed in injured vascular tissue [Giachelli et al., 1993, 1995a,b; Jain et al., 1996] and in angiogenic vessels [Giachelli et al., 1993; Griffioen et al., 1997]. In addition, both molecules have been implicated in adhesive and migratory functions; two processes important for vascular disease.

Although the potential interaction of vascular smooth muscle cell (SMC) CD44 with OPN has not been investigated, our own studies have demonstrated that SMCs, which express high levels of CD44 [Jain et al., 1996], do not interact with osteopontin through the CD44 receptor. Attachment and migration of SMCs to OPN can be blocked with $\alpha_v\beta_3$-integrin neutralizing antibodies and RGD peptides suggesting that this interaction is mediated through integrins [Liaw et al., 1994, 1995b]. Moreover, adhesion of SMC to OPN does not take place in the absence of cations [Liaw et al., 1995b]; a requirement for integrin interactions, but not CD44 interactions. The lack of CD44-mediated attachment of SMC to OPN may not be all that surprising since there are multiple CD44 isoforms that are differentially expressed on various cell types. It is possible that only particular splice variant(s) of CD44, not expressed on vascular SMCs can interact with OPN.

This study was undertaken to determine if osteopontin is a ligand for the standard CD44 receptor and some of the other CD44 variants thought to be upregulated during disease. We used the standard form of CD44-immunoglobulin (CD44-hIg) fusion proteins and several CD44 splice variants in enzyme-linked immunosorbant assays (ELISA) to examine the CD44-osteopontin interactions. We found that although the CD44-hIg proteins could interact with HA as expected, there was no interaction between CD44H, CD44E, CD44v3, v8-v10, or CD44v3 with osteopontin.

MATERIALS AND METHODS

Cell Lines

COS cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Gaithersburg, MD) with 7% FBS, penicillin (100 u/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine. WEHI-3B cells were provided to us by Dr. Alan Sartorelli (New Haven, CT) and were maintained in DMEM containing 10% fetal calf serum.

Construction of CD44-Ig Expression Vectors

CD44-hIg constructs hCD44H-hIg and hCD44H-R41A-hIg mutant were previously described [Peach et al., 1993]. CD44-hIg constructs hCD44E-hIg and hCD44 V3,V8-V10-hIg were also previously described [Bennett et al., 1995b]. CD44 exon V3 construct was generated by PCR using CD44 V3-FP-SpeI: ACTAGTACGTCTTCAAATACCATCTCAG and CD44 V3-RP-BamHI: GGGATCCAGGGTGCTGGAGATAAAATCTTC. PCR reaction conditions were as follows: 94°C for 5 min, with 35 cycles of 94°C for 30 sec, 57°C for 1 min, and 72°C for 1 min and 45 sec. PCR products were purified with Qiaquick spin PCR purification kit (Qiagen Corp., Santa Clarita, CA). Purified products were digested with restriction enzymes SpeI and BamHI (Boehringer Mannheim Corp., Indianapolis, IN), gel purified and ligated into

SpeI/BamHI cut vector CDM7B- with CD5 signal sequence 5′ and human Ig 3′ of CD44 insert as described [Bennett et al., 1995a]. All DNA constructs were sequenced to verify correct inserts.

CD44-hIg Fusion Protein Expression

Transient expression of fusion proteins in COS cells was previously described [Aruffo et al., 1990]. Briefly, semi-confluent COS cells were transfected with constructs using DEAE dextran. Cells were trypsinized after 12 h, seeded onto fresh plates, and grown for an additional 7–10 days. Fusion protein supernatants were harvested and purified over protein-A sepharose column, eluted in 4 M imidizole with 1 mM each $MgCl_2$ and $CaCl_2$. Eluted protein was dialyzed extensively in 1 × PBS. Protein concentrations were determined using Pierce BCA Assay as described by manufacturer (Pierce, Rockford, IL). The molecular weights of the proteins were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The proteins migrated as follows: CD44v3,8–10 (150–200 kD), CD44v3 (46–108 kD), CD44H (80–90 kD), and CD44E (120–150 kD). Variability in molecular weights is due to the heterogeneity of glycosylation on these proteins.

Osteopontin

Full-length recombinant osteopontin was generated as a histidine-tagged protein (his-OPN) as previously described [Smith et al., 1996]. Briefly, the full-length splice variant of human osteopontin (OP10) [Young et al., 1990] was cloned into pQE30 vector (Qiagen, Chatsworth, CA). *Escherichia coli* transformed with the his-OPN plasmid was grown in LB with 100 µg/ml ampicillin and induced with isopropyl-1-thio-β-D-galactopyranoside for 4 h. The His-OPN protein was purified from the bacterial cells according to the manufacturer's instructions. (QIAexpressionist kit, Qiagen). The molecular weight for his-OPN is 36 kD.

Recombinant osteopontin N-terminal and C-terminal fragments were generated by thrombin cleavage of GST-30N, GST-10N, and GST-10C fusion proteins as described previously [Smith et al., 1996]. The recombinant fragments were separated from the GST by cleaving with biotinylated-thrombin (Novagen, Madison, WI; 10 units/mg protein) at room temperature for 2 h. Biotinylated-thrombin was then removed with UltraLink Immobilized NeutraAvidin (Pierce) and separated from the protein by centrifugation. Protein determinations were done using the Micro BCA Protein Assay Reagent Kit (Pierce). The molecular weights for these proteins are 20 kD for 30N, 19 kD for 10N, and 18 kD for 10C.

Native osteopontin derived from the conditioned medium of aortic smooth muscle cell cultures and human urinary osteopontin were purified as previously described [Liaw et al., 1994]. Briefly, human urine or conditioned media were dialyzed against PBS and fractionated over a DEAE-sepharose column with a linear salt gradient (0.15 M–1 M NaCl). Fractions containing osteopontin were adsorbed to barium citrate and eluted with 0.2 mol/L sodium citrate The protein was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and it migrated at 70 kd in a 12.5% gel.

Osteopontin Antibody

Anti-human osteopontin antibody (OP189) was produced in a goat immunized with 50 µg human urinary osteopontin. The osteopontin was purified as described above and mixed with Freund's adjuvant. Two booster injections, 50 µg each, in incomplete Freund's adjuvant, were given 3 weeks apart after the initial immunization. Thirteen weeks after the initial immunization, serum was collected and the IgGs were purified by caprylic acid precipitation and sepharose column chromatography [Russo et al., 1983]. The antibody was then dialyzed and stored frozen. Specificity was tested by western blot analysis (not shown) and enzyme-linked immunosorbant assay (see below). Immunocytochemistry with 189 antibody, stains human and primate kidney and cross reacts weakly with rat kidney.

HA and Osteopontin Binding Assay

The ability of CD44-Ig fusion proteins to bind hyaluronic acid and osteopontin was analyzed by ELISA. Maxisorp microtiter plates (Nunc Inc., Naperville, IL) were coated overnight at room temperature with human umbilical cord hyaluronic acid (Sigma) in 50 mM sodium bicarbonate buffer (pH 9.6) at a concentration of 10 µg/ml or the indicated amount of osteopontin in PBS. Previous results show that under these conditions, the coating efficiency of OPN is 38% [Liaw et al., 1994] and that the OPN coated in this fashion can support adhesion of a variety of cell types [Liaw et al., 1994, 1995a; Smith et al.,

1996]. Wells were washed three times with PBS containing 0.05% Tween-20 and blocked with 1× Specimen Diluent (Genetic Systems, Redmond, WA) for 1–2 h at room temperature. Wells were then washed and incubated with CD44-Ig fusion proteins at various concentrations for 1 h room temperature. After washing, wells were incubated with HRP-conjugated $(Fab')^2$ goat anti-human Ig gamma chain (Biosource, Camarillo, CA) at 1:5,000 dilution in 1× specimen diluent for 1 h at room temperature. After washing, bound HRP-antibody was detected using Chromagen-TMB diluted 1:100 in citrate buffered substrate (both from Genetic Systems). The absorbance was measured at wavelength 450 nm. To detect osteopontin on the wells, anti-osteopontin antibody (OP189) was added to wells followed by anti-goat HRP.

Cell Adhesion Assays

These assays were performed as described elsewhere [Liaw et al., 1994]. Briefly, matrix proteins were coated onto 96-well Maxisorp microtiter plates (Nunc, Inc.) overnight at 4°C and blocked 1 h with PBS containing 10 mg/ml bovine serum albumin (BSA). Cells were resuspended in DMEM containing 1 mg/ml BSA and preincubated with or without EDTA for 15 min at 37°C. WEHI-3B cells (100,000) were added to the wells and allowed to incubate for 45 min at 37°C. Attached cells were stained with toluidine blue, solubilized and quantitated by reading the absorbance at 595 nm. Under these conditions, absorbance was proportional to cell number [Liaw et al., 1994].

RESULTS

CD44-hIg Fusion proteins do not bind human recombinant osteopontin. In a recent report, CD44 transfected cells expressing the CD44 v7-v10 variant was shown to interact with certain forms of osteopontin [Weber et al., 1996]. To determine if the standard form of CD44 or other variants of CD44 can interact with this protein, we examined the ability of CD44 receptor human immunoglobulin fusion proteins (CD44-hIg) to directly bind osteopontin in an ELISA assay. Several different CD44-hIgG isoforms were available for use in this study. The CD44H-hIg is a chimeric protein that contains all the CD44 extracellular common exons, E1-E5, E15-E16, in frame with the hinge, CH2 and CH3 domains of a human IgG. CD44E-hIg is a similar construct which contains three additional exons (v8-v10). CD44 v3,v8-v10 contains v3, v8-v10 in addition to the common exons, and CD44 v3 contains just the v3 exon. A schematic diagram of the CD44 splice variants used in these studies is shown in Figure 1.

To measure CD44 interaction with certain forms of OPN, soluble CD44-hIg fusion proteins (20 µg/ml) were incubated with 1 µg/ml osteopontin immobilized on 96-well plates. Bound CD44-hIg was detected by adding HRP-labeled anti-human IgG antibodies which recognize the Ig portion of the fusion protein. Hyaluronic acid, which was used as a control ligand, supported binding of CD44H and CD44E and reduced binding to the CD44 v3 and CD44 v3, v8-v10 (Fig. 2A) as has been previously shown

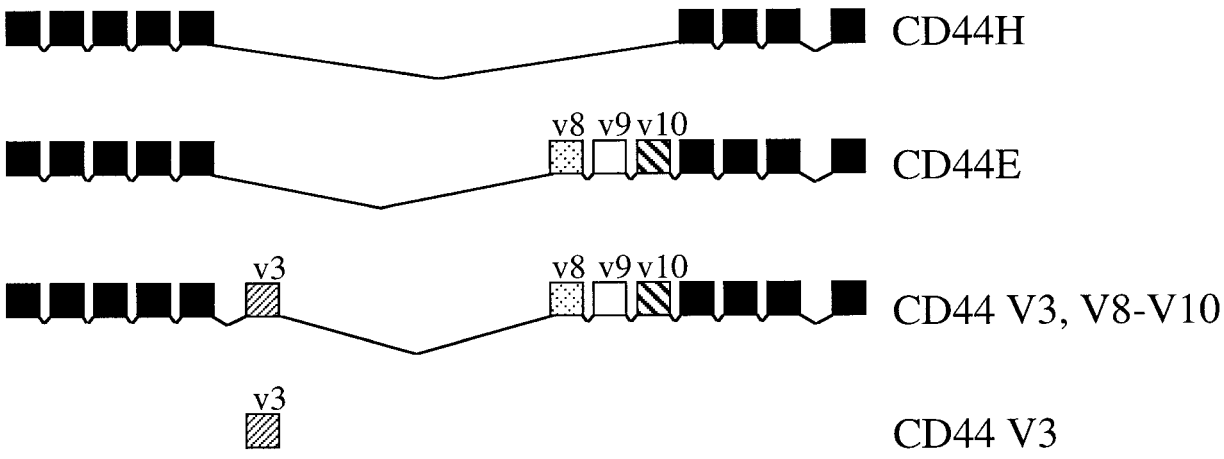

Fig. 1. Schematic diagram of CD44-hIg variants used for ELISA. Four CD44-Ig constructs were used for these studies; CD44H-Ig, CD44E-Ig, CD44v3, v8-v10-Ig, and CD44 v3.

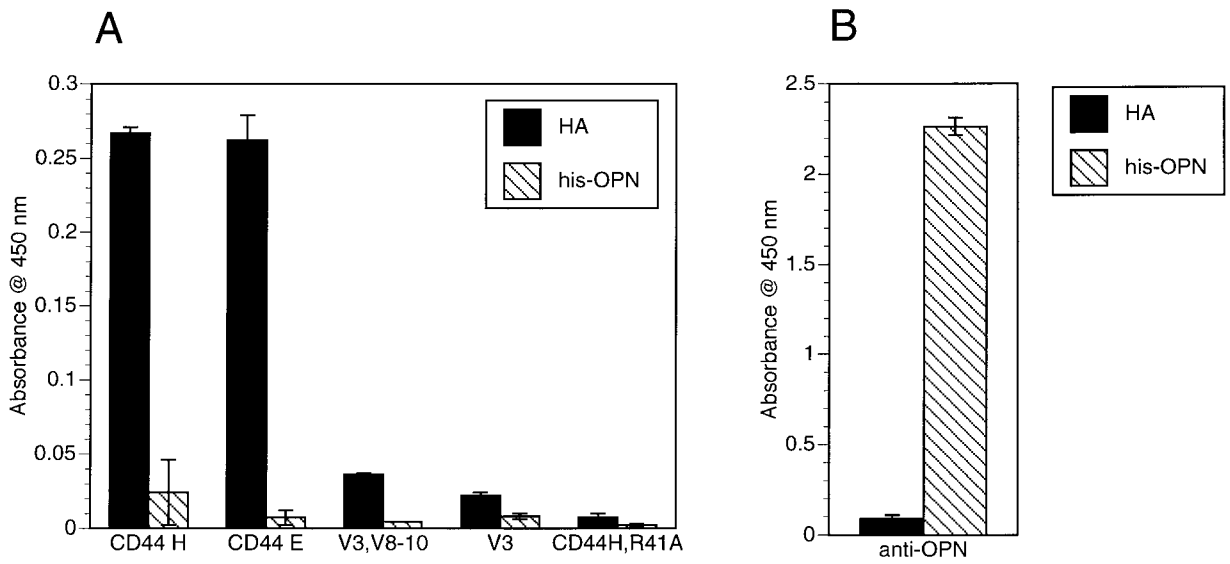

Fig. 2. ELISA of CD44 interaction with human recombinant osteopontin and hyaluronic acid. **A**: CD44-hIg fusion proteins (20 ug/ml) were allowed to bind for 1 h to wells coated with 10 ug/ml hyaluronic acid (HA) or 1 µg/ml human recombinant his-tagged osteopontin (his-OPN). The attached CD44-hIg proteins were detected by adding HRP-labeled anti-human IgG antibodies followed by substrate as described in Materials and Methods. **B**: Anti-OPN antibodies were allowed to bind for 1 h to wells coated with 10 ug/ml hyaluronic acid (HA) or 1 µg/ml human recombinant his-tagged osteopontin (his-OPN). The attached anti-osteopontin antibody was detected by adding HRP-labeled anti-goat IgG antibodies followed by substrate. Each data point represents the mean ± S.D. of triplicate samples.

[Bartolazzi et al., 1995; Bennett et al., 1995b; Jackson et al., 1995]. Reduced HA binding to CD44 variants containing v3 is due to glycosylation which inhibits the interaction with HA [Bartolazzi et al., 1996; Jackson et al., 1995]. In addition, HA failed to bind a mutant CD44-hIg fusion protein that contains a point mutation in the extracellular domain (CD44HR41A) that has previously been shown to be important for HA interactions [Peach et al., 1993]. The CD44H form, as well as the CD44E, CD44 v3, v8-v10, and CD44 v3 all failed to bind recombinant human his-OPN. Similar results were also seen using 50 µg/ml CD44-hIg proteins (not shown) and 2 µg/ml, 10 µ g/ml, and 20 µg/ml osteopontin. To rule out the possibility that osteopontin was not successfully coated on the plate, goat anti-OPN antibodies were added in the place of CD44-hIg proteins to detect the OPN. The results demonstrate that there is significant binding of anti-OPN antibodies to OPN-coated, but not HA-coated wells indicating that the OPN coating is efficient (Fig. 2B).

CD44-hIg Fusion proteins do not bind native osteopontin or recombinant osteopontin fragments. Native osteopontin can be both heavily phosphorylated and glycosylated [Giachelli et al., 1995a; Kasugai et al., 1991; Sorensen and Petersen, 1994; Sorensen and Petersen, 1995]. In most cases, post-translational modification of OPN does not affect its function since recombinant OPN behaves similar to the native protein. There are however, several reports suggesting that glycoslyation and phosphorylation can be critical for some activities [Boskey et al., 1993; Ek Rylander et al., 1994; Hunter et al., 1994; Shanmugam et al., 1997]. To determine if post-translational modifications are important for CD44 interactions, we examined the ability of CD44-hIg proteins to bind several different native OPN preparations including human OPN purified from urine and rat OPN derived from pup rat SMCs (pup OPN). Smooth muscle cell-derived OPN has previously been shown to be both glycoslyated and phosphorylated [Giachelli et al., 1995a; Saavedra, 1994]. In addition to native OPN, we also tested the N- and C-terminal recombinant osteopontin fragments expected to be formed following thrombin cleavage at the Arg169-Ser170 site. The N-terminal domain of OPN included amino acids 17–169 (10N and 30N). 30N and 10N fragments are identical except the 30N includes the alternative splice exon 5. Both of these fragments have previously been shown to support both $\alpha_v\beta_3$- and $\alpha_9\beta_1$-mediated adhesion. The C-terminal fragment (10C), contains amino acids 170–317. A

function for this fragment has not yet been found. A schematic diagram of these proteins are shown in Figure 3. In each experiment, HA was used as a control ligand. The results clearly show that CD44-hIg does not interact with any of the osteopontin preparations tested (Fig. 4). All forms of osteopontin were sufficiently coated onto the wells as detected by anti-OPN antibodies (not shown). In addition all preparations of OPN except for 10C, were found to be biologically active by adhesion assays. As expected the C-terminal OPN recombinant fragment which doesn't contain the RGD site fails to interact with any of the cell lines we have tested.

Divalent cations are required for adhesion of WEHI-3B cells to osteopontin. The CD44 molecules used in the ELISA experiments were all CD44-IgG fusion proteins. It is possible that the IgG portion of the fusion protein sterically hinders binding to OPN, therefore, we investigated the interaction of cell surface expressed CD44 with osteopontin. The monocytic line, WEHI-3B, was shown by Weber et al. [1996] to bind osteopontin in a CD44-dependent manner using ligand binding assays. To determine if WEHI-3B could interact with immobilized osteopontin, we performed cell attachment assays with full-length recombinant osteopontin. A small amount of WEHI-3B adhesion to osteopontin was seen (Fig. 5), however, adhesion was completely inhibited in the presence of EDTA. Since divalent cations are necessary for integrin binding, the inhibition by EDTA suggests adhesion of WEHI-3B to plate-bound OPN is mediated through integrin receptors rather then CD44.

DISCUSSION

This study examined the potential interaction of CD44 with the RGD-containing adhesive protein, osteopontin. Using four different splice variant CD44-hIg fusion proteins, we have shown that CD44H, CD44E, CD44 v3,v8-v10, and CD44 v3 could not interact with human recombinant his-tagged OPN. Further analysis demonstrated that several additional

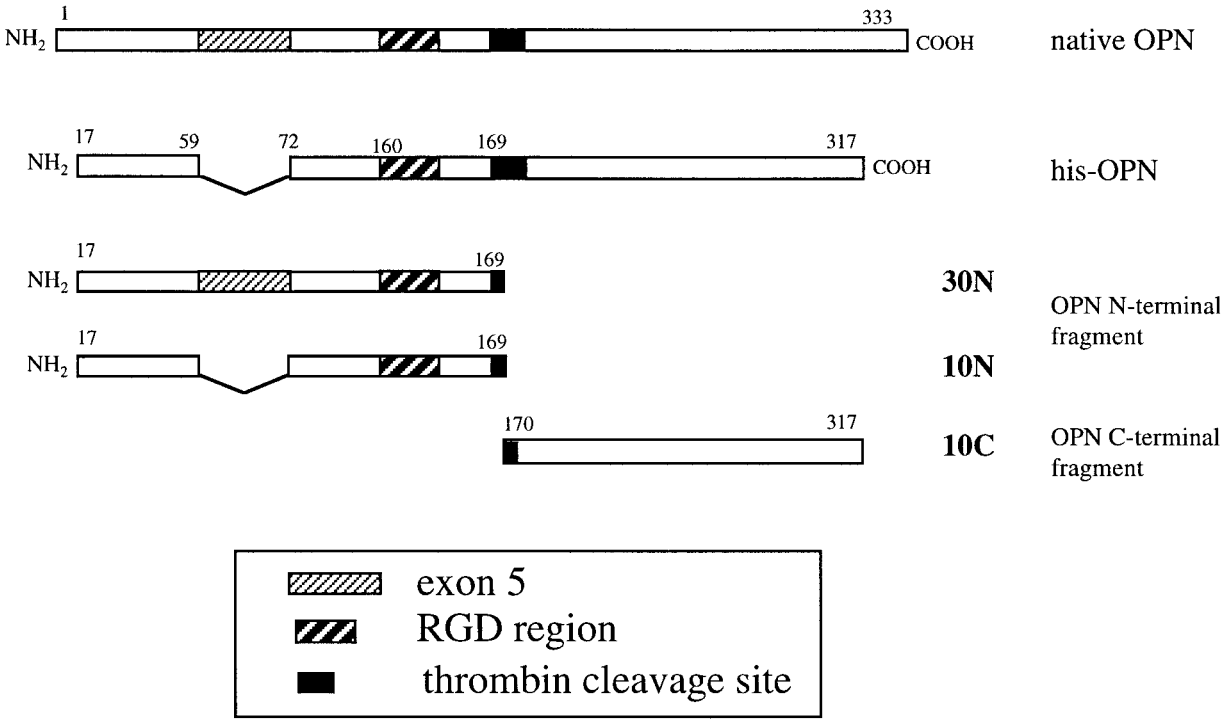

Fig. 3. Schematic diagram of native osteopontin and human recombinant osteopontin fragments used for ELISA and adhesion assays. The native, full-length osteopontin preparations used in these studies was purified from rat pup smooth muscle cells and human urine. The full-length recombinant OPN was prepared as a His-tagged protein (His-OPN). All other osteopontin molecules were prepared as human recombinant GST-fusion proteins. The N- and C-terminal domains are fragments that are expected to be formed following thrombin cleavage. The C-terminal osteopontin domain (10C) contains amino acids 170–317. The N-terminal OPN fragments (30N and 10N) are alternatively spliced and include amino acids 17–169. the 30N splice variant contains an additional 14 amino acids (NAVSSEETND-FKQE), which correspond to exon 5. Only the native OPN from SMC and urine are glycosylated and phosphorylated.

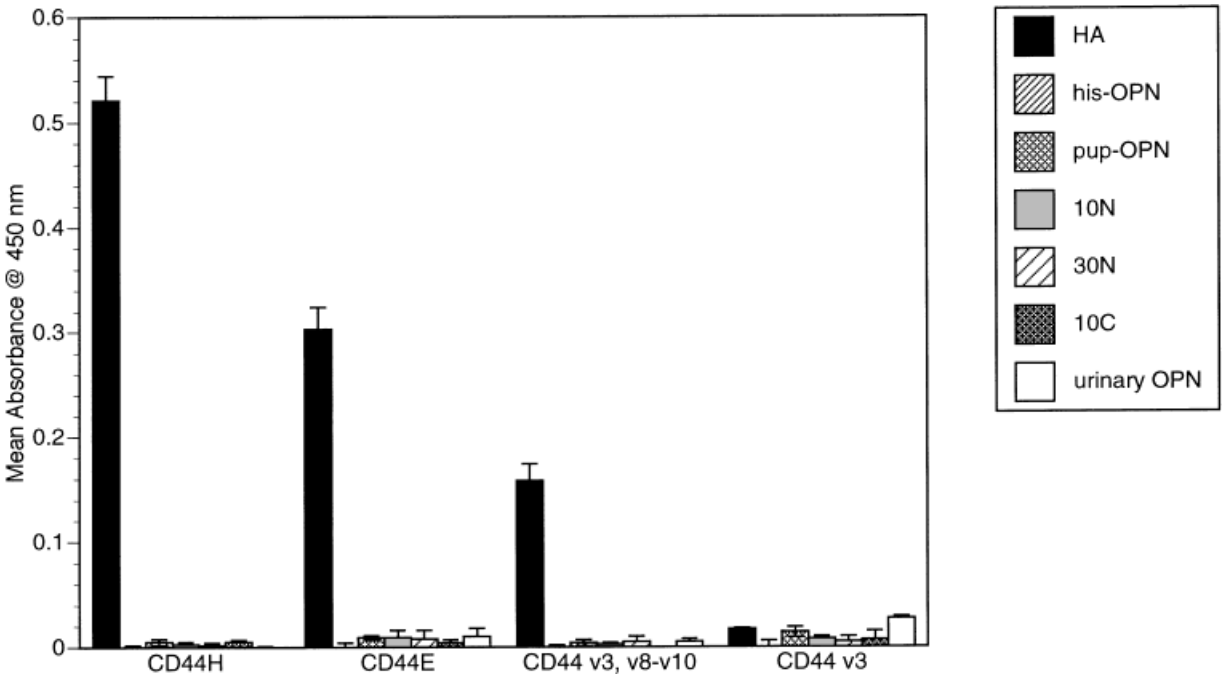

Fig. 4. ELISA of CD44 interaction with native osteopontin and recombinant osteopontin fragments. CD44-hIg fusion proteins (20 µg/ml) were allowed to bind for 1 h to wells coated with 10 µg/ml hyaluronic acid (HA),5 µg/ml human recombinant his-tagged osteopontin (his-OPN), 5 µg/ml native rat pup smooth muscle-derived osteopontin (pup-OPN), 5 µg/ml human urinary osteopontin, and 5 µg/ml recombinant osteopontin fragments (30N, 10N, and 10C). The attached CD44-hIg proteins were detected by adding HRP-labeled anti-human IgG antibodies followed by substrate as described in Materials and Methods. Each data point represents the mean ± S.D. of triplicate samples.

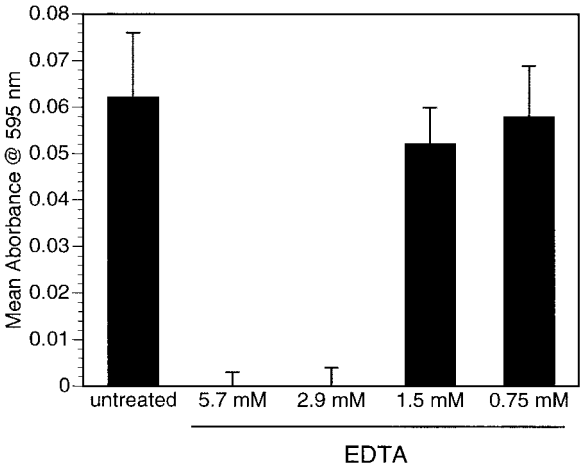

Fig. 5. Adhesion of WEHI-3B cells to osteopontin in the presence of EDTA. WEHI-3B cells were preincubated with and without EDTA at the indicated concentrations for 15 min at 37°C before plating on wells coated with 100 nM human recombinant osteopontin. The attached cells were fixed and stained with toluidine blue as described under Materials and Methods. Each data point represents the mean ± S.D. of triplicate samples. Nonspecific cell adhesion as measured on BSA-coated wells was subtracted.

preparations of osteopontin, including recombinant fragments of human OPN formed following thrombin cleavage, native OPN derived from rat SMCs and native OPN derived from human urine, also did not support the binding of any CD44 isoforms used as measured by ELISA. Furthermore, WEHI-3B cells which were previously shown to bind osteopontin, failed to adhere to our osteopontin preparations in the presence of EDTA. These results are contradictory to those published by Weber et al. [1996] that showed a CD44 transfected cell line could mediate cation-independent adhesion and migration to OPN.

One explanation for the lack of CD44-mediated attachment to OPN in this study is that the interaction may depend on a particular CD44 splice variant. Specific isoforms has previously been shown to be important for the binding of CD44 to other ligands. For example, the binding of CD44 to HA is dependent on the glycosylation state, which varies according to the alternative splice variant expressed [Bartolazzi et al., 1996; Bennett et al., 1995b; Katoh et al., 1995; Lesley et al., 1995]. In addition, the ability of CD44 to bind and present heparin-

binding growth factors is dependent on the v3-containing isoforms [Bennett et al., 1995a]. In the study by Weber et al. [1996], cells had been transfected with a CD44 isoform derived from an osteosarcoma cell line which contains v7-v10. The CD44 v7-v10 variant has been shown by Bartolazzi et al. [1995] to have weak binding to hyaluronan coated surfaces. In addition, Namalwa cells expressing v7-v10 show delayed tumor formation when injected into nude mice. However, other CD44 molecules which contain variant exon 7 (CD44 v4-v7) were shown to promote tumor metastasis in a rat model [Gunthert et al., 1991]. For our study, we used four different CD44 variants. CD44H is the most common form and is found on hemopoietic and mesoderm cells [Haynes et al., 1989; Stamenkovic et al., 1989]. This variant was also upregulated in vascular tissue following balloon injury The other variants include CD44E, CD44v3, v8-v10, and CD44v3. CD44E is the epithelial form of CD44. CD44 variants that contain v3 have been shown to bind heparin binding growth factors through the heparin sulfate side chain [Bennett et al., 1995a]. Since none of the CD44-Ig molecules tested in this study contain exon 7, it is possible that this exon contains the osteopontin binding site. We did, however, obtain the CD44-transfected A31 cells which express v7-v10 used by Weber et al. [1996]. These cells failed to interact with our osteopontin preparations in adhesion assays (data not shown).

In the study by Weber et al. [1996], a CD44-specific binding of OPN derived from K8 osteosarcoma cells to a monocytic cell line, WEHI-3B, was demonstrated. It is unclear from that study what particular splice variant(s) are expressed by these cells. In our own experiments, we found that WEHI-3B could attach to recombinant human his-OPN, but failed to bind SMC derived OPN. The adhesion to recombinant OPN was eliminated by the addition of 2.3 mM EDTA suggesting that the interaction is mediated through integrin receptors or other calcium-dependent receptors rather then CD44. One possible explanation for the discrepancy between these two studies, is that a particular source of OPN is required for CD44 binding. For example, Weber's study used cell-derived OPN for WEHI-3B binding where our study measured WEHI-3B adhesion using recombinant OPN. Osteopontin has a number of post-translational modifications including phosphorlyation and glycosylation. In most cases, these post-translational modifications do not alter its biological activity, since bacterial expressed recombinant proteins behave in a similar manner to native OPN purified from several different sources [Liaw et al., 1995a; Xuan et al., 1994]. There are however, several reports where post-translational modifications have been shown to alter OPN function. For example, sialylation of OPN is crucial for receptor-mediated binding to tsB77 cells. In addition, the phosphorylation state of OPN affects its interaction with osteoclasts [Ek Rylander et al., 1994] and normal rat kidney cells [Singh et al., 1990], as well as its ability to inhibit hydroxyapatite formation [Boskey et al., 1993; Hunter et al., 1994]. In the experiments described by Weber et al. [1996], the source of OPN was derived from K8 osteosarcoma cells, which is both glycoslated and phosphorylated. In the experiments described in this paper, we used recombinant OPN for WEHI-3B adhesion, and recombinant OPN and native OPN previously shown to be phosphorlyated and glycosylated [Giachelli et al., 1995a] for ELISAs. All preparations failed to interact with CD44, in the assays used in this study. We can not however, rule out the possibility that a post-translationally modified form of OPN not used in this study could interact with CD44. For example, other studies have reported different OPN functions depending on its source. Bone OPN or OPN derived from certain tumors could potentially contain a modified form of OPN able to recognize CD44. Several other explanations for the discrepancy between the two studies include: 1) the CD44 interaction with immobilized and soluble osteopontin may differ. For example, binding to osteopontin without clustering may fail to initiate adhesion; 2) immobilization of OPN on maxisorb plates used for attachment assays, may mask sites important for CD44 binding; 3) The affinity of the CD44 for OPN may be too low to detect using ELISA where repeated washings could potentially disrupt the interaction.

In conclusion, we have found that the standard CD44 and three CD44 splice variants do not interact with several different osteopontin preparations. These studies suggest that CD44-OPN interactions may not be a common event in vivo, and may be limited to a specific CD44 splice variant(s), and/or a particular modified form of osteopontin.

ACKNOWLEDGMENTS

The authors thank Hsueh-Ying Yang for purifying native osteopontin and Dr. Hung-Kam Cheung and Dr. Leona Ling (Biogen) for providing the GST-OPN and his-OPN constructs, and Dr. Samy Ashkar for providing the CD44 transfected murine embryonic cell line A31. C.M.G. is an established investigator of the American Heart Association.

REFERENCES

Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. 1990. CD44 is the principal cell surface receptor for hyaluronate. Cell 61:1303–1313.

Bartolazzi A, Jackson D, Bennett K, Aruffo A, Dickinson R, Shields J, Whittle N, Stamenkovic I. 1995. Regulation of growth and dissemination of a human lymphoma by CD44 splice variants. J Cell Sci 108:1723–1733.

Bartolazzi A, Nocks A, Aruffo A, Spring F, Stamenkovic I. 1996. Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronan. J Cell Biol 132: 1199–1208.

Bennett KL, Jackson DG, Simon JC, Tanczos E, Peach R, Modrell B, Stamenkovic I, Plowman G, Aruffo A. 1995a. CD44 isoforms containing exon V3 are responsible for the presentation of heparin-binding growth factor. J Cell Biol 128:687–698.

Bennett KL, Modrell B, Greenfield B, Bartolazzi A, Stamenkovic I, Peach R, Jackson DG, Spring F, Aruffo A. 1995b. Regulation of CD44 binding to hyaluronan by glycosylation of variably spliced exons. J Cell Biol 131:1623–1633.

Boskey AL, Maresca M, Ullrich W, Doty SB, Butler WT, Prince CW. 1993. Osteopontin-hydroxyapatite interactions in vitro: Inhibition of hydroxyapatite formation and growth in a gelatin-gel. Bone Miner 22:147–159.

Butler WT, Ridall AL, McKee MD. 1996. Osteopontin. In: Bilezikian JP, Raisz LG, Rodan GA, editors. Principles of bone biology. San Diego, CA: Academic Press. p 167–181.

Cooper DL, Dougherty GJ. 1995. To metastasize or not? Selection of CD44 splice sites. Nat Med 1:635–7.

Culty M, Miyake K, Kincade PW, Sikorski E, Butcher EC, Underhill C, Silorski ESS. 1990. The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins [published erratum appears in J Cell Biol 1991 Feb;112(3):following 513]. J Cell Biol 111: 2765–2774.

D'Errico JA, Sauk JJ, Prince CW, Somerman MJ. 1995. Osteopontin adhesion receptors on gingival fibroblasts. J Periodontal Res 30:34–41.

Denhardt DT, Chambers AF. 1994. Overcoming obstacles to metastasis—defenses against host defenses: Osteopontin (OPN) as a shield against attack by cytotoxic host cells. J Cell Biochem 56:48–51.

Denhardt DT, Guo X. 1993. Osteopontin: A protein with diverse functions. FASEB J 7:1475–1482.

Denhardt DT, Lopez CA, Rollo EE, Hwang SM, An XR, Walther SE. 1995 Osteopontin-induced modifications of cellular functions. Ann NY Acad Sci 760:127–142.

Ek Rylander B, Flores M, Wendel M, Heinegard D, Andersson G. 1994. Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro. J Biol Chem 269:14853–14856.

Flores ME, Heinegard D, Reinholt FP, Andersson G. 1996. Bone sialoprotein coated on glass and plastic surfaces is recognized by different beta 3 integrins. Exp Cell Res 227:40–46.

Freed E, Gailit J, van der Geer P, Ruoslahti E, Hunter T. 1989. A novel integrin beta subunit is associated with the vitronectin receptor alpha subunit (alpha v) in a human osteosarcoma cell line and is a substrate for protein kinase C. EMBO J 8:2955–2965.

Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM.1993. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. J Clin Invest 92:1686–1696.

Giachelli CM, Liaw L, Murry CE, Schwartz SM, Almeida M. 1995a. Osteopontin expression in cardiovascular diseases. Ann NY Acad Sci 760:109–126.

Giachelli CM, Pichler R, Lombardi D, Denhardt DT, Alpers CE, Schwartz SM, Johnson RJ. 1994. Osteopontin expression in angiotensin II-induced tubulointerstitial nephritis. Kidney Int 45:515–524.

Giachelli CM, Scatena M, Wada T. 1997. Osteopontin: Potential roles in vascular function and dystrophic calcification. J Bone Miner Metab 15:179–183.

Giachelli CM, Schwartz SM, Liaw L. 1995b. Molecular and cellular biology of osteopontin: Potential role in cardiovascular disease. Trends Cardiovas med 5:88–95.

Griffioen AW, Coenen MJ, Damen CA, Hellwig SM, van Weering DH, Vooys W, Blijham GH, Groenewegen G. 1997. CD44 is involved in tumor angiogenesis; an activation antigen on human endothelial cells. Blood 90:1150–1159.

Gunthert U. 1996. CD44 in malignant disorders. Curr Top Microbiol Immunol 213:271–285.

Gunthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann I, Matzku S, Wenzel A, Ponta H, Herrlich P. 1991. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65:13–24.

Haynes BF, Telen MJ, Hale LP, Denning SM. 1989. CD44—a molecule involved in leukocyte adherence and T-cell activation. Immunol Today 10:423–428.

Hofmann M, Rudy W, Zoller M, Tolg C, Ponta H, Herrlich P, Gunthert U. 1991. CD44 splice variants confer metastatic behavior in rats: Homologous sequences are expressed in human tumor cell lines. Cancer Res 51:5292–5297.

Hu DD, Lin EC, Kovach NL, Hoyer JR, Smith JW. 1995. A biochemical characterization of the binding of osteopontin to integrins alpha v beta 1 and alpha v beta 5. J Biol Chem 270:26232–26238.

Hunter GK, Kyle CL, Goldberg HA. 1994. Modulation of crystal formation by bone phosphoproteins: Structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochem J 300:723–728.

Hwang SM, Lopez CA, Heck DE, Gardner CR, Laskin DL, Laskin JD, Denhardt DT. 1994. Osteopontin inhibits induction of nitric oxide synthase gene expression by inflammatory mediators in mouse kidney epithelial cells. J Biol Chem 269:711–715.

Jackson DG, Bell JI, Dickinson R, Timans J, Shields J, Whittle N. 1995. Proteoglycan forms of the lymphocyte homing receptor CD44 are alternatively spliced variants containing the v3 exon. J Cell Biol 128:673–685.

Jain M, He Q, Lee WS, Kashiki S, Foster LC, Tsai JC, Lee ME, Haber E. 1996. Role of CD44 in the reaction of vascular smooth muscle cells to arterial wall injury. J Clin Invest 97:596–603.

Jalkanen S, Jalkanen M. 1992. Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. J Cell Biol 116:817–825.

Jalkanen ST, Bargatze RF, Herron LR, Butcher EC. 1986. A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. Eur J Immunol 16:1195–202.

Kasugai S, Todescan R, Jr., Nagata T, Yao KL, Butler WT, Sodek J. 1991. Expression of bone matrix proteins associated with mineralized tissue formation by adult rat bone marrow cells in vitro: Inductive effects of dexamethasone on the osteoblastic phenotype. J Cell Physiol 147:111–120.

Katoh S, Zheng Z, Oritani K, Shimozato T, Kincade PW. 1995. Glycosylation of CD44 negatively regulates its recognition of hyaluronan. J Exp Med 182:419–429.

Koopman G, Heider KH, Horst E, Adolf GR, van den Berg F, Ponta H, Herrlich P, Pals ST. 1993. Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. J Exp Med 177:897–904.

Labarriere N, Piau JP, Otry C, Denis M, Lustenberger P, Meflah K, Le Pendu J. 1994. H blood group antigen carried by CD44V modulates tumorigenicity of rat colon carcinoma cells. Cancer Res 54:6275–6281.

Lesley J, English N, Perschl A, Gregoroff J, Hyman R. 1995. Variant cell lines selected for alterations in the function of the hyaluronan receptor CD44 show differences in glycosylation. J Exp Med 182:431–437.

Lesley J, Hyman R, Kincade PW. 1993. CD44 and its interaction with extracellular matrix. Adv Immunol 54: 271–335.

Liaw L, Almeida M, Hart CE, Schwartz SM, Giachelli CM. 1994. Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. Circ Res 74:214–224.

Liaw L, Birk DE, Ballas CB, Whitsitt JS, Davidson JM, Hogan BLM. 1998. Altered wound healing in mice lacking a functional osteopontin gene. J Clin Invest 101:1468–1478.

Liaw L, Lindner V, Schwartz SM, Chambers AF, Giachelli CM. 1995a. Osteopontin and beta 3 integrin are coordinately expressed in regenerating endothelium in vivo and stimulate Arg-Gly-Asp-dependent endothelial migration in vitro. Circ Res 77:665–672.

Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, Giachelli CM. 1995b. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. J Clin Invest 95:713–724.

Matsumura Y, Tarin D. 1992. Significance of CD44 gene products for cancer diagnosis and disease evaluation [see comments]. Lancet 340:1053–1058.

Miyake K, Underhill CB, Lesley J, Kincade PW. 1990. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. J Exp Med 172:69–75.

Murry CE, Giachelli CM, Schwartz SM, Vracko R. 1994. Macrophages express osteopontin during repair of myocardial necrosis. Am J Pathol 145:1450–1462.

Nasu K, Ishida T, Setoguchi M, Higuchi Y, Akizuki S, Yamamoto S. 1995. Expression of wild-type and mutated rabbit osteopontin in *Escherichia coli*, and their effects on adhesion and migration of P388D1 cells. Biochem J 307: 257–265.

O'Brien KD, Kuusisto J, Reichenbach DD, Ferguson M, Giachelli C, Alpers CE, Otto CM. 1995. Osteopontin is expressed in human aortic valvular lesions. Circulation 92:2163–2168.

Oates AJ, Barraclough R, Rudland PS. 1997. The role of osteopontin in tumorigenesis and metastasis. Invas Metast 17:1–15.

Patarca R, Saavedra RA, Cantor H. 1993. Molecular and cellular basis of genetic resistance to bacterial infection: The role of the early T-lymphocyte activation-1/osteopontin gene. Crit Rev Immunol 13:225–246.

Peach RJ, Hollenbaugh D, Stamenkovic I, Aruffo A. 1993. Identification of hyaluronic acid binding sites in the extracellular domain of CD44. J Cell Biol 122:257–264.

Russo C, Callegaro L, Lanza E, Ferrone S. 1983. Re.: Purification of IgG monoclonal antibody by caprylic acid precipitation. J Immunol Meth 65:269–271.

Saavedra RA. 1994. The roles of autophosphorylation and phosphorylation in the life of osteopontin. BioEssays 16:913–918.

Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. 1992. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc Natl Acad Sci U S A 89:12160–12164.

Shanmugam V, Chackalaparampil I, Kundu GC, Mukherjee AB, Mukherjee BB. 1997. Altered sialylation of osteopontin prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells. Biochemistry 36:5729–5738.

Singh K, DeVouge MW, Mukherjee BB. 1990. Physiological properties and differential glycosylation of phosphorylated and nonphosphorylated forms of osteopontin secreted by normal rat kidney cells. J Biol Chem 265:18696–18701.

Smith LL, Cheung HK, Ling LE, Chen J, Sheppard D, Pytela R, Giachelli CM. 1996. Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by alpha9beta1 integrin. J Biol Chem 271:28485–28491.

Sorensen ES, Petersen TE. 1994. Identification of two phosphorylation motifs in bovine osteopontin. Biochem Biophys Res Commun 198:200–205.

Sorensen ES, Petersen TE. 1995. Phosphorylation, glycosylation, and transglutaminase sites in bovine osteopontin. Ann NY Acad Sci 760:363–366.

Stamenkovic I, Amiot M, Pesando JM, Seed B. 1989. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell 56: 1057–1062.

Taher TE, Smit L, Griffioen AW, Schilder Tol EJ, Borst J, Pals ST. 1996. Signaling through CD44 is mediated by tyrosine kinases. Association with p56lck in T lymphocytes. J Biol Chem 271:2863–2867.

Tanabe KK, Ellis LM, Saya H. 1993. Expression of CD44R1 adhesion molecule in colon carcinomas and metastases. Lancet 341:725–726.

Terpe HJ, Koopmann R, Imhof BA, Gunthert U. 1994. Expression of integrins and CD44 isoforms in non-Hodgkin's lymphomas: CD44 variant isoforms are preferentially expressed in high-grade malignant lymphomas. J Pathol 174:89–100.

Tyrrell DJ, Ishihara M, Rao N, Horne A, Kiefer MC, Stauber GB, Lam LH, Stack RJ. 1993. Structure and biological activities of a heparin-derived hexasaccharide with high affinity for basic fibroblast growth factor. J Biol Chem 268:4684–4689.

Weber GF, Ashkar S, Glimcher MJ, Cantor H. 1996. Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science 271:509–512.

Xuan JW, Hota C, Chambers AF. 1994. Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. J Cell Biochem 54:247–255.

Xuan JW, Hota C, Shigeyama Y, D'Errico JA, Somerman MJ, Chambers AF. 1995. Site-directed mutagenesis of the arginine-glycine-aspartic acid sequence in osteopontin destroys cell adhesion and migration functions. J Cell Biochem 57:680–690.

Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW, Fisher LW. 1990. cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomics 7:491–502.

Yue TL, McKenna PJ, Ohlstein EH, Farach Carson MC, Butler WT, Johanson K, McDevitt P, Feuerstein GZ, Stadel JM. 1994. Osteopontin-stimulated vascular smooth muscle cell migration is mediated by beta 3 integrin. Exp Cell Res 214:459–464.