Published in final edited form as:

Matrix Biol. 2015 January ; 41: 19–25. doi:10.1016/j.matbio.2014.11.005.

Osteopontin binding to the alpha 4 integrin requires highest affinity integrin conformation, but is independent of post-translational modifications of osteopontin

Tommy Hui¹, Esben S. Sørensen², and Susan R Rittling^{1,3,*}

¹Department of Immunology and Infectious Disease, The Forsyth Institute, Cambridge MA

²Department of Molecular Biology and Genetics, Aarhus University, Aarhus Denmark

³Department of Developmental Biology, Harvard School of Dental Medicine, Boston MA

Abstract

Osteopontin (OPN) is a ligand for the $\alpha 4$ integrin, but the physiological importance of this binding is not well understood. Here, we have assessed the effect of posttranslational modifications on OPN binding to the $\alpha 4$ integrin on cultured human leukocyte cell lines, and compared OPN interaction with $\alpha 4$ integrin to that of VCAM and fibronectin. Jurkat cells, whose $\alpha 4$ integrins are inherently activated, adhered to different preparations of OPN in the presence of Mn^{2+} : the EC50 of adhesion was not affected by phosphorylation or glycosylation status. Thrombin cleavage of OPN at the C-terminus of the $\alpha 4$ integrin binding site also did not affect binding affinity. THP-1 cells express a low affinity conformation of the integrin and adhered to OPN only in the presence of Mn^{2+} plus PMA or an activating antibody. This was in contrast to VCAM and fibronectin: THP-1 cells adhered to these ligands without integrin activation. Studies with ligand induced binding site antibodies demonstrated that the SVVYGLR peptide of OPN bound to the $\alpha 4$ integrin with a similar affinity as the LDV peptide of fibronectin, suggesting that a high off-rate is responsible for the reduced binding of OPN to the low affinity forms of this integrin. Together, the results suggest OPN has very low affinity for the $\alpha 4$ integrin on human leukocytes under physiological conditions.

Keywords

Osteopontin; Adhesion; Integrin Alpha4; VLA-4; Phosphorylation; VCAM

1. Introduction

The $\alpha 4\beta 1$ integrin is expressed on many leukocytes, and is an important mediator of extravasation of leukocytes from the circulation to sites of inflammation through its binding to VCAM expressed on endothelial cells (Rose et al., 2002). The importance of this interaction in the maintenance of inflammation is illustrated by the effectiveness of natalizumab, a monoclonal antibody that blocks the $\alpha 4\beta 1$ integrin, in suppressing the

*To whom correspondence should be addressed sritting@foryth.org 617-892-8450.

symptoms of multiple sclerosis by inhibiting extravasation of myelin-reactive T cells, thereby limiting the associated inflammation (Engelhardt and Briskin, 2005). The $\alpha 4\beta 1$ integrin can be found in a series of activation states comprising a resting state, several intermediate states and a fully activated, unbent conformation (Chigaev et al., 2001; Chigaev and Sklar, 2012). Chemokines, such as SDF and FMLP, regulate $\alpha 4$ activation state, generating high affinity binding to its ligand at sites of inflammation, where these chemokines are produced, and enhancing leukocyte tethering, adhesion and extravasation at such sites (Sanz-Rodriguez et al., 2001). Thus, regulation of $\alpha 4$ integrin affinity represents an important mechanism for the regulation of inflammation.

Osteopontin (OPN) is a secreted phosphorylated glycoprotein that binds to several distinct integrins. While OPN is matrix associated in bone (McKee and Nanci, 1996), its association with the extracellular matrix in soft tissues remains controversial (Rittling et al., 2002). OPN is a high affinity ligand for the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins (Hu et al., 1995a; Hu et al., 1995b) and binds the $\alpha 5\beta 1$ integrin (Barry et al., 2000b), all through its RGD sequence. Adjacent to the RGD, the SVVYGLR sequence of human OPN mediates binding to both $\alpha 4\beta 1$ and $\alpha 9\beta 1$ integrins. OPN is glycosylated in mammalian cells, and is variably phosphorylated, with up to 36 phosphorylation sites identified on milk osteopontin (Christensen et al., 2005), while tumor cell-produced OPN averages only four phosphates per molecule; the degree of phosphorylation can in some cases regulate cell adhesion. Further, OPN is a substrate for thrombin and other proteases that cleave OPN just C-terminal to the $\alpha 4$ binding site (Christensen et al., 2007; Christensen et al., 2010). Osteopontin has also been implicated in the development of multiple sclerosis (Steinman, 2009), suggesting that its $\alpha 4\beta 1$ interaction may be important in this disease.

The physiological role of the OPN- $\alpha 4$ interaction is still unclear. Since OPN is present physiologically with varying degrees of phosphorylation, we have asked if post-translational modification regulates the ability of OPN to interact with the $\alpha 4$ integrin on human leukocytic cell lines. Furthermore, we compared OPN binding to this integrin to the binding of the well-studied $\alpha 4$ ligands VCAM and fibronectin. We find that thrombin cleavage and post-translational modification do not regulate the affinity of the OPN $\alpha 4$ - integrin interaction, but that the affinity of OPN is significantly lower than that of VCAM. Importantly, OPN only binds to the $\alpha 4\beta 1$ integrin when the integrin is in its highest affinity state, and OPN at physiological concentrations cannot induce the high affinity conformation. We conclude that while most forms of OPN can interact with the $\alpha 4$ integrin, any physiological function of OPN as a ligand for the $\alpha 4$ integrin would be limited to situations where the integrin is in the highest affinity state.

2. Results

2.1 Protein Preparations

To determine whether post-translational modifications affect the binding of OPN to the $\alpha 4$ integrin, we selected a series of OPN forms with different modifications. Human milk OPN was used as the most highly phosphorylated form (mOPN (Christensen et al., 2005)). The phosphorylation status of commercially available recombinant OPN prepared in mammalian cells (rmOPN, from R&D or Peprotech) is unknown, but it is likely a low level of

phosphorylation; in addition, both these proteins are expected to be glycosylated. Bacterially produced OPN (rbOPN,) is expected to be neither glycosylated nor phosphorylated. The mutated recombinant bacterially produced protein, RAA OPN, has the RGD sequence mutated to RAA, and includes only the N-terminal half of the protein, terminating at the C-terminal arginine residue of the $\alpha 4$ binding sequence (Ito et al., 2009). To further isolate the SVVYGLR sequence we prepared SVVYGLR peptide crosslinked to BSA (SVV-BSA), and its corollary for fibronectin, EILDVPST-BSA (LDV-BSA). Full length OPN protein was cleaved with thrombin which cuts OPN into two parts with similar molecular weight (N-terminal half = 17094; C-terminal half = 16831). The purity and migration patterns of these proteins were analyzed by SDS-PAGE (Figure 1). Although OPN has a molecular weight of about 35,000 daltons, it migrates more slowly on SDS PAGE than expected, largely because of its low Pi, and this migration difference is exacerbated by phosphorylation. mOPN migrates more slowly than recombinant OPN made in cultured cells (rmOPN), suggesting that rmOPN is not highly phosphorylated. This difference in migration is also seen in the thrombin cleaved forms of these molecules. Bacterially produced OPN, which is expected to be neither phosphorylated nor glycosylated migrates at a similar position as rmOPN, further supporting a low level of phosphorylation of rmOPN. RAA OPN migrates at around 33 KD: lower bands are degradation products while the 66Kd band is a contaminating bacterial protein (determined by mass spectrometry). The peptide-BSA conjugates migrate as a smear more slowly than BSA confirming the presence of multiple copies of the peptides (calculated ratio = average of 14 peptides/BSA molecule).

To confirm the glycosylation status of rmOPN, proteins were separated by gel electrophoresis and stained for glycosylations using a modified periodic acid-schiff technique. Strong staining of mOPN was observed with weaker but clearly detectable staining of rmOPN. rbOPN as expected did not stain under these conditions (Figure 1B).

The expression of the $\alpha 4$ and other OPN-binding integrins on Jurkat and THP-1 cells was confirmed by flow cytometry. Both cell lines express the $\alpha 4$ and $\alpha 5$ integrins, but no expression of αv or $\alpha 9$ integrins could be detected (Figure 1 B and C). Thus the only integrin that can bind OPN through the RGD sequence on these cells is the $\alpha 5$ integrin.

2.2 $\alpha 4$ integrin interacts with different forms of OPN

The Jurkat T cell leukemia cell line (Schneider et al., 1977) has been previously shown to bind to recombinant N-terminal OPN, and adhesion of these cells to this form of OPN was exclusively through the $\alpha 4$ integrin (Pepinsky et al., 2002). Therefore, this cell line was used to determine the structural requirements for OPN binding to the $\alpha 4$ integrin by using a cell adhesion assay. Although Jurkat cells were reported to bind to OPN in the presence of Ca^{2+} and Mg^{2+} only (Pepinsky et al., 2002), in our hands, there was no adhesion of these cells to any of these forms of OPN unless the integrins were activated with either MnCl_2 (1 mM) or PMA (50 $\mu\text{g/ml}$) or both (Figure 2A and data not shown). This is in contrast to the well characterized $\alpha 4$ ligands VCAM and fibronectin, which can both support robust adhesion in the absence of integrin activation. In the presence of MnCl_2 , however, Jurkat cells bind efficiently to all forms of OPN and VCAM, as well as to SVV-BSA and RAA OPN, which lack RGD sequences (Figure 2B). In the presence of MnCl_2 , adhesion to OPN is completely

abolished by the HP2/1 $\alpha 4$ blocking antibody (Figure 2C) confirming that these cells bind to OPN exclusively through the $\alpha 4$ integrin under these conditions. Addition of PMA appears to increase adhesion of Jurkat cells to all forms of OPN, but the differences are not significant. In addition, adhesion to OPN in the presence of PMA is not completely blocked by HP2/1 (Figure 2C), suggesting that PMA activates adhesion to OPN through the $\alpha 5\beta 1$ integrin. The efficiency with which each protein preparation was able to bind to the wells during the coating process was determined as described in methods, and was similar for all form of OPN, although the bacterially produced OPNs (rbOPN and RAA OPN) bound slightly less efficiently (Figure 2D).

2.3 OPN post-translation modifications and thrombin cleavage do not regulate binding to the $\alpha 4$ integrin

The experiment of Figure 2A indicates that all forms of OPN tested can bind to the $\alpha 4$ integrin, but this experiment was performed at saturating concentrations of OPN. In order to determine if there is any modest effect of OPN modification on the affinity of binding to the $\alpha 4$ integrin, dose response experiments were conducted, and EC50s calculated for forms of OPN with different modifications. For these experiments, the concentration of OPN in each preparation was determined by amino acid analysis and adhesion was performed in the presence of MnCl₂. Figure 3A shows a representative experiment, comparing rmOPN with and without thrombin cleavage. In Figure 3B, the EC50 values for different forms of OPN are compared. The measured EC50s vary over a narrow range, from 5–18 nM, with no significant effect of thrombin cleavage or post-translational modifications. Only VCAM has a significantly lower EC50 value as compared to rmOPN and RAA OPN. We conclude that the SVVYGLR- $\alpha 4$ integrin interaction is not affected by phosphorylation or glycosylation of OPN, and that exposure of the C-terminus of the SVVYGLR sequence by thrombin cleavage does not modify affinity of this sequence for the $\alpha 4$ integrin. However, even the highest affinity form of the $\alpha 4$ integrin binds more avidly to VCAM than to OPN.

2.4 OPN binds exclusively to high affinity forms of the $\alpha 4$ integrin

$\alpha 4$ integrins can adopt a series of different conformations, resulting in different binding affinities for its ligands (Chigaev et al., 2001; Chigaev and Sklar, 2012). On Jurkat cells, the $\alpha 4$ integrins are inherently activated, while those expressed on the monocytic cell line THP-1 are in a lower affinity conformation (Yednock et al., 1995). Since we established that there is no effect of phosphorylation or thrombin cleavage on OPN- $\alpha 4$ binding, we tested adhesion of THP-1 cells to the less phosphorylated form of mammalian OPN, rmOPN and to SVV-BSA, which contains just the $\alpha 4$ binding sequence, comparing adhesion to these substrates to that to VCAM and fibronectin. FN and VCAM can support limited adhesion of THP-1 cells in the absence of any activators, and MnCl₂ alone increases adhesion of these cells to VCAM (Figure 4A). rmOPN, on the other hand cannot support adhesion of THP-1 cells under these conditions. Adhesion of THP-1 cells to OPN only occurs if MnCl₂ is combined with PMA or the activating antibody TS2/16, demonstrating that THP-1 cells adhere to OPN only when the $\alpha 4$ integrin is in the highest affinity conformation. Even under these highest affinity conditions, moreover, adhesion to OPN is less than to VCAM, when both are present at saturating concentrations, consistent with the higher affinity of the VCAM- $\alpha 4$ integrin interaction. SVV-BSA on the other hand, can support limited adhesion

of THP-1 cells in the presence of MnCl₂ or PMA only, with highest level adhesion seen under conditions of full activation. These results suggest that the increased concentration of α 4 ligands in this molecule (nominally 14 peptides/BSA molecule) may partially compensate for the lower affinity of the less open conformations. Unlike Jurkat cells, PMA in THP-1 cells seems to stimulate adhesion only through the α 4 integrin, since SVV-BSA lacks the RGD sequence and cannot bind the RGD-binding integrin α 5 β 1. This is confirmed by the observation that adhesion to rmOPN in the presence of MnCl₂ and PMA is completely blocked with the HP2/1 antibody (Figure 4B). THP-1 cells also bind VCAM exclusively through the α 4 integrin, while much of these cells' adhesion to FN is not blocked by an anti α 4 antibody (Figure 4B), likely representing α 5 binding.

2.5 Using the LIBS assay, OPN and CS-1 peptides show similar binding to α 4 β 1

The binding of the LIBS (ligand-induced binding site) antibody B44, which recognizes an epitope in the β 1 subunit of α 4 β 1, is induced when the integrin is bound to its ligands, reflecting the ability of these ligands to induce a conformational change in the integrin exposing the antibody binding site. We found that binding of a sub-saturating dose of B44 to THP-1 cells is increased in a dose dependent manner by VCAM, as shown previously (Vanderslice et al., 2010), confirming that soluble VCAM, even at a relatively low concentration (5×10^{-8} M) is able to bind to the α 4 integrin in untreated THP-1 cells and induce the active conformation (Figure 5A). Soluble OPN (mOPN or the RAA N-terminal recombinant protein) at a maximal dose of 5×10^{-7} M on the other hand, was unable to induce antibody binding (Figure 4A), indicating that soluble OPN at physiological concentrations is unable to bind to the low affinity state of the α 4 β 1 integrin. As expected, MnCl₂, PMA and LDV peptide were all able to induce the high affinity state of the integrin, as assessed by B44 binding (Figure 5B and data not shown). Interestingly, the SVVYGLR peptide of human OPN was able to induce B44 binding, although not as efficiently as the LDV peptide (Figure 5B). The EC₅₀s for LDV and SVVYGLR were similar (3.11×10^{-4} M and 5.57×10^{-4} M, respectively), while the maximal binding of SVVYGLR was about 50% that of LDV. Substitution of alanine for leucine in SVVYGAR reduced the EC₅₀ by more than 15-fold to 8.8×10^{-3} M in agreement with previous determination of the importance of this amino acid in α 4 binding (Green et al., 2001; Ito et al., 2009).

3. Discussion

While the ability of OPN to bind to the α 4 integrin has long been recognized (Barry et al., 2000a; Bayless et al., 1998), the physiological importance of this binding remains unclear. We have for the first time compared directly the α 4 integrin binding ability of OPN to that of its other well characterized ligands, VCAM and the CS-1 sequence (Katoh et al., 1998) of fibronectin. We show that integrin activation is absolutely required for OPN binding to the α 4 integrin. We found no evidence for soluble or immobilized OPN binding to the low affinity integrin, in contrast to VCAM, which can bind to unstimulated cells in solution, and can support adhesion of cells under unstimulated conditions. Further, we show that OPN binds to this integrin independently of its post-translational modifications, and that thrombin cleavage does not affect α 4 β 1-OPN interaction.

The regulation of the affinity of the $\alpha 4$ integrin for a standard peptide ligand LDV has been extensively studied (reviewed in (Chigaev and Sklar, 2012)). Binding affinity is regulated by both the conformation of the integrin, in transitioning from a bent to an extended conformation, as well as the affinity of the ligand binding site. In the resting state, which is found in cells in low concentrations of Ca^{2+} , the integrin is in a bent conformation, and the ligand binding site is low affinity. PMA can activate the ligand binding site to high affinity, but does not result in unbending. Mn^{2+} on the other hand is able to promote the unbent state (Chigaev et al., 2003), while LIBS antibodies bind to the hinge region, which is exposed upon activation or ligand binding (Chigaev et al., 2009). In THP-1 cells, activation of the $\alpha 4$ integrin by PMA or Mn^{2+} alone does not induce a conformation that is able to bind OPN, and OPN at physiological concentrations cannot cause the conformational change required for LIBS antibody binding. Thus, we conclude that OPN can only bind to the very highest affinity state of the integrin, where the integrin is both unbent and the ligand binding site is in high affinity conformation.

In contrast to these results with the intact proteins, we found that the SVVYGLR peptide of OPN (at 20–20,000 fold higher concentration than full length OPN) can induce LIBS antibody binding with similar kinetics to that of the LDV-containing peptide. This apparent paradox can be explained by consideration of the mechanism of the LIBS antibody assay. Rather than detecting peptide-integrin binding directly, this assay detects binding of the B44 antibody to its binding site on the $\beta 1$ integrin. Since antibody binding is typically high affinity and relatively stable, the B44 antibody will bind to activated integrins once the binding site is exposed regardless of ligand occupancy: for instance, Mn^{2+} induces B44 binding. Thus, this assay measures the association of the peptides with the integrin, but not the dissociation, or off-rate: if the ligand dissociates, the antibody will remain bound. Taken together, therefore, our results support a model where the k_{on} for LDV and SVVYGLR binding to the $\alpha 4$ integrin are similar, while the k_{off} is much higher for SVVYGLR. Similarly, the observation that SVV-BSA with its high density of ligands is able to support adhesion of cells with lower affinity forms of the $\alpha 4$ integrin than can the whole molecule (Figure 4) is also consistent with a high off rate for the SVVYGLR peptide interaction with low affinity integrins. In the presence of this high concentration of ligands, after dissociation of integrin-peptide binding, new binding interactions could be rapidly established. These results suggest that at concentrations that can be achieved with intact OPN (up to 5×10^{-7} M, or 20 $\mu\text{g/ml}$), the SVVYGLR sequence is unable to establish a stable association with all but the very highest affinity conformations of the $\alpha 4$ integrin.

While the inherently activated $\alpha 4$ integrins on cells such as Jurkat are useful for studying the ligand binding under optimal conditions, the lower affinity form of the integrin as found on THP-1 cells is a more physiologically relevant model. Indeed, the activation state of $\alpha 4$ integrins on normal blood monocytes are found in a similar affinity state as on THP-1 cells (Yednock et al., 1995), suggesting that OPN would not bind to resting blood monocyte $\alpha 4$ integrins. While chemokines can increase the affinity of integrins during leukocyte extravasation, the Mn^{2+} induced integrin has a lower K_d than the integrin on chemokine-treated cells (Chigaev et al., 2003). Physiological effects of OPN mediated by $\alpha 4$ integrins have been suggested for mouse bone marrow macrophages (Lund et al., 2013) and hematopoietic stem cells (Grassinger et al., 2009). The regulation of the affinity of the $\alpha 4$

integrin on these cell types is unknown, and our results suggest that the $\alpha 4$ integrins on these cell types may be in the open, high affinity conformation for OPN to mediate functional effects. It is also possible that the requirements for integrin activation may be different in these mouse systems.

Our results demonstrate further that there is no effect of OPN post-translational modifications, including both phosphorylation and glycosylation, on the binding ability of the $\alpha 4$ integrin on Jurkat cells. The EC₅₀ for adhesion of Jurkat cells to OPN was not significantly different between the most highly phosphorylated form of OPN – milk OPN – and the non-phosphorylated form rbOPN. The similar EC₅₀ of RAAOPN, rbOPN and rmOPN demonstrates further that glycosylation does not regulate this interaction. These results are of interest because the post-translational modifications, primarily phosphorylation, vary on different forms of OPN (Christensen et al., 2007), and have not been characterized on OPN in most situations, such as that expressed by various immune cells. In some cases, these modifications have been shown to affect cell adhesion through the $\alpha_v\beta_3$ integrin (Christensen et al., 2007; Christensen et al., 2012), but our data indicate that this is not the case for the $\alpha 4$ integrin. The role of thrombin cleavage in OPN binding to $\alpha 4$ integrin has been controversial. In experiments using peptides to inhibit this interaction, the C-terminal COOH group was required for efficient SVVYGLR inhibition of Jurkat cell adhesion to OPN (Green et al., 2001). In addition, thrombin cleavage was reported to modestly increase the ability of Jurkat cells to adhere to OPN, when OPN was cleaved with thrombin after binding to the plate (Myles et al., 2003). However, thrombin cleavage of OPN is not required for $\alpha 4\beta 1$ binding (Ito et al., 2009), and our results demonstrate that thrombin cleavage does not affect the affinity of the interaction under our experimental conditions. Additional work will be required to resolve these different results.

In summary, we report here the first direct comparison of OPN binding to the $\alpha 4$ integrin with that of other physiological ligands, and demonstrate that only the highest affinity conformation of this integrin can interact with OPN, likely due to a high off-rate for this ligand for lower affinity forms of the integrin. In addition, we demonstrate that post-translational modifications and cleavage by thrombin do not affect the affinity of OPN for the $\alpha 4$ integrin. The physiological role of the OPN- $\alpha 4$ integrin interaction should be considered carefully in light of these findings.

4. Experimental Procedures

4.1 Osteopontin Preparations

OPN from human milk was purchased from BD, Lee Biotech or purified as previously described (Christensen et al., 2005). Human OPN (isoform A) and murine OPN – N-terminal half (aa 1–142)(RAA mutant, (Ito et al., 2009) were cloned into pGEX-6P-1 and purified from bacterial lysates by chromatography on glutathione-sepharose followed by elution with Precision Protease (GE Life Sciences) as described (Ito et al., 2009). There was no difference in the ability of full length mouse and human rbOPN to support adhesion of Jurkat cells in MnCl₂ (data not shown). 12 μ g of OPN was cleaved with 0.5 unit of thrombin (Millipore, restriction grade) in a total volume of 300 μ l of thrombin cleavage buffer supplied by the manufacturer. Recombinant human mammalian OPN and VCAM

were purchased from Peprotech or R&D systems; plasma fibronectin, PMA and BCECF were obtained from Sigma. CGGGSVVYGLR peptide and CGGGEILDVPST were conjugated via cysteine to BSA (Genscript): an equal weight of BSA was used and about 14 peptides are expected to be coupled per BSA molecule. SDS Page was performed using AnyKd precast gels in tris glycine buffer (Biorad); non-ammoniacal silver staining was performed as described (Ausubel, 1995). Pro-Q Emerald (Invitrogen) staining for glycoproteins was performed according to the supplier's instructions.

4.2 Adhesion Assays

Corning 3590 96 well plates were coated overnight at 4°C with proteins diluted in PBS at 1000 ng/well or as indicated except for VCAM which was used at 250 ng/well. To compare the ability of the different protein preparations to bind to the plate, 100 µl of a 10 µg/ml solution of each preparation was added to triplicate wells and incubated overnight at 4°C. After washing the wells with PBS, the amount of protein bound was determined by BCA assay, and expressed as a percentage of the total protein added to the well determined by BCA assay of the soluble protein. A modified BCA assay was used, with a total assay volume of 0.1 ml, incubated at 60°C for 2 hours. The limit of detection of this assay was calculated at 0.1 µg/ml protein using BSA as a standard. The concentration of OPN preparations used for EC50 experiments was determined by amino acid analysis. THP-1 (ATCC) and Jurkat cells were cultured in suspension in RPMI 1640 (ATCC) in 10% FBS (Hyclone) with 0.05 M β-mercaptoethanol added for THP-1 cells. Cells were harvested by centrifugation and resuspended in HBS buffer (25mM Hepes, 5 mM glucose, 150 mM NaCl, 2.5 mM KCl, 1 mg/ml BSA, pH 7.4 with 1 mM each CaCl₂ and MgCl₂) containing BCECF (2.5 µg/ml), and incubated for 30 min at 37°C. After washing with PBS, labeled cells were added to coated wells in HBS; MnCl₂ (1 mM) PMA (50 ng/ml), and/or antibodies were added as indicated. Cells were allowed to adhere for 1 hour at 37°C, and total fluorescence determined using an Optima plate reader (excitation 485, emission 520 nm), with gain adjustment for maximal fluorescent detection. Plates were centrifuged upside down for 5 minutes at 48 × g, culture medium removed, and remaining fluorescence in each well was determined using the same gain settings. Percent adhesion was calculated by dividing remaining fluorescence by original fluorescence after subtraction of background fluorescence from empty wells. In some cases, percent fluorescence was more than 100%, so the data were normalized to bring all values to 100% or less. Data shown are representative of 2–3 independent experiments, with significance determined by ANOVA as indicated.

4.3 Flow Cytometry

Expression of OPN-binding integrins on THP and Jurkat cells was determined by flow cytometry using antibodies to αv (clone 23C6, Biolegend), α9 (Y9A2, Biolegend), α5 (P1D6, Santa Cruz) and α4 (P1H4, Millipore) integrins. HP2/1 (Serotec) was used for blocking at 10 µg/ml; the activating antibody TS2/16 (Biolegend) was used at 5 µg/ml. For LIBS experiments, THP-1 cells were incubated with the indicated proteins or peptides in the presence of 3 µg/ml B44 antibody (Millipore) as described (Vanderslice et al., 2010) in HBS with 1 mM MgCl₂ and CaCl₂. Cells were incubated at room temperature (RT) for 1 hr., then washed and FITC anti- mouse IgG antibody was added. After an additional 30 minutes

incubation at RT, cells were washed and flow cytometry used to determine the mean fluorescence intensity (MFI) of each sample. MFI of cells incubated with antibody alone was subtracted and data were fit to a sigmoidal dose response curve using Graph-Pad Prism, and the EC50 was calculated.

4.4 Statistical Analyses

Significance of difference among groups was determined by one-way ANOVA with Bonferroni's post-test using Graph Pad Prism. For dose response experiments, data were fit to a sigmoidal dose response curve and EC50 calculated using Graph Pad Prism. Data are shown as mean $\pm$ SEM.

Acknowledgments

The authors thank Dr. Toshimitsu Uede for the kind gift of the RAA OPN construct, Dr. Aritsune Matsui for help with the FACS analysis, Dr. Toshi Kawai for the gift of Jurkat cells, and Dr. Markus Hardt for mass spectrometry. Research reported in this publication was supported by the NIDCR of the NIH under award # RO1DE022380. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations

OPN	osteopontin
MFI	mean fluorescence intensity
mOPN	milk osteopontin
rmOPN	recombinant mammalian OPN
rbOPN	recombinant bacterial OPN
RAA OPN	N-terminal OPN half with RGD mutated to RAA
SVV-BSA	CGGSVVYGLR peptide crosslinked to BSA
LDV-BSA	CGGGEILDVPST peptide crosslinked to BSA
FN	fibronectin
PMA	phorbol-12-myristate-13-acetate
BCECF	2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein
LIBS	ligand-induced binding site
BCA	bicinchoninic acid assay
CS-1	connecting segment-1 peptide of fibronectin.

References

Ausubel, F.; B, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K. Short protocols in molecular biology. John Wiley & Sons; New York: 1995.

- Barry ST, Ludbrook SB, Murrison E, Horgan CM. Analysis of the $\alpha 4 \beta 1$ integrin-osteopontin interaction. *Exp Cell Res*. 2000a; 258:342–351. [PubMed: 10896785]
- Barry ST, Ludbrook SB, Murrison E, Horgan CM. A regulated interaction between $\alpha 5 \beta 1$ integrin and osteopontin. *Biochem Biophys Res Commun*. 2000b; 267:764–769. [PubMed: 10673366]
- Bayless KJ, Meininger GA, Scholtz JM, Davis GE. Osteopontin is a ligand for the $\alpha 4 \beta 1$ integrin. *J Cell Sci*. 1998; 111:1165–1174. [PubMed: 9547293]
- Chigaev A, Blenc AM, Braaten JV, Kumaraswamy N, Kepley CL, Andrews RP, Oliver JM, Edwards BS, Prossnitz ER, Larson RS, Sklar LA. Real time analysis of the affinity regulation of $\alpha 4$ -integrin. The physiologically activated receptor is intermediate in affinity between resting and $Mn(2+)$ or antibody activation. *J Biol Chem*. 2001; 276:48670–48678. [PubMed: 11641394]
- Chigaev A, Buranda T, Dwyer DC, Prossnitz ER, Sklar LA. FRET detection of cellular $\alpha 4$ -integrin conformational activation. *Biophys J*. 2003; 85:3951–3962. [PubMed: 14645084]
- Chigaev A, Sklar LA. Aspects of VLA-4 and LFA-1 regulation that may contribute to rolling and firm adhesion. *Front Immunol*. 2012; 3:242. [PubMed: 22876249]
- Chigaev A, Waller A, Amit O, Halip L, Bologna CG, Sklar LA. Real-time analysis of conformation-sensitive antibody binding provides new insights into integrin conformational regulation. *J Biol Chem*. 2009; 284:14337–14346. [PubMed: 19251697]
- Christensen B, Kazanekki CC, Petersen TE, Rittling SR, Denhardt DT, Sorensen ES. Cell type-specific post-translational modifications of mouse osteopontin are associated with different adhesive properties. *J Biol Chem*. 2007; 282:19463–19472. [PubMed: 17500062]
- Christensen B, Klaning E, Nielsen MS, Andersen MH, Sorensen ES. C-terminal modification of osteopontin inhibits interaction with the $\alpha V \beta 3$ -integrin. *J Biol Chem*. 2012; 287:3788–3797. [PubMed: 22179617]
- Christensen B, Nielsen MS, Haselmann KF, Petersen TE, Sorensen ES. Post-translationally modified residues of native human osteopontin are located in clusters: identification of 36 phosphorylation and five O-glycosylation sites and their biological implications. *Biochem J*. 2005; 390:285–292. [PubMed: 15869464]
- Christensen B, Schack L, Klaning E, Sorensen ES. Osteopontin is cleaved at multiple sites close to its integrin-binding motifs in milk and is a novel substrate for plasmin and cathepsin D. *J Biol Chem*. 2010; 285:7929–7937. [PubMed: 20071328]
- Engelhardt B, Briskin MJ. Therapeutic targeting of $\alpha 4$ -integrins in chronic inflammatory diseases: tipping the scales of risk towards benefit? *Eur J Immunol*. 2005; 35:2268–2273. [PubMed: 16052610]
- Grassinger J, Haylock DN, Storan MJ, Haines GO, Williams B, Whitty GA, Vinson AR, Be CL, Li S, Sorensen ES, Tam PP, Denhardt DT, Sheppard D, Choong PF, Nilsson SK. Thrombin-cleaved osteopontin regulates hemopoietic stem and progenitor cell functions through interactions with $\alpha 9 \beta 1$ and $\alpha 4 \beta 1$ integrins. *Blood*. 2009; 114:49–59. [PubMed: 19417209]
- Green PM, Ludbrook SB, Miller DD, Horgan CM, Barry ST. Structural elements of the osteopontin SVVYGLR motif important for the interaction with $\alpha 4$ integrins. *FEBS Lett*. 2001; 503:75–79. [PubMed: 11513858]
- Hu DD, Hoyer JR, Smith JW. Calcium suppresses cell adhesion to osteopontin by attenuating binding affinity for integrin $\alpha v \beta 3$. *J Biol Chem*. 1995a; 270:9917–9925. [PubMed: 7537271]
- Hu DD, Lin ECK, Kovach NL, Hoyer JR, Smith JW. A biochemical characterization of the binding of osteopontin to integrins $\alpha v \beta 1$ and $\alpha v \beta 5$. *J Biol Chem*. 1995b; 270:26232–26238. [PubMed: 7592829]
- Ito K, Kon S, Nakayama Y, Kurotaki D, Saito Y, Kanayama M, Kimura C, Diao H, Morimoto J, Matsui Y, Uede T. The differential amino acid requirement within osteopontin in $[\alpha] 4$ and $[\alpha] 9$ integrin-mediated cell binding and migration. *Matrix Biol*. 2009; 28:11–19. [PubMed: 19000758]
- Katoh K, Mohri H, Ogawa K, Okubo T. Human Plasma Fibronectin Mediates Adhesion of U937 Cells by RGD and CS1. *J Thromb Thrombolysis*. 1998; 5:125–134. [PubMed: 10767107]

- Lund SA, Wilson CL, Raines EW, Tang J, Giachelli CM, Scatena M. Osteopontin mediates macrophage chemotaxis via $\alpha 4$ and $\alpha 9$ integrins and survival via the $\alpha 4$ integrin. *J Cell Biochem.* 2013; 114:1194–1202. [PubMed: 23192608]
- McKee MD, Nanci A. Osteopontin at mineralized tissue interfaces in bone, teeth, and osseointegrated implants: ultrastructural distribution and implications for mineralized tissue formation, turnover, and repair. *Microsc Res Tech.* 1996; 33:141–164. [PubMed: 8845514]
- Myles T, Nishimura T, Yun TH, Nagashima M, Morser J, Patterson AJ, Pearl RG, Leung LL. Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation. *J Biol Chem.* 2003; 278:51059–51067. [PubMed: 14525995]
- Pepinsky RB, Mumford RA, Chen LL, Leone D, Amo SE, Riper GV, Whitty A, Dolinski B, Lobb RR, Dean DC, Chang LL, Raab CE, Si Q, Hagmann WK, Lingham RB. Comparative assessment of the ligand and metal ion binding properties of integrins $\alpha 9\beta 1$ and $\alpha 4\beta 1$. *Biochemistry.* 2002; 41:7125–7141. [PubMed: 12033947]
- Rittling SR, Chen Y, Feng F, Wu Y. Tumor-derived osteopontin is soluble, not matrix associated. *J Biol Chem.* 2002; 277:9175–9182. [PubMed: 11741994]
- Rose DM, Han J, Ginsberg MH. $\alpha 4$ integrins and the immune response. *Immunol Rev.* 2002; 186:118–124. [PubMed: 12234367]
- Sanz-Rodriguez F, Hidalgo A, Teixido J. Chemokine stromal cell-derived factor-1 α modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. *Blood.* 2001; 97:346–351. [PubMed: 11154207]
- Schneider U, Schwenk HU, Bornkamm G. Characterization of EBV-genome negative “null” and “T” cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *Int J Cancer.* 1977; 19:621–626. [PubMed: 68013]
- Steinman L. Shifting therapeutic attention in MS to osteopontin, type 1 and type 2 IFN. *Eur J Immunol.* 2009; 39:2358–2360. [PubMed: 19681057]
- Vanderslice P, Woodside DG, Caivano AR, Decker ER, Munsch CL, Sherwood SJ, Lejeune WS, Miyamoto YJ, McIntyre BW, Tilton RG, Dixon RA. Potent in vivo suppression of inflammation by selectively targeting the high affinity conformation of integrin $\alpha 4\beta 1$. *Biochem Biophys Res Commun.* 2010; 400:619–624. [PubMed: 20807504]
- Yednock TA, Cannon C, Vandevert C, Goldbach EG, Shaw G, Ellis DK, Liaw C, Fritz LC, Tanner LI. $\alpha 4\beta 1$ integrin-dependent cell adhesion is regulated by a low affinity receptor pool that is conformationally responsive to ligand. *J Biol Chem.* 1995; 270:28740–28750. [PubMed: 7499396]

Figure 1.

(A) SDS Page analysis of OPN proteins used in this work. Approximately 1 ug of each OPN preparation was separated on a gradient gel and stained with non-ammoniacal silver stain. mOPN -milk OPN, rmOPN – recombinant mammalian OPN, rbOPN – recombinant bacterial OPN, M-molecular weight marker. Proteins were cleaved with thrombin (Thr) as indicated. The OPN bands of interest in each lane are indicated by boxes. For rbOPN and RAA OPN, bands migrating below the main band are OPN fragments, while the protein migrating just above 66 kD is a bacterial protein (determined by mass spectrometry). The lane containing rbOPN is from a different gel. B) Staining of OPN proteins for glycosylation. mOPN, rmOPN and rbOPN as indicated were separated on SDS-PAGE gels and stained with coomassie blue (right panel) or with Pro-Q Emerald stain, which stains glycoproteins (left panel). C) FACS analysis of OPN-binding integrin expression on Jurkat

and THP-1 cells. Cells were stained with antibodies to integrins α_v (.....), α_9 —, α_5 (—), α_4 (dark grey shading), isotype control, light grey shading. Secondary antibody was anti-mouse labeled with FITC (for Jurkat cells) or PE (for THP-1 cells).

Figure 2.

Jurkat cells adhere to different forms of OPN. A: Wells were coated with fibronectin (FN, 1 ug/well, 2.2 pmol/well), VCAM (250 ng/well, 3.3pmol/well) or various forms of OPN, all at 1 ug/well (30 pmol/well,). Abbreviations as for Figure 1. Thr= thrombin cleaved. Cells were treated with nothing (open bars), 1 mM MnCl₂ (grey bars), or 1 mM MnCl₂ plus 50 ng/ml PMA (black bars) as indicated. B: Wells were coated with α4-specific ligands VCAM (250 ng/well), LDV-BSA, SVV-BSA or RAA OPN each at 1 ug/well (10, 12 and 67 pmol/well respectively). Bars as in A. C: α4 antibody blocks adhesion of Jurkat cells to different

substrates. Cells were incubated with HP2/1 anti- α 4 antibody for 45 minutes before adhesion assay in the presence of 1 mM MnCl₂ and/or 50 ug/ml PMA as indicated. A and B: ***, p<0.001 compared to BSA; ** p<0.01 compared to BSA; * p<0.05 compared to BSA. C: ***p<0.001, * p<0.05 compared to isotype control. Significance determined by one-way ANOVA. D. The efficiency of coating of each of the proteins was determined by BCA assay of coated wells, normalized to total protein added per well to calculate % coating efficiency.

Figure 3.

Affinity of Jurkat cell binding to different OPN preparations. A: Dose response analysis of Jurkat cell binding to recombinant mammalian OPN (rmOPN), either intact or thrombin cleaved, in the presence of 1 mM MnCl_2 . A sigmoidal dose response curve was fit to the data. B: EC50 values were calculated from curves generated as in A. *: $p < 0.05$ by one way ANOVA, n =average of 3 independent experiments. All other differences were not significant. Protein concentrations are those used for coating at 0.1 ml/well.

Figure 4.

Adhesion of THP-1 cells to $\alpha 4$ ligands under different conditions. Wells were coated with FN (1 μ g/well), VCAM (250 ng/well) or OPN preparations (1 μ g/well). A. Cells were treated with 1 mM MnCl₂, 50 ng/ml PMA, or both, or with 10 μ g/ml TS2/16 activating antibody with 1 mM MnCl₂. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ all compared to BSA; a= $p < 0.001$; b= $p < 0.01$. B. Cells were treated with MnCl₂ plus PMA and adhesion to the indicated substrates determined after treatment with HP2/1 neutralizing antibody or isotype control. ***, $p < 0.001$ compared to IgG.

Figure 5.

$\alpha 4$ integrin ligand binding in solution detected by LIBS antibody binding A. VCAM, rmOPN or RAA OPN in the indicated concentrations was incubated with THP-1 cells in the absence of MnCl₂. Flow cytometry was used to measure the mean fluorescence intensity of the LIBS antibody B44 bound to cells with or without ligand. MFI of cells without ligand was subtracted from MFI of cells with ligands as indicated. B. Peptides EILDVPST (LDV), SVVYGLR or SVVYGAR were added at increasing concentrations to THP-1 cells, and MFI

of B44 binding was determined as in A. Results were plotted and fitted to a sigmoidal dose-response curve. Combined results from 2–3 independent experiments are shown.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript