Osteopontin: A Novel Non-Microbial Ligand for Dectin-1

Deena H. Elsori, Satya P. Yadav, and Martha K. Cathcart

***Abstract*—**Dectin-1 is a well known receptor for β-glucan rich microbial ligands. To date non-microbial endogenous ligands for Dectin-1 have not yet been identified. This study supports the role of an endogenous glycoprotein, osteopontin (OPN), as a novel ligand for Dectin-1. OPN is an acidic phosphorylated adhesion protein. It is a multi-functional molecule expressed by different immune cells including monocytes, macrophages, neutrophils and T-lymphocytes and it is also highly expressed in chronic inflammatory diseases including atherosclerosis. In search for potential novel non-microbial Dectin-1 ligands, OPN was one of the candidates that we wanted to test due to its highly repetitive structural characteristics (featuring a pattern of numerous phosphates and sugars). In addition, the fact that osteopontin is expressed by inflammatory cells and that it promotes the pathogenesis of atherosclerosis made this protein an even more tantalizing candidate

In this study we show that osteopontin signals though Dectin-1 for the activation of NADPH oxidase and superoxide anion production in human monocytes and this activation is inhibited by laminarin, a lectin blocking reagent. Using the method of surface plasmon resonance (SPR), we show direct binding of human recombinant OPN to Dectin-1 immobilized on a sensor chip.

CD44, a cell-surface glycoprotein and hyaluronic acid receptor, is also known to bind to OPN. To show that laminarin specifically blocks OPN-induced superoxide anion through Dectin-1 and not CD44, we used SPR to exclude laminarin binding to CD44. In addition we also show that laminarin does not bind to CD36, another pattern recognition receptor.

Taken together, our results suggest that OPN is a novel ligand for Dectin-1. We show for the first time that OPN signals through Dectin-1 for the activation of NADPH oxidase in primary human monocytes and demonstrate selective binding between the two proteins.

***Keywords*—**Kinematic viscosity, McAllister model , specific gravity, petroleum fraction.

I. Introduction

THE β-glucan receptor Dectin-1 was first identified by Brown and Gordon (1). It is expressed on monocytes, macrophages, neutrophils and on subsets of dendritic cells and T-cells (2). It is a type II transmembrane receptor with a C-type lectin domain at the C-terminus and an immunoreceptor tyrosine-based activation motif (ITAM)-like signaling motif in the intracellular N-terminus domain (4). Dectin-1 recognizes intact yeast such as Saccharomyces cerevisiae, fungal pathogens including Candida albicans and Pneumocystis carinii, and β-glucan containing particles such as Zymosan (yeast cell wall preparation). Soluble β-glucans such as laminarin and glucan phosphate block the binding of Dectin-1 to Zymosan. Dectin-1 binds specifically to β-1,3-linked or both β-1,3- and β-1,6-linked glucose monomers through the lectin-like domain (3). Dectin-1 doesn't have a typical lectin domain in that it binds to glucans in a calcium independent manner (1). Dectin-1 also recognizes intact fungal and yeast pathogens through glucan binding. (1, 42, 48)

Non-microbial endogenous ligands recognized by Dectin-1 have yet been determined. All of the ligands used to study Dectin-1 function are exogenous β-glucan rich particles and intact microorganisms. It is noteworthy to mention that in addition to binding to glucans, Dectin-1 binds to a subset of T-lymphocytes and triggers their proliferation (1, 4). Soluble glucans do not inhibit Dectin-1 binding to T-cells (1) and this endogenous ligand is still not identified.

In this study we investigate the role of a non-microbial phosphorylated protein, osteopontin, as novel endogenous ligand for Dectin-1. Osteopontin (OPN) is an acidic secreted glycoprotein with an approximate molecular weight of 32 kDa (7). Osteopontin is post-translationally modified through glycosylation (5-6 O-linked and one N-linked oligosaccharides) and variable phosphorylation (12 phospho-Ser and one phospho-Thr) and it is also sulphated. Interestingly, in rat kidney cells (8) and calcitrol-stimulated mouse JB6 epidermal cells (9) OPN is secreted in a non-phosphorylated form. OPN has a calcium, hydroxyapatite, RGD and a thrombin/MMP cleavage site (7). Cleaved fragments of OPN expose new domains and may induce different functions. The amino acid sequence of OPN is conserved throughout different species including human, rat, mouse and pig. At first, OPN was thought to be synthesized by cells of the osteoblastic lineage and deposited on mineralized matrix; however, it was later shown that several bone marrow-derived cells including dendritic cells, macrophages, smooth muscle cells and endothelial cells also produce OPN.

OPN binds to different members of the integrin family, αv and β1 integrins, through its RGD binding domain. In

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addition, OPN has been shown to bind to a non-integrin receptor, namely the hyaluronan-receptor CD44 (13). Binding of OPN to CD44 was shown to be essential for bone resorption (14).

Osteopontin (OPN) is expressed by different immune cells including monocytes/macrophages, neutrophils and lymphocytes. It has different functions in different cells. OPN regulates immune responses by acting as a chemokine and promoting cell recruitment to inflammatory sites (15). It triggers cell attachment through its integrin binding domain (RGD) by binding to different members of the integrin family (16). In addition it has been shown to be essential for wound healing (17). OPN mediates cytokine production and cell activation (18). Finally, this protein was shown to play an important role in cell survival by regulating apoptosis (18, 19). It is highly expressed in chronic inflammatory diseases including atherosclerosis (20, 49) and it is also over-expressed in autoimmune diseases (50).

During chronic inflammation, OPN accumulates in and around inflammatory cells. OPN has been shown to regulate atherogenesis by acting as a chemokine, by recruiting monocytes and macrophages to inflammatory sites, and through its adhesive properties (RGD sequence) which promotes cell attachment through integrin binding. In addition, it modulates cytokine production in dendritic cells, monocytes/macrophages and T-cells during chronic inflammation (20). OPN plasma levels have been used clinically to diagnose different inflammatory diseases including cardiovascular disease. In addition to its role as a proinflammatory molecule, OPN is an inhibitor of mineralization and vascular calcification (49).

The reason for choosing OPN as a possible ligand for Dectin-1 is mainly due to its highly repetitive structural characteristics (featuring a pattern of numerous phosphates and sugars). In addition, OPN is expressed in human monocytes and plays a role in the pathogenesis of atherosclerosis.

We investigated the role of OPN in regulating superoxide anion production in a Dectin-1 dependent manner by using the SOD-inhibitable Cytochrome C reduction assay. In addition, using SPR (Surface Plasmon Resonance) we tested the binding of recombinant osteopontin to Dectin-1 immobilized on a chip and excluded laminarin binding to CD44 and CD36, another pattern recognition receptor.

II. Materials And Methods

Reagents

Zymosan was purchased from MP Biomedicals (Solon, OH). Laminarin and human CD44 monoclonal antibody were purchased from Sigma (St. Louis, MO). Human Dectin-1 monoclonal antibody (clone 259931), Dectin-1, osteopontin and CD44 recombinant proteins were purchased from R&D Systems (Minneapolis, MN).Human recombinant CD36 was a generous gift from Dr M.Febbraio and Dr R.Silverstein (Cleveland Clinic, Cleveland, OH).

Preparation of Zymosan

Zymosan was reconstituted in PBS and boiled for 1 hour. Zymosan was pelleted at 931 x g and then washed two times with PBS and resuspended in PBS to a final concentration of 40 mg/ml and aliquots were stored at -20ºC (22).

Isolation of human monocytes and cell culture

Human monocytes were isolated and purified from whole blood as described previously (32). PBS-diluted whole blood was layered over a Ficoll-Paque density solution and centrifuged. The mononuclear cell layer was collected and washed twice with PBS, and contaminating platelets were removed by centrifugation (280xg) through bovine calf serum (BCS) after overlaying the serum with the mononuclear cells. This serum spin was repeated twice. Monocytes were isolated from the platelet-free mononuclear cells by adherence to flasks precoated with BCS and containing DMEM and 10% BCS (BCS/DMEM). The flasks were incubated for 2 h at 37°C in 10% CO_2. Non-adherent cells were removed by washing the flasks with BCS/DMEM. Adherent cells were detached with PBS containing 5 mM EDTA. The monocytes were collected, washed three times with BCS/DMEM, resuspended in BCS/DMEM, and incubated at 37°C in 10% CO_2 for at least 2 h before their use in experiments. In some of the experiments monocytes were isolated from human peripheral blood using a countercurrent centrifugal elutriation method (33, 34). Monocyte preparations purified by both methods were very similar in response to Zymosan or opsonized Zymosan (ZOP) and are consistently >90% $CD14^+$. ZOP or Zymosan were used to activate the monocytes by protocols previously described (22).

Surface plasmon resonance studies (SPR)

The interaction between Dectin-1 and various ligands was measured using surface plasmon resonance (SPR) using a Biacore 3000 instrument (Biacore, Uppsala, Sweden). Dectin-1 was covalently coupled via primary amines, at a concentration of 5000 response units, to the dextran matrix of CM5 sensor chips. Different concentrations of recombinant human osteopontin in Hank's (HBSS) buffer containing 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 1 mM $MgCl_2$ were flowed over flow cells on the sensor chip containing Dectin-1 or nothing (reference cell). All data were corrected for the response obtained using a blank reference flow cell that was activated with EDC/NHS and then blocked with ethanolamine. Experiments were performed by injecting the analytes at 20 µl/min for 2 minutes. The chip surface was regenerated using 2 M NaCl plus 50 mM NaOH. Data were analyzed using the BIAevaluation 3.1 program (Biacore, Uppsala, Sweden)

III. Results

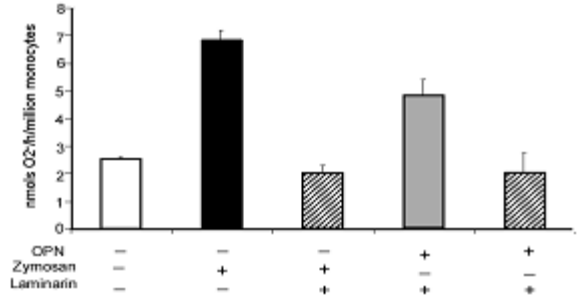
Osteopontin triggers superoxide anion production through Dectin-1

Non-microbial endogenous ligands for Dectin-1 have not yet been identified. We have already shown in chapter II that a microbial ligand, yeast Zymosan, induces NADPH oxidase activation through Dectin-1 in primary human monocytes. In

this experiment we tested the role of a non-microbial pathophysiological ligand, osteopontin, in regulating the activity of monocyte NADPH oxidase through Dectin-1.

The effect of laminarin on Zymosan and OPN-stimulated cells is shown in Figure 1A. Monocytes were pre-treated with laminarin for 1 hour followed by stimulation with Zymosan or OPN for 1 hour, which is the time frame we use when measuring $O_2^{\cdot-}$ release in human monocytes. Our results demonstrate that Dectin-1 regulates OPN- induced $O_2^{\cdot-}$ production in primary human monocytes. Zymosan signaling through Dectin-1 is supportive of our results from chapter II. In Figure 1B we carried out a dose response experiment using increasing levels of OPN. OPN concentrations used in this experiment are similar to OPN plasma levels found in patients with chronic inflammatory disease. Results indicate that increasing OPN concentration increases the level of $O_2^{\cdot-}$ release in monocytes and laminarin showed inhibition in all three doses.

A)



7) B)

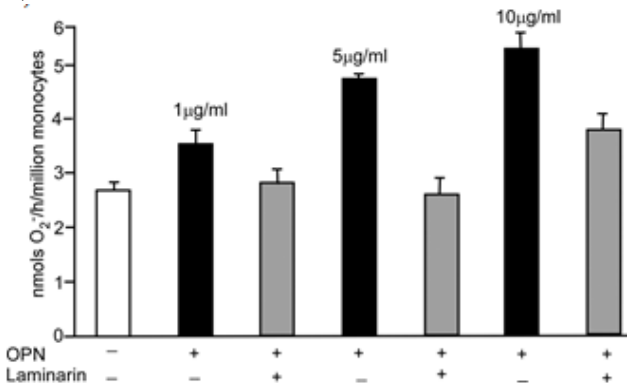


Fig. 1 Osteopontin stimulates superoxide anion production through Dectin-1

Primary human monocytes were plated in 24-well plates (500µl; $1x10^6$/ml). Monocytes were activated with Zymosan (100µg/ml), OPN (5µg/ml) in the presence or absence of laminarin (500µg/ml). Cells were pretreated with laminarin for 1 hour. Superoxide anion production was measured during the first hour of activation. Data represent the mean ± SD (n=3).

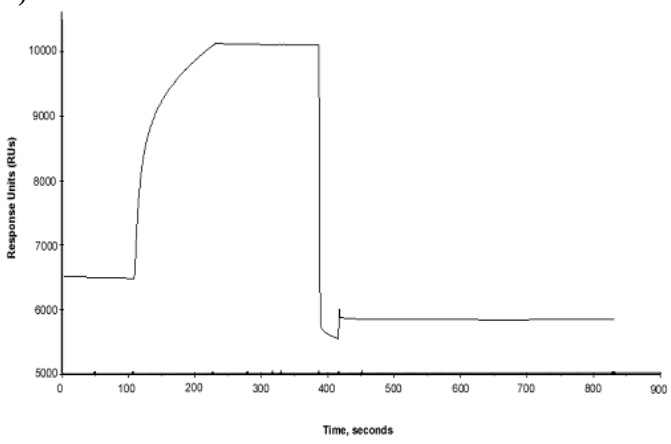
Osteopontin binds to Dectin-1

We wanted to see if Osteopontin binds to Dectin-1 directly. We examined the ability of recombinant human Osteopontin to interact with Dectin-1. A mature recombinant human Osteopontin was obtained commercially and based on the N-terminal sequencing, the protein encompasses residues IIe 17-Asn 314 and has calculated molecular mass of ~ 33 kDa. OPN is post-translationally modified and due to glycosylation it migrates at ~ 65 kDa in SDS-PAGE under reducing conditions. The DNA sequence used to generate recombinant human Dectin-1 codes for the extracellular domain of Dectin-1 (amino acid residues 66-201). Both recombinant proteins were expressed in a mouse myeloma cell line, NS0.

The capacity of Dectin-1 to bind osteopontin and other ligands was tested using SPR. Dectin-1 was coupled to the CM5 chip. First we wanted to test the ability of Dectin-1 to binding its own antibody. Figure 2A shows a sensogram that represent strong binding of Dectin-1 to human Dectin-1 monoclonal antibody. This was done to confirm the efficiency of Dectin-1 immobilization and activation on the chip. Then we tested the binding of osteopontin to Dectin-1. A representative set of SPR profiles across a range of osteopontin concentrations flowed over Dectin-1 surface was also determined (Figure 2B). Increasing the concentration of OPN increases its binding to Dectin-1. The average dissociation constant (K_d) value of OPN binding to Dectin-1 which is the maximal responses achieved at equilibrium for each OPN concentration was 3.9 nM. In figure 2C we show that posttranslational modification of OPN is required for its efficient binding to Dectin-1. De-glycosylated and De-phosphorylated OPN do not bind to Dectin-1 as observed in the sensogram of figure 2C.

In Figure 2D we show laminarin binding to Dectin-1, we then injected CD44 and CD36 to test their binding to laminarin. No binding was detected between CD44 or CD36 and laminarin. This was done to confirm exclusive binding of laminarin to Dectin-1 which confirms OPN signaling through Dectin-1 for the activation of NADPH oxidase in monocytes. Finally, we tested the binding of OPN to CD44 and CD36. In Figure 2E, we show direct binding of OPN to CD44, a known receptor of OPN, but no binding to CD36 (used as a negative control).

A)



B)

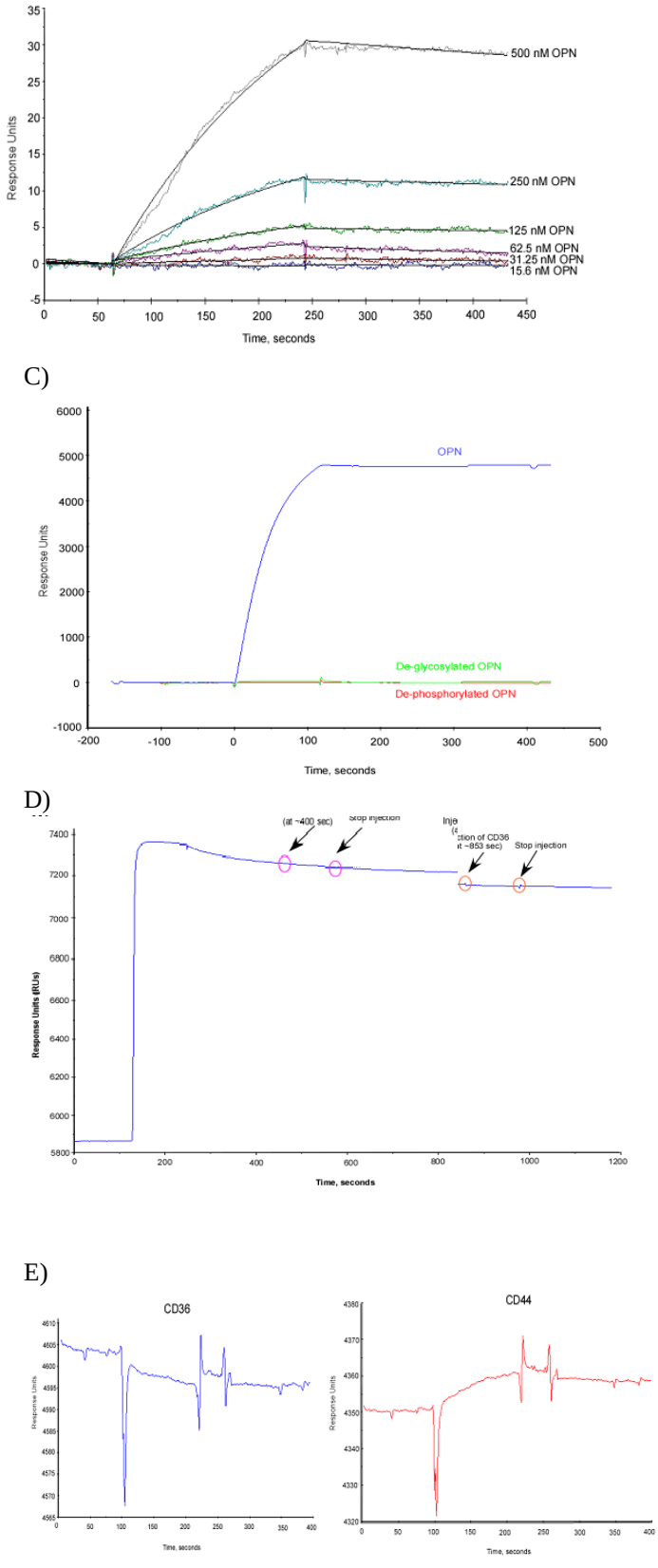


Fig. 2 Dectin-1 binds directly to OPN

A) A sensorgram presenting binding of Dectin-1 antibody (2nM) to immobilized Dectin-1. B) Representative profiles of the SPR responses for osteopontin binding (concentrations ranging from 15.6 nM to 500 nM) to Dectin-1in Hank's BSS media. RU indicates response/resonance units. C) Comparing OPN, de-lgycosylated OPN and de-phophosrylated OPN (all at 250 nM) binding to Dectin-1. D) Upper panel shows binding of laminarin (2nM) to Dectin-1 followed by injection of CD44 and CD36. E) Testing OPN binding to CD44 and CD36. Studies were done using Hank's (HBSS) buffer supplemented with 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 1 mM $MgCl_2$

IV. Discussion

Dectin-1 is a pattern recognition receptor that binds to β-glucan rich pathogens. The present experiments show that Dectin-1 can also bind other types of ligands that are non-microbial in nature. We show that osteopontin (OPN), a phosphorylated glycoprotein adhesion molecule, is a different type of ligand for Dectin-1. Our studies demonstrate that laminarin, a Dectin-1 inhibitor, blocks osteopontin-induced superoxide anion production. Osteopontin binds to Dectin-1 recombinant protein and adheres to monocytes after activation of additional receptors.

Lai and coworkers (51) have shown in a recent study that OPN regulates the production of superoxide anion production and NADPH oxidase (NOX2) subunit accumulation in aortic vascular smooth muscle cells. They show that OPN-derived superoxide anion and oxylipids promote upregulation of MMP-9 during high glucose conditions in vitro. In this study we show data supporting the role of OPN in activating NADPH oxidase. Our experiments were done using primary human monocytes and we were more interested to know whether Dectin-1 is involved in OPN-induced superoxide production. The fact that OPN signals through Dectin-1 for the activation of NADPH oxidase and ROS production in human monocytes is very relevant because OPN promotes the development and progression of atherosclerosis and vascular remodeling (49). It is also highly expressed in atherosclerotic lesions and found in association with monocyte-derived macrophages and foam cells. It would be interesting to do further experiments to identify downstream signaling molecules that regulate OPN signaling through Dectin-1 and NADPH oxidase activation.

Binding of osteopontin to Dectin-1 was confirmed using the SPR method. Surface Plasmon Resonance is a powerful method used to measure interactions between biomolecules in real-time without the need to label the interactants. The concept is to immobilize one of the interactants (ligand) on the sensor chip and pass the other molecule (analyte) in solution over the surface. Binding and dissociation are presented in a graph known as the sensogram and levels of association are presented as response/resonance units (RUs). In our experiments Dectin-1 was immobilized on the sensor chip and osteopontin at different concentrations was passed over the surface. Binding between the two proteins is shown by the sensograms in Figure 18. In the de-glycosylated and de-phosphorylated form, OPN does not bind to Dectin-1. We confirmed Dectin-1 immobilization and activation by showing binding to laminarin and Dectin-1 monoclonal antibody. Furthermore, OPN binds to CD44 but not to CD36. We also show that laminarin does not bind to CD44 or CD36. This information is required to confirm that laminarin specifically blocked OPN-induced superoxide through Dectin-1 and not CD44 or other pattern recognition receptors such as CD36. Additional studies are required to determine the exact

binding site for OPN recognition on Dectin-1 as our data indicates that the β-glucan site is required which is demonstrated by laminarin inhibition but other sites are yet to be investigated.

Taken together, our study provides new insights into Dectin-1 ligands in primary human monocytes and introduces OPN as a novel non-microbial ligand for Dectin-1. This study highlights novel findings that are relevant for understanding this pattern recognition receptor and its role in the innate immunity and in chronic inflammatory diseases.

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