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Characterization of Osteopontin Binding Kinetics in MDA-MB231 Breast and SK-Hep-1 Liver Cancer Cells

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Abstract

Osteopontin (OPN) is a secreted phosphoprotein which plays a critical role in metastasis of colon, liver, and breast cancers. The canonical pathway for OPN signaling focuses on its binding interactions with integrin and CD44 cell surface receptors. However, the binding characteristics of OPN to integrin and CD44 receptors has not been previously examined. In this paper, using MDA-MB231 breast cancer and SK-Hep-1 liver cancer cells, we determine the relative binding characteristics of the OPN ligand to its integrin and CD44 cell surface receptors. The apparent K_D's for binding to CD44 was 56 μM and 49 μM and to integrin was 18 μM and 17 μM, in SK-Hep-1 and MDA-MB231, respectively. The CD44/Integrin ratio of OPN bound was 1.3 and 3.8 in SK-Hep-1 and MDA-MB231, respectively. Our results indicate that OPN binds to it recognized receptors with substantially different affinities, receptor expression varies between cell types, and significant OPN cell surface interactions that are integrin- and CD44-independent. These uncharacterized interactions may reveal important insights into OPN's role in cancer metastasis.

Introduction

Cancer metastasis requires physiological changes which are regulated by cell signaling molecules. One such molecule, osteopontin (OPN), is a secreted phosphoprotein which functions as a cell attachment protein and cytokine (Ashkar et al., 2000; Wai and Kuo, 2008). A substantial body of evidence indicates that OPN is a major regulatory factor in increased cellular migratory and invasive behavior, increased metastasis, protection from apoptosis, promotion of colony formation and 3D growth ability, induction of tumor-associated inflammatory cells, and induction of expression of angiogenic factors (Ashkar et al., 2000; Wai and Kuo, 2008). Gain- and loss-of function assays have demonstrated a critical role for OPN in tumor metastatic function in colon, liver, and breast cancers.

The study of OPN signaling has focused on interactions with the integrin ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$ and $\alpha_9\beta_1$) and CD44 cell adhesion molecules. OPN signaling through integrins modulates the phosphorylation of kinases (NIK, IKKβ) involved in NFκB activation, while OPN signaling through CD44 promotes cell survival by activating the PI3K/Akt pathway (Wai and Kuo, 2008). In addition, using MDA-MB231 breast cancer cells, we have demonstrated that global ablation of OPN cell surface receptor binding is associated with significant alteration in gene and protein expression critical in apoptosis, vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), interleukin-10 (IL-10), granulocyte-macrophage colony stimulating factor (GM-CSF) and proliferation signaling pathways. (Unpublished data.). Clearly, binding of OPN to its cell surface receptors plays a critical role in OPN-dependent metastatic functions. However, despite the large amount of research focused on OPN expression in cancer, the binding characteristics of OPN to integrin and CD44 receptors has not been previously examined. In this paper, using MDA-MB231 breast cancer and SK-Hep-1 liver cancer cells, we determine the relative binding characteristics of the OPN ligand to its integrin and CD44 cell surface receptors. Our results indicate that OPN binds to its recognized receptors with substantially different affinities, receptor expression varies between cell types, and there are significant OPN cell surface interactions that are integrin- and CD44-independent. The parsing of these critical OPN interactions has not been previously addressed.

Methods

Cell culture

MDA-MB-231 and SK-Hep-1 human cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and maintained at 37°C in a humidified atmosphere of 5% CO_2.

Plasmids and recombinant human OPN purification

The human full length OPN-b cDNA with 6 His tag codons was cloned into pEYFP (BD Biosciences Clontech). His tagged YFP served as a measure of nonspecific binding. Wild type OPN-b, mutant OPN with deletion of the integrin RGD binding domain (R159-D161; ΔRGD) or deletion of the CD44 binding domain (D298-I305; ΔCD44) were cloned with 6 His tag codons into pcDNA3.1 vector (Invitrogen, Carlsbad, CA). The plasmids were transfected into COS7 cells; OPN proteins were purified from the cell lysates by using the MagneHis Protein Purification System (Promega, Madison, WI) and quantified with Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Competitive binding assays

Cells were cultured on coverslips and then washed with PBS

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(137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, and a pH of 7.4) 3 times, treated for 4 h with 500uM recombinant human OPN-YFP in DMEM culture medium at 37°C in a humidified atmosphere of 5% CO_2. Competitive binding assays were performed at 37°C and 4°C, by adding 1μM to 5 mM wild-type OPN-b, OPN(ΔRGD) or OPN(ΔCD44). His-tagged YFP was used as a measure of nonspecific binding. After 4 h incubation, the coverslips were rinsed three times with ice-cold PBS followed by fixation for 15 min with 1% (wt/vol) paraformaldehyde. Coverslips were rinsed three times with PBS and mounted onto a microscope slide using 50ul mounting medium (Calbiochem, San Diego, CA). Leica TCS SP2 confocal microscope was used for image acquisition. YFP emission spectrum was optimized at 514nm. Fluorescence intensity was quantified by MetaMorph Premier Software (Molecular Devices, PA) by using the integrated fluorescence weighting measurement for 12-bit images. In all experiments, specific binding was defined as the difference between total binding and nonspecific binding. By applying nonlinear regression curve fitting analysis (mathematics equation: $Y = Bmax \cdot X / (kd + X)$, where X is the concentration of variety competitors of OPN mutants, Y is specific binding fluorescent intensity), apparent binding affinity was calculated with Prism5 software (GraphPad Software, San Diego, CA) under homologous competitive binding modeling.

Data analysis

Data are expressed as the mean of measurements taken in 20 different cells in each of three separate experiments.

Results and Discussion

To determine the potential for cell surface binding of OPN-YFP to SK-Hep- and MDA-MB231 cells, fluorescence was measured over an OPN-YFP concentration range of 10^{-5} to 10^{-2} M (Figure 1a). His-tagged YFP was used as a measure of nonspecific binding and subtracted in all experiments. Nonspecific binding accounted for 2-4% of total binding in all experiments. The results indicate that OPN-YFP binds to the cell surface, maximal binding is approximately 2-fold greater in MDA cells and is saturable in both cell types. Competition assays in the presence of excess cold wild-type OPN (10^{-5} to 10^{-2} M) indicate that OPN-YFP cell surface binding is specific and can be competed off by wild-type OPN (Figure 1b).

OPN mutants containing deletions of the RGD-integrin binding domain (ΔRGD) or the CD44 binding domain (ΔCD44) were then constructed. Binding studies were then performed using these mutants as competitor ligands with OPN-YFP. Three populations of OPN receptors were considered: integrin, CD44 and non-CD44/non-integrin receptors. As the non-CD44/non-integrin receptor group represents a heterogeneous population, binding studies were first completed in the presence of His-tagged YFP to correct for nonspecific binding. Competition binding studies were then performed in the presence of both ΔRGD and ΔCD44 mutants (Figure 2a). This would allow correction for all OPN binding to non-CD44 and non-integrin receptors. Saturation of the OPN binding occurred at an OPN concentration of 500 μM and this concentration was used in competition with mutant OPN. At 37°C, in the presence of both ΔRGD and ΔCD44, binding of OPN-YFP was decreased to a level that was 35% and 39% of fully saturated binding in MDA-MB231 and

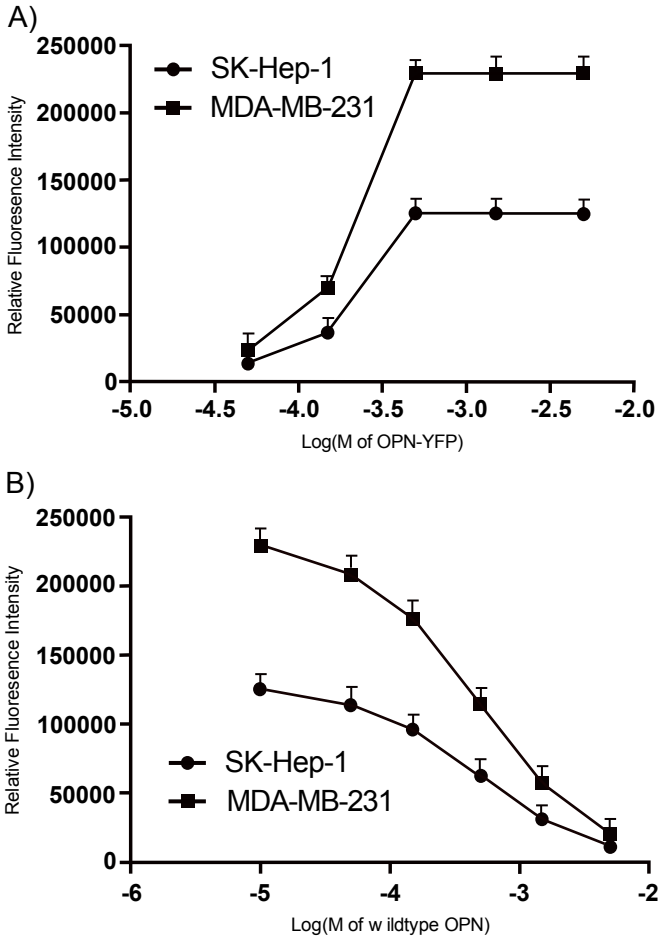


Figure 1: Binding kinetics of OPN-YFP to SK-Hep-1 and MDA-MB231 cells.

A: Saturation binding curve of OPN-YFP

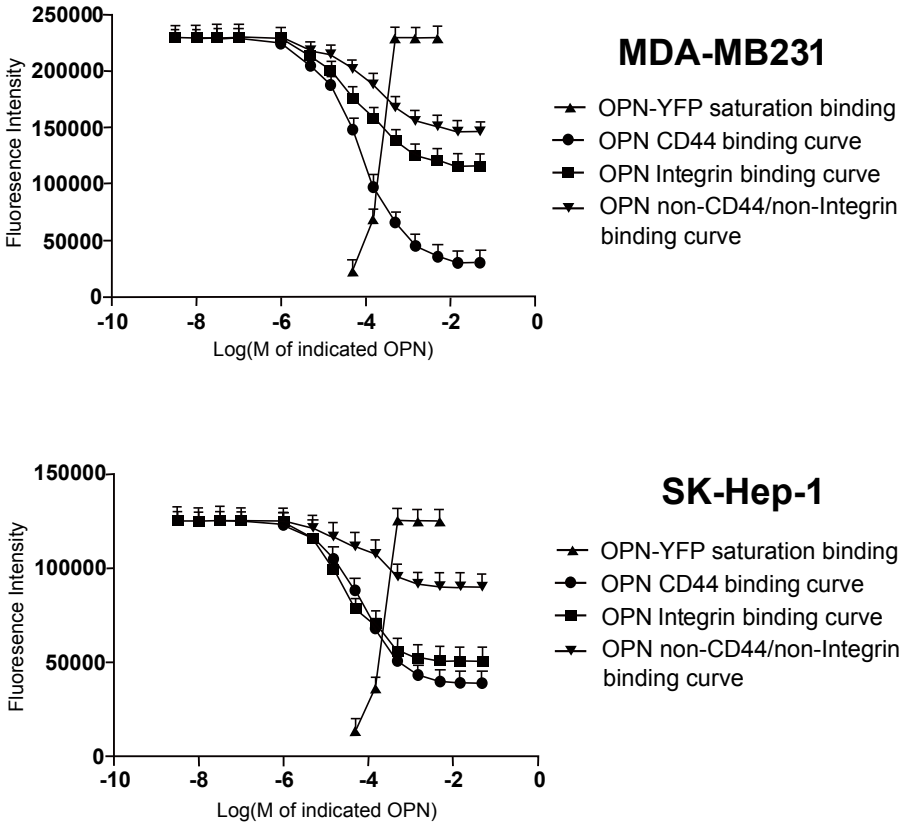
Fluorescence was measured over an OPN-YFP concentration range of 10^{-5} to 10^{-2} M. His-tagged YFP was used as a measure of nonspecific binding and subtracted in all experiments. Nonspecific binding accounted for 2-4% of total binding in all experiments. Leica TCS SP2 confocal microscope was used for image acquisition. YFP emission spectrum was optimized at 514nm. Fluorescence intensity was quantified by MetaMorph Premier Software (Molecular Devices, PA) by using the integrated fluorescence weighting measurement for 12-bit images. Data are expressed as the mean of measurements taken in 20 different cells. Figure is representative of three experiments.

B: Competitive binding curve of OPN-YFP and wild-type OPN

Competition assays in the presence of excess cold wild-type OPN (10^{-5} to 10^{-2} M) were performed. Data are expressed as the mean of measurements taken in 20 different cells. Figure is representative of three experiments.

SK-Hep-1, respectively. This suggests that a substantial fraction of OPN binding can be integrin- and CD44-independent. Competition binding studies were then performed in the presence of either ΔRGD or ΔCD44; these values were then corrected for OPN binding that was integrin- and CD44-independent. In the presence of either competitor ΔRGD or competitor ΔCD44, there was displacement of bound OPN-YFP. The CD44/Integrin ratio of OPN bound was approximately 1.3 and 3.8 in SK-Hep-1 and MDA-MB231, respectively. Apparent affinity binding constants for interaction with CD44, integrin and non-CD44/integrin receptors were then calculated under homologous competitive binding modeling (Figure 2b). At 37°C, the apparent K_D for OPN binding to CD44 was 56 μM and 49 μM in SK-Hep-1 and MDA-MB231, respectively; in contrast, the apparent K_D for OPN bind-

A)



B)

Calculated OPN Binding Parameters for MDA-MB231 and SK-Hep-1 Cells

	One site – Homologous Binding Best-fit values	CD44 binding SK-Hep-1	Integrin binding SK-Hep-1	CD44 binding MDA-MB-231	Integrin binding MDA-MB-231
37 deg C	Log K_d	-4.251	-4.749	-4.311	-4.782
	Bmax	50653	39261	115787	30515
	Kd	5.610e-005	1.782e-005	4.887e-005	1.652e-005
	Std. Error	<1%	<1%	<1%	<1%
4 deg C	Log K_d	-4.232	-4.734	-4.310	-4.770
	Bmax	33187	25581	75434	25581
	Kd	5.861e-005	1.845e-005	4.898e-005	1.698e-005
	Std. Error	<1%	<1%	<1%	<1%

Figure 2: Characterization of CD44, integrin, and non-CD44/non-integrin binding OPN-YFP to SK-Hep-1 and MDA-MB231 cells.

A. Competition binding curves of OPN-YFP were performed at 37° C in the presence of 1μM to 5 mM mutant OPN(ΔRGD), OPN(ΔCD44) or OPN(ΔRGD) + OPN(ΔCD44). OPN mutants containing deletions of the RGD-integrin binding domain (ΔRGD) or the CD44 binding domain (ΔCD44) were constructed. Binding studies were performed using these mutants as competitor ligands with OPN-YFP. Three populations of OPN receptors were considered: integrin, CD44 and non-CD44/non-integrin receptors. As the non-CD44/non-integrin receptor group represents a heterogeneous population, binding studies were first completed in the presence of His-tagged YFP to correct for nonspecific binding. Competition binding studies were then performed in the presence of both ΔRGD and ΔCD44 mutants. This would allow correction for all OPN binding to non-CD44 and non-integrin receptors. Saturation of the OPN binding occurred at an OPN concentration of 500 μM and this concentration was used in competition with mutant OPN. His-tagged YFP was used as a measure of nonspecific binding. Data are expressed as the mean of measurements taken in 20 different cells. Figure is representative of three experiments.

B. OPN binding kinetics in SK-Hep-1 and MDA-MB231 cells

Apparent binding affinity was calculated with Prism5 software (GraphPad Software, San Diego, CA) under homologous competitive binding modeling by applying nonlinear regression curve fitting analysis (mathematics equation: $Y = Bmax \cdot X / (kd + X)$, where X is the concentration of variety competitors of OPN mutants, Y is specific binding fluorescent intensity). Data are representative of three experiments.

ing to integrin was 18 μM and 17 μM in SK-Hep-1 and MDA-MB231, respectively. At 4°C, the apparent K_D for OPN binding to CD44 was 59 μM and 49 μM in SK-Hep-1 and MDA-MB231, respectively; the apparent K_D for OPN binding to integrin was 17 μM and 19 μM in SK-Hep-1 and MDA-MB231, respectively. This indicates that OPN binds greater avidity to integrin receptors over CD44. Given, the potential for multiple additional receptors in the setting of competition with both ΔRGD and ΔCD44, calculation of an apparent K_D was not deemed relevant. These data indicate that OPN binds to its CD44 and integrin receptors with substantially different affinities, receptor expression varies between cell types, and significant fraction of OPN cell surface interactions are integrin- and CD44-independent that have yet to be fully characterized.

OPN is rich in aspartate, glutamate and serine residues and contains functional domains for calcium-binding, glycoslyation, phosphorylation and extra-cellular matrix adhesion. Functional domains include NH2 and COOH-terminal regions, a thrombin cleavage site (RSK) and a Glycine-Arginine-Glycine-Aspartate-Serine (GRGDS) sequence (Wai and Kuo, 2008; Weber et al., 2001). The GRGDS sequence contains a functional Arginine-Glycine-Asparate (RGD) binding motif that ligates cell surface $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ integrin (Hijiya et al., 1994; O'Regan and Berman, 2000) Six amino acids away from the RGD motif resides a thrombin cleavage site (O'Regan and Berman, 2000; Senger et al., 1996). Thrombin cleaves OPN efficiently at this protease-hypersensitive site, produces two fragments of approximately equivalent size and reveals the integrin and CD44 binding domains (Senger et al., 1996; Senger and Perruzzi, 1988). Efficient engagement of the integrin receptor by OPN requires thrombin cleavage and phosphorylation of the N-terminal fragment, to enhance cell attachment and migration as compared to uncleaved OPN (Ashkar et al., 2000; Senger et al., 1994). Thrombin cleavage of human OPN also uncovers the SVVYGLR adhesion sequence which is a ligand for the $\alpha_9\beta_1$ integrins (Senger et al., 1994).

To date, many of the signaling pathways mediated by OPN are thought to be activated by ligation of the $\alpha_v\beta$ integrin and CD44 families of receptors. Integrins are heterodimeric cell surface glycoproteins with non-covalently associated α and β subunits (Giancotti and Ruoslahti, 1999). This receptor family has broad binding specificity with vitronectin, fibronectin, fibrinogen, thrombospondin and OPN acting as ligands. OPN binds integrin $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$ and $\alpha_9\beta_1$ (Wai and Kuo, 2008). OPN-integrin binding directly mediates migration and invasion of tumor cells (Angelucci et al., 2002). Ligation of OPN induces neovascularization by upregulating endothelial cell migration, survival and lumen formation during angiogenesis (Brooks et al., 1994). Supporting this pro-angiogenic role of OPN is the observation that vascular endothelial growth factor (VEGF) induces OPN and $\alpha_v\beta_3$ expression in microvascular endothelial cells (Senger et al., 1996).

The CD44 glycoproteins are ubiquitously expressed, cell-surface adhesion molecules that mediate cell-matrix and cell-cell interactions (Goodison et al., 1999). The principal ligand for CD44 is hyaluronic acid (HA) but other extracellular-matrix (ECM) proteins, such as OPN also bind CD44. OPN-CD44 interactions appear to be RGD independent (Weber et al., 1996). The CD44 receptor has multiple isoforms in addition to standard CD44, which arise from differential splicing of 10 variant exons in the extracellular domain and they are designated CD44v6-15 (Goodison et al., 1999). CD44 variants, especially CD44v6, have been identified as protein markers for metastatic behavior in hepatocellular, breast, lung, pancreatic, colorectal and gastric cancers and in lymphomas. Functional studies show that CD44v7-10 ligation of OPN mediates chemotaxis and adhesion of fibroblasts, T-cells and bone marrow cells, downregulates the host-inflammatory response in a IL-10 mediated manner, and confers metastatic potential when overexpressed through plasmid vectors in a mouse model of pancreatic cancer (Ashkar et al., 2000; Weber et al., 1996). Studies also suggest that OPN and CD44 interact with the ezrin, radixin and moesin proteins to alter cytoskeletal dynamics, cell adhesion, and motility through the cortical actin filaments (Denhardt et al., 2001).

Based on our results, there also exist significant OPN cell surface interactions that are integrin- and CD44-independent. In the presence of both ΔRGD and ΔCD44, binding of OPN-YFP was decreased to a level that was 35% and 39% of fully saturated binding in MDA-MB231 and SK-Hep-1, respectively. In 4T1 and 4T07 murine breast cancer cells, we have previously shown that the COOH-terminal fragment of OPN binds with cyclophilin C (or rotamase) to the CD147 cell surface glycoprotein to activate Akt1/2 and MMP-2 (Mi et al., 2007). Our results indicate that the OPN + cyclophilin C complex binds to the CD147 with an apparent K_d of 5 nM, suggesting a high degree of avidity, even greater than that found for CD44 or integrin binding in the present study. Thus, CD147 represents an additional OPN cell surface receptor and signal transduction pathway (among potentially many others) that has yet to be fully characterized. These additional receptors may play key functions in OPN's critical role in cancer metastasis.

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