Osteopontin is a ligand for the $\alpha_4\beta_1$ integrin

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SUMMARY

Recent work has shown that osteopontin expression is upregulated at sites of cardiovascular injury. It has been hypothesized that osteopontin provides an adhesive matrix for endothelial and smooth muscle cells during remodeling of the vascular wall following injury. Osteopontin has also been found to be synthesized by monocytes and macrophages within injury sites. Here, we present data showing that osteopontin can promote leukocyte adhesion through the $\alpha_4\beta_1$ integrin. In the presence of physiologic concentrations of Mg^{2+} and Ca^{2+}, osteopontin purified from bovine milk promoted cell-substrate adhesion of HL-60 and Ramos cells, two model leukocyte cell lines. As with other adhesive ligands, adhesion to osteopontin required leukocyte activation. Under these conditions, no adhesion to control substrates such as bovine serum albumin was observed. Leukocyte adhesion was inhibited by anti-integrin antibodies directed at either the α_4 or β_1 integrin subunits but not by control antibodies directed to other integrins. Further adhesion experiments revealed that leukocyte binding to osteopontin was completely inhibited by an $\alpha_4\beta_1$-binding peptide containing the leucine-aspartate-valine (LDV) sequence, while a control, non-binding peptide containing leucine-glutamate-valine (LEV) had minimal effects. Affinity chromatography using either surface labeled HL-60 or Ramos cell extracts revealed that the $\alpha_4\beta_1$ integrin specifically bound to osteopontin. Immunoprecipitation of eluted fractions from these columns positively identified the $\alpha_4\beta_1$ integrin. In order to localize potential $\alpha_4\beta_1$-binding sites within osteopontin, the protein was proteolytically cleaved with thrombin. A 30 kDa N-terminal osteopontin fragment purified using fast protein liquid chromatography promoted $\alpha_4\beta_1$ dependent leukocyte adhesion in a manner similar to that of the intact protein. These data collectively demonstrate that the $\alpha_4\beta_1$ integrin is a new adhesion receptor for osteopontin and that an $\alpha_4\beta_1$ binding site exists in the NH_2-terminal thrombin fragment of osteopontin.

Key words: Osteopontin, $\alpha_4\beta_1$, Integrin, Leukocyte, Adhesion, Injury

INTRODUCTION

Osteopontin (OPN) is an acidic, phosphorylated matrix protein that contains an Arg-Gly-Asp (RGD) cell attachment sequence (Oldberg et al., 1986) and has been identified as an adhesive and migratory substrate for several cell types. OPN has previously been shown in vitro to promote adhesion and migration of vascular smooth muscle (Liaw et al., 1994, 1995b; Bayless et al., 1997) and endothelial cells (Liaw et al., 1994, 1995a,b; Bayless et al., 1997). Peritoneal or subcutaneous injection of OPN results in mononuclear cell and/or neutrophil (PMN) accumulation (Singh et al., 1990), suggesting that OPN may provide migratory and adhesive signals for leukocytes. OPN expression is observed at the intimal edge of a balloon-injured artery (Giachelli et al., 1991, 1993; Liaw et al., 1995a) and its expression correlates with the presence of macrophages in several types of renal injury (Giachelli et al., 1994; Pichler et al., 1994). This evidence indicates that OPN may participate in smooth muscle cell, endothelial cell and leukocyte adhesion and/or migration within injury sites.

Most cells adhere to OPN through integrins. The $\alpha_v\beta_1$ (Liaw et al., 1995b; Hu et al., 1995a), $\alpha_v\beta_3$ (Miyauchi et al., 1991;

Ross et al., 1993; Liaw et al., 1994, 1995b; Hu et al., 1995b) and $\alpha_v\beta_5$ (Liaw et al., 1995b; Hu et al., 1995a) integrins bind OPN in an RGD-dependent manner. Recent evidence suggests that OPN is capable of promoting cell adhesion through sites other than its RGD site (Van Dijk et al., 1993; Nasu et al., 1995; Katagiri et al., 1996; Smith et al., 1996). Other integrins such as $\alpha_4\beta_1$ (Nasu et al., 1995), $\alpha_5\beta_1$ (Nasu et al., 1995) and $\alpha_9\beta_1$ (Smith et al., 1996) have been suggested as potential OPN receptors that may bind these alternative sites. However, further work is necessary to investigate the role of these other integrins in OPN-mediated cellular events.

The $\alpha_4\beta_1$ integrin is expressed on leukocytes (Lobb and Hemler, 1994; Springer, 1994; Carlos and Harlan, 1994), differentiated vascular smooth muscle cells (Duplaá et al., 1997) and tumor cells (Taichman et al., 1991; Qian et al., 1994). The $\alpha_4\beta_1$ integrin has been shown to mediate cell-cell attachment as well as cell-substrate adhesion to extracellular matrix and other proteins (Hynes, 1992; Lobb and Hemler, 1994; Springer, 1994; Carlos and Harlan, 1994; Kilger and Holzmann, 1995; Davis et al., 1997). This integrin appears to play a critical role in the control of the inflammatory response in disease states such as diabetes, encephalomyelitis (Yednock

et al., 1992; Yang et al., 1993; Lobb and Hemler, 1994) and graft rejection (Molossi et al., 1995) in that administration of anti-$\alpha_4\beta_1$ blocking reagents inhibited the development of the diseases. The $\alpha_4\beta_1$ integrin has been reported to bind the CS-1 and CS-5 alternatively spliced domains of fibronectin (Humphries et al., 1987; Guan and Hynes, 1990; Mould et al., 1991) as well as VCAM-1, an inducible leukocyte-endothelial cell adhesion molecule (Elices et al., 1990). Known binding sequences for the $\alpha_4\beta_1$ integrin are Leu-Asp-Val (LDV) in the CS-1 region of fibronectin (Guan and Hynes, 1990; Komoriya et al., 1991; Wayner and Kovach, 1992) and Gln-Ile-Asp-Ser-Pro-Leu (QIDSPL) in VCAM-1 (Osborn et al., 1994; Vonderheide et al., 1994; Clements et al., 1994; Jones et al., 1995). Other identified recognition sites for $\alpha_4\beta_1$ within fibronectin include Ile-Asp-Ala-Pro-Ser (IDAPS) (Mould and Humphries, 1991), Arg-Glu-Asp-Val (REDV) (Mould et al., 1991) and Arg-Gly-Asp (RGD) (Sanchez-Aparicio et al., 1994).

In this study, we show that leukocytes can adhere to OPN through the $\alpha_4\beta_1$ integrin. We have shown this interaction by utilizing anti-integrin blocking antibodies directed to the α_4 or β_1 integrin subunits and $\alpha_4\beta_1$-specific binding peptides which blocked leukocyte binding to OPN. Affinity chromatography experiments demonstrated direct binding of $\alpha_4\beta_1$ to OPN. Leukocyte adhesion to an NH_2-terminal thrombin fragment of OPN was also mediated through $\alpha_4\beta_1$. Collectively, these data show that the $\alpha_4\beta_1$ integrin can mediate cellular adhesive events through OPN.

MATERIALS AND METHODS

Cell adhesion assays

Cell adhesion assays were performed to determine the ability of isolated OPN to promote leukocyte adhesion. Polystyrene microwells (Corning-Costar, Cambridge, MA) were coated with 50 μl of bovine OPN purified as previously described (Bayless et al., 1997) at a concentration of 20 μg/ml in Tris-buffered saline (TBS) overnight at 4°C. OPN was purified from bovine milk using sequential ion-exchange and hydrophobic chromatographic steps yielding ~8 mg of OPN per liter of milk. Purity (>95%) was assessed using Sypro-Orange staining of SDS-PAGE gels (showing a single 60 kDa band) and a single NH_2-terminal sequence as previously described (Bayless et al., 1997). After blocking with 10 mg/ml BSA (bovine serum albumin) (Sigma, St. Louis, MO) in TBS, wells were rinsed with Puck's Saline A (PSA) (Gibco-BRL, Grand Island, NY). HL-60 promyelocytic leukemia cells or Ramos lymphoblastoid cells (ATCC, Rockville, MD) were grown in RPMI-1640 (Gibco-BRL) and 10% fetal calf serum. Cells were rinsed and resuspended in PSA at a density of 100,000 cells/well. Medium for adhesion in all HL-60 cell experiments contained a final concentration of 100 μg/ml BSA in PSA. Concentrations of $CaCl_2$ and $MgCl_2$ were varied as described in the results. HL-60 cells were activated with the β_1-activating antibody, 8A2 (Kovach et al., 1992) at a concentration of 1 μg/ml and a phorbol ester, 12-0-tetradecanoyl phorbol 13-acetate (TPA) at a concentration of 50 ng/ml. Ramos cells were activated with the 8A2 monoclonal antibody alone at 1 μg/ml. After plating, cells were allowed to adhere for one hour at which time they were fixed with formalin. Plates were stained with 0.1% Amido Black for 30 minutes, rinsed and solubilized with 2 N NaOH to obtain an absorbance reading at 595 nm which corresponds directly to the number of cells stained in each well (Davis and Camarillo, 1993).

To determine which integrins were expressed on the cell surface of Ramos and HL-60 cells, cell binding experiments were performed using wells coated with anti-integrin antibodies. The cells' ability to attach to an antibody is governed by whether or not the cells express a given antigen. Antibodies tested include HP2/1 (α_4), Mab13 (β_1), Fib 504 ($\alpha_4\beta_7$), Mab16 (α_5) and TS1/22 (α_L). Control (blank) wells were coated with 10 mg/ml BSA. Wells were blocked with BSA and rinsed. Cells were added at 100,000 cells per well in the presence of 100 μg/ml BSA and the absence of cations. These experiments were performed with HL-60, Ramos and RPMI 8866 cells. The RPMI 8866 cell line is known to express $\alpha_4\beta_7$ (Erle et al., 1994) and was kindly provided by Drs David Erle and Russell Pachynski (University of California, San Francisco). Cells were allowed to adhere for 30 minutes and quantitated as described above. Fluorescence-activated cell sorting (FACS) was also performed with the same mouse anti-human antibodies used in the antibody binding experiments. Approximately 10^6 leukocytes per group were labeled with 5 μg of primary antibody for 30 minutes on ice and rinsed twice with 500 μl PBS. Goat anti-mouse secondary antibody conjugated to FITC (Dako, Glostrup, Denmark) was then added (10 μl) for 30 minutes and rinsed twice with 500 μl of PBS. FACS analysis was conducted using FACSCalibur (Becton-Dickinson) at the Center for Flow Cytometry and Image Analysis of the Institute for Molecular Pathogenesis and Therapeutics, Texas A&M University.

To determine which integrins were responsible for leukocyte adhesion to OPN, HL-60 and Ramos cells were incubated with various concentrations of divalent cations in combination with anti-integrin monoclonal antibodies or synthetic peptides specific for various integrins. Following a 15 minute preincubation period at 37°C with either antibodies or peptides, cells were activated with 1 μg/ml 8A2 and (for HL-60 cells) 50 ng/ml TPA, plated and allowed to adhere for one hour before fixing. In the antibody blocking experiments, HL-60 cells were incubated with the following monoclonal antibodies directed to integrin subunits at a concentration of 20 μg/ml: α_4 (HP2/1; supernatant used at 1:10 dilution; Francisco Sanchez-Madrid, Spain) (Sanchez-Madrid et al., 1986), α_4 (Immunotech, Westbrook, ME), β_1 (Mab13; Becton Dickinson, Bedford, MA) (Akiyama et al., 1989), $\alpha_4\beta_7$ (Fib 504; Pharmingen, San Diego, CA) (Andrew et al., 1994), α_L (TS1/22; ATCC, purified as in the method of Larson, 1989) and α_5 (Mab16; Becton Dickinson) (Akiyama et al., 1989). To further investigate the involvement of the $\alpha_4\beta_1$ integrin in adhesion to OPN, the Glu-Ile-Leu-Asp-Val-Ser-Pro (EILDVSP) synthetic peptide (Peninsula Labs, Belmont, CA) (Komoriya et al., 1991) and its control Glu-Ile-Leu-Glu-Val-Ser-Pro (EILEVSP) were pre-incubated with cells at a concentration of 250 μg/ml. Other peptides tested included the Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP, Gibco BRL) and its control, Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) (Pierschbacher and Ruoslahti, 1984). These peptides were also added at 250 μg/ml. Following the incubation period, cells were seeded and the assay performed as described above.

Isolation of integrins using osteopontin-Sepharose

To illustrate the integrin-binding capacity of OPN, purified OPN was coupled to cyanogen-bromide 4B (Sigma) at 2 mg/ml according to the manufacturer's instructions. HL-60 and Ramos cells were surface biotinylated as described (Davis, 1992). A 50 μl pellet of cells was extracted with 1 ml 3% octylglucoside (ICN, Irvine, CA) in TBS containing 1.5 mM Mg^{2+}, 1.5 mM Mn^{2+} and 10^{-3} M phenylmethanesulfonic acid. The cell extracts were mixed at 5-10 minute intervals with OPN-Sepharose (0.9 ml) over a 2 hour period at 0°C. The column was washed with 20 ml of 1% octylglucoside plus cations and 0.5 ml fractions were eluted with 3 ml of 1% octylglucoside + 10 mM EDTA. 30 μl of each fraction were loaded and run under non-reducing conditions on a 7% acrylamide gel and transferred to polyvinylidene diflouride (PVDF) membrane (Bio-Rad, Hercules, CA). The membranes were blocked overnight at 4°C with 5% non-fat dry milk in water and developed for alkaline phosphatase activity as previously described (Bayless et al., 1997).

Integrin immunoprecipitation

Integrins which bound to the OPN-Sepharose column were identified using immunoprecipitation. Sepharose beads conjugated with goat anti-mouse IgG (Sigma) or with Protein A (Sigma), were rinsed and suspended 1:1 with 0.5% Triton X-100 in TBS. In 1.5 ml microcentrifuge tubes, 200 μl of the bead mixture was added to 5 μg of monoclonal antibodies against several human integrin subunits including α_4 (HP2/1, Immunotech), α_4 (P4C2, Gibco-BRL), β_1 (LM534), α_5 (MAB$_{16}$) and α_L (TS1/22) or 10 μl of a rabbit polyclonal antibody directed to the α_4 subunit cytoplasmic domain (Chemicon, Temecula, CA). These mixtures were then combined with 300 μl of pooled EDTA eluate from OPN-Sepharose and 700 μl of 0.5% Triton X-100 in TBS. This mixture was rotated continuously at 4°C overnight after which time tubes were centrifuged and rinsed six times with 1 ml of 0.5% Triton X-100 in TBS. 75 μl of 2× sample buffer was added to the beads and this mixture was boiled for 5 minutes. 30 μl samples were run on 7% SDS-PAGE under non-reducing conditions and transferred to PVDF. The blots were probed for biotin using streptavidin-alkaline phosphatase as described above.

Thrombin cleavage of OPN

Bovine thrombin (American Diagnostica, Greenwich, CT) was coupled to cyanogen-bromide 4B at 200 μg/ml according to the manufacturer's instructions. OPN was incubated with the beads for 2 hours at 37°C and gently mixed every 10 minutes. Following cleavage, the beads were pelleted and supernatant containing thrombin-cleaved OPN was aliquoted and frozen at −20°C.

Purification of an N-terminal thrombin fragment of OPN using fast protein liquid chromatography (FPLC)

The fragments of OPN produced after thrombin treatment were isolated and purified using FPLC. Approximately 100 μg of thrombin-cleaved OPN in PBS was dissolved in 6 M urea and 10 mM Tris (200 μl total) and applied to a Q-Sepharose column (Pharmacia-Biotech; Alameda, CA) at a flow rate of 0.8 ml per minute. The column was washed with 5 ml of buffer A (0 M NaCl, 10 mM Tris, 6 M urea). Following the wash, bound proteins were eluted using a 0-1.0 M NaCl gradient in 6 M urea and 10 mM Tris-HCl over 20 minutes. Fractions (600 μl) were collected and analyzed using SDS-PAGE and visualized with a copper staining method (Bio-Rad). The N-terminal OPN fragment eluted consistently at 0.5 M NaCl. The purified fragment was subjected to amino-terminal sequencing using the Edman method on a Hewlett Packard G1005A protein sequencing system at the Biotechnology Instrumentation Facility in the Department of Entomology at Texas A&M University.

RESULTS

Leukocyte adhesion to OPN occurs in the presence of physiologic levels of divalent cations

A model leukocyte cell line, HL-60, was used to investigate whether OPN promoted leukocyte adhesion. Fig. 1 shows the adhesion of HL-60 cells to OPN in the presence of various concentrations of Ca^{2+} and Mg^{2+} and in the presence or absence of leukocyte activation using TPA and 8A2. Cell attachment to OPN was greatest with 3 mM Mg^{2+} alone and decreased with increasing Ca^{2+}/Mg^{2+} ratios. However, substantial adhesion to OPN substrates occurred with physiologic concentrations of divalent cations (2 mM Ca^{2+}, 1 mM Mg^{2+}). Adhesion to the control substrate, BSA was insignificant in all groups, regardless of activation. A second leukocyte cell line, Ramos, also attached to OPN in the presence of 2 mM Ca^{2+}, 1 mM Mg^{2+} (see later on) but not in
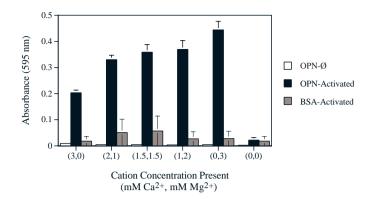

Fig. 1. Leukocyte adhesion to OPN requires activation and the presence of divalent cations. HL-60 cells were activated with the β_1-activating antibody 8A2 (1 μg/ml) and with a phorbol ester, TPA at 50 ng/ml. The divalent cations Mg^{2+} and Ca^{2+} were then added at the concentrations indicated and allowed to adhere to wells coated with either OPN (20 μg/ml) or bovine serum albumin (BSA) (10 mg/ml). Experiments were performed and quantitated as described in Materials and Methods. The data shown are from a representative experiment (*n*=3) performed in triplicate wells and values shown are mean absorbance ± s.e.m.
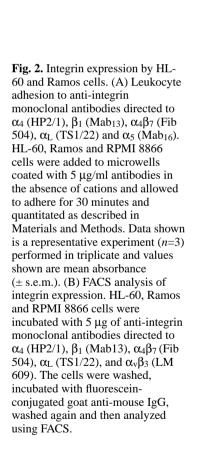
the absence of divalent cations (not shown). Ramos cell attachment required activation with only the 8A2 antibody. No adhesion of either leukocyte cell line occurred in the absence of divalent cations, supporting the concept that this adhesion was mediated through integrins. Also, the activating influence of 8A2 (Kovach et al., 1992) strongly suggested the involvement of a β_1 integrin in leukocyte adhesion to OPN.
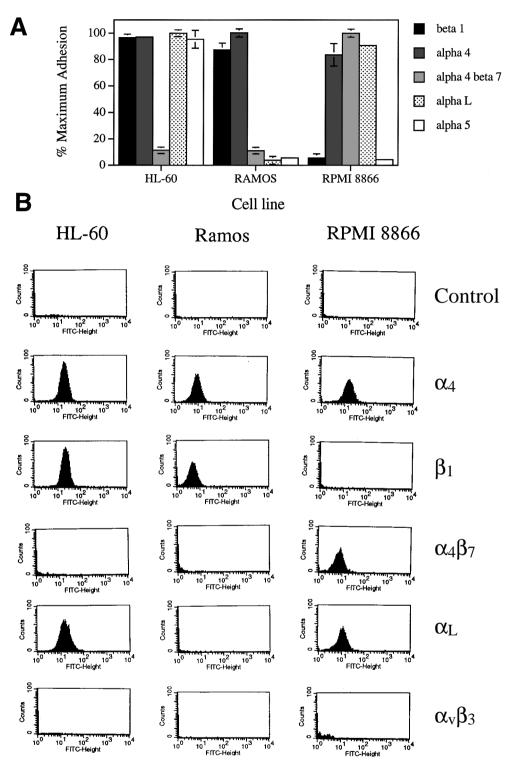
Integrin expression by HL-60 and Ramos cell lines

To assess which integrins were responsible for leukocyte adhesion to OPN, adhesion experiments to substrate bound anti-integrin antibodies and fluorescence-activated cell sorting (FACS) were performed. As shown in Fig. 2A, HL-60 and Ramos cells adhered to microwells coated with anti-integrin antibodies directed to α_4 and β_1 but not $\alpha_4\beta_7$. In contrast, a control cell line which is known to express $\alpha_4\beta_7$, RPMI 8866, bound to anti-α_4 and anti-$\alpha_4\beta_7$ coated microwells but not anti-β_1 wells. Also shown are the binding of these cells to anti-α_L and -α_5 integrin antibodies. A FACS experiment in Fig. 2B confirms the cell adhesion data. HL-60 cells express $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_L\beta_2$ while Ramos cells express $\alpha_4\beta_1$. The only integrin commonly expressed between the two cell lines is $\alpha_4\beta_1$. Neither cell line expresses $\alpha_4\beta_7$ or $\alpha_v\beta_3$ (Fig. 2).

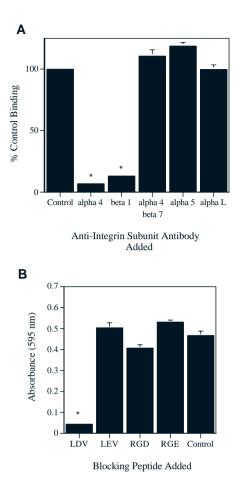### The $\alpha_4\beta_1$ integrin mediates leukocyte adhesion to OPN

Cell adhesion experiments with integrin blocking reagents were performed to identify the integrins responsible for the adhesion of HL-60 and Ramos cells to OPN. Cells were incubated with antibodies directed to HL-60 or Ramos cell integrin subunits and plated on OPN. The results are shown in Figs 3A (HL-60) and 4A (Ramos). The addition of either anti-α_4 or anti-β_1 antibodies markedly reduced leukocyte adhesion to OPN compared to control. Antibodies directed toward $\alpha_4\beta_7$, α_5 and α_L had no effect. To further confirm that the $\alpha_4\beta_1$ integrin is involved in leukocyte adhesion to OPN, integrin-binding peptides were utilized (Figs 3B and 4B). The LDV

Fig. 2. Integrin expression by HL-60 and Ramos cells. (A) Leukocyte adhesion to anti-integrin monoclonal antibodies directed to α_4 (HP2/1), β_1 (Mab13), $\alpha_4\beta_7$ (Fib 504), α_L (TS1/22) and α_5 (Mab16). HL-60, Ramos and RPMI 8866 cells were added to microwells coated with 5 μg/ml antibodies in the absence of cations and allowed to adhere for 30 minutes and quantitated as described in Materials and Methods. Data shown is a representative experiment (*n*=3) performed in triplicate and values shown are mean absorbance (± s.e.m.). (B) FACS analysis of integrin expression. HL-60, Ramos and RPMI 8866 cells were incubated with 5 μg of anti-integrin monoclonal antibodies directed to α_4 (HP2/1), β_1 (Mab13), $\alpha_4\beta_7$ (Fib 504), α_L (TS1/22), and $\alpha_v\beta_3$ (LM 609). The cells were washed, incubated with fluorescein-conjugated goat anti-mouse IgG, washed again and then analyzed using FACS.

peptide is a competitive inhibitor of $\alpha_4\beta_1$ integrin binding to fibronectin (Komoriya et al., 1991). The RGD peptide is a known inhibitor of cell attachment occurring through RGD sites (Pierschbacher and Ruoslahti, 1984). The LEV and RGE peptides serve as inactive controls. As shown in Fig. 3B, the LDV peptide completely abolished adhesion to OPN compared to control, while LEV and RGE had no effect. An RGD peptide showed slight inhibitory activity. Previous work has indicated

that RGD peptides can bind $\alpha_4\beta_1$ under some conditions of leukocyte activation and block its function (Sanchez-Aparicio et al., 1994). The antibody and peptide data combined show that $\alpha_4\beta_1$ mediates leukocyte adhesion to OPN.

To ensure that leukocyte adhesion to OPN observed in the presence of different levels of Ca^{2+} and Mg^{2+} (Fig. 1) occurred through $\alpha_4\beta_1$, separate blocking experiments were performed in the presence of varying cation levels. Fig. 5 illustrates that

A

B

incubation, the columns were washed in the presence of divalent cations and eluted with EDTA (Fig. 6A). For the HL-60 cell extracts, integrins eluted in fractions 3 and 4, while for Ramos cell extracts, fractions 2 and 3 contained integrins. The molecular masses of these bands, 150 and 130 kDa, match that of the α_4 and β_1 subunits, respectively. To identify these integrins, immunoprecipitations were performed using anti-integrin antibodies (Fig. 6B). For both cell types, α_4 and β_1 subunits were observed in the immunoprecipitates. Three different anti-α_4 antibodies were capable of immunoprecipitating the band identified as the α_4 subunit (Fig. 6B, Ramos) while control antibodies did not. Interestingly, the $\alpha_5\beta_1$ integrin was also identified from the HL-60 cell experiment, suggesting it may have an affinity for OPN coupled to Sepharose, presumably through its RGD site. However, there is no evidence that $\alpha_5\beta_1$ participates in the adhesion of HL-60 cells to OPN based on our adhesion data where addition of anti-α_5 blocking antibodies had no effect on adhesion and RGD peptides showed minimal inhibitory effects. The interaction of $\alpha_5\beta_1$ with OPN-Sepharose may be related to the presence of Mn^{2+} in the buffers used during affinity chromatography. In contrast, the adhesion assays were

the LDV peptide markedly blocked HL-60 cell adhesion in the presence of Ca^{2+} and Mg^{2+} or with either Ca^{2+} or Mg^{2+} alone. In contrast, the LEV peptide showed no effect.

The $\alpha_4\beta_1$ integrin directly binds OPN

Further investigation showed that $\alpha_4\beta_1$ directly bound OPN in affinity chromatography experiments. HL-60 and Ramos cells were surface-labeled with biotin, extracted with detergent, and the extracts were incubated with OPN-Sepharose in the presence of 1.5 mM Mg^{2+} and 1.5 mM Mn^{2+}. After

A

B

Fig. 5. The effects of LDV on leukocyte adhesion in the presence of various concentrations of Ca^{2+} and Mg^{2+}. Activated HL-60 cells were preincubated for 15 minutes at 37°C with both LDV and LEV peptides (250 µg/ml) and with various combinations of Mg^{2+} and Ca^{2+} before plating. For all groups, control (Ø) indicates the absence of peptide during incubation. Values represent actual absorbance readings from a representative experiment in triplicate wells (± s.e.m.). *$P<0.001$ compared to control using Student's t-test.

performed with Mg^{2+} and Ca^{2+} which may alter the ability of $\alpha_5\beta_1$ to bind.

An NH₂-terminal thrombin fragment of OPN promotes $\alpha_4\beta_1$-dependent HL-60 cell adhesion

As an initial step to locate the $\alpha_4\beta_1$-binding site within OPN, the native protein was proteolytically cleaved with thrombin. The fragments were tested for activity and found to promote

dose-dependent adhesion of leukocytes comparable to the native protein (not shown). Separation of fragments was accomplished using fast protein liquid chromatography (FPLC) and anion exchange utilizing a Q-Sepharose column. This experiment was performed in the presence of 6 M urea to prevent the fragments from aggregating. SDS-PAGE analysis of eluted fractions is shown in Fig. 7A. Copper staining of the elution profile revealed a single 30 kDa band in fractions 15

Fig. 6. Affinity chromatography data illustrating the binding of $\alpha_4\beta_1$ to OPN-Sepharose. (A) OPN-Sepharose was incubated with labeled HL-60 and Ramos cell lysates as described in Materials and Methods. 0.5 ml EDTA elution fractions were collected, run on SDS-PAGE gels and blots were developed. Left: HL-60 cell integrin elution pattern is shown (lanes 1-5). Right: Ramos cell integrin elution pattern (lanes 1-4). Upper arrows denote the α subunit, while lower arrows denote the β subunit. (B) Immunoprecipitations were performed for both cell types as described in Materials and Methods. Monoclonal or polyclonal antibodies directed to HL-60 and Ramos cell integrin subunits are indicated below each figure. Note that three different α_4-subunit specific antibodies were utilized in the Ramos cell immunoprecipitation experiment. These are α_4 subunit cytodomain, P4C2 and HP2/1 in lanes 2, 3 and 4, respectively. Upper arrows denote the α subunit, while lower arrows denote the β subunit.
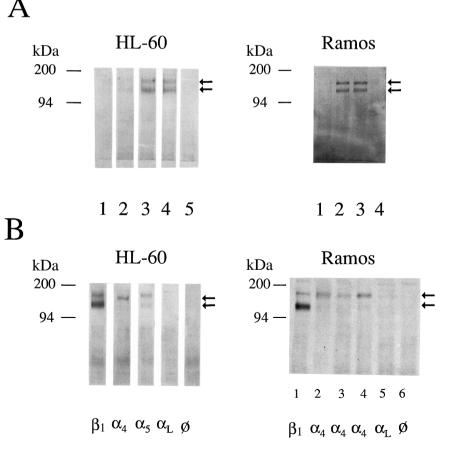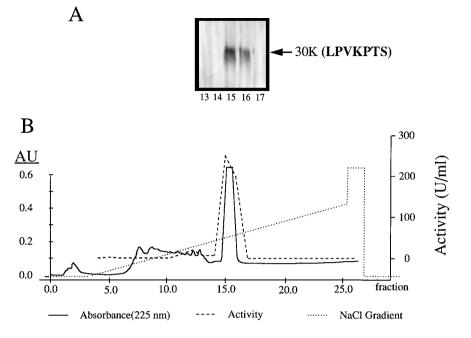
A

← 30K (**LPVKPTS**)

13 14 15 16 17

B

AU

Fig. 7. Purification of a 30 kDa, NH_2-terminal fragment of OPN. OPN was cleaved using thrombin-Sepharose and injected onto a fast protein liquid chromatography column, Q-Sepharose in the presence of 6 M urea. A linear NaCl gradient (0-1.0 M) was performed over a 30 minute period ($n=5$). Fractions (600 μl) were collected and analyzed using SDS-PAGE, shown in A. Amino-terminal sequence analysis of the 30 kDa band revealed the sequence L-P-V-K-P-T-S, matching exactly the N terminus of bovine OPN. (B) Tracings indicating the elution profile, leukocyte adhesive activity and linear NaCl gradient for the Q-Sepharose column. To identify which fractions were active in promoting HL-60 cell adhesion, wells were coated with serial two-fold dilutions of each fraction and cell adhesion assays were performed with activated cells in the presence of 2 mM Ca^{2+}, 1 mM Mg^{2+}. The resulting activity units are expressed as U/ml where a unit corresponds to the dilution at which half-maximal adhesion occurred.

and 16. Fig. 7B shows the NaCl gradient utilized and the resulting elution and activity profiles. A single major peak consistently eluted at 0.5 M NaCl. Single fractions from the elution pattern shown were tested for their ability to promote cell adhesion. Although a small amount of activity was present in fractions 11-13 (Fig. 7B), by far the majority of the activity was seen in fractions 15-16, corresponding to the major elution peak. Amino-terminal sequence analysis of the 30 kDa fragment revealed LPVKPTS, matching the N terminus of bovine OPN. In addition, the 30 kDa fragment stained poorly with Coomassie blue and could only be visualized using a copper stain. These characteristics of the 30 kDa fragment are consistent with those previously reported for an N-terminal fragment of human OPN (Senger and Perruzzi, 1996). Although we did successfully purify an N-terminal fragment of bovine OPN, we were unable to isolate any defined C-terminal fragments in sufficient quantities. This may be due to heterogeneity in the thrombin proteolysis of the C-terminal region of OPN or to further proteolytic degradation of C-terminal fragments.

Dose-response curves illustrating cell adhesion for the N-terminal fragment versus native OPN are shown in Fig. 8. The efficacies of the two OPN preparations in promoting HL-60 cell adhesion were similar. Both species promoted dose-dependent adhesion of leukocytes in the presence of 2 mM Ca^{2+} and 1 mM Mg^{2+}. Native OPN and the N-terminal fragment also promoted dose-dependent adhesion of human endothelial cells (not shown). Additionally, leukocyte adhesion to the N-terminal fragment was blocked by LDV peptides but not by the control LEV peptide, indicating that adhesion occurred through the $\alpha_4\beta_1$ integrin (Fig. 8B).

DISCUSSION

We present here data showing that OPN is a new ligand for the $\alpha_4\beta_1$ integrin. This work was facilitated by the use of two leukocyte cell lines, HL-60 and Ramos, which do not express the $\alpha_v\beta_3$ integrin that recognizes the RGD site in OPN. Cell adhesion experiments identified $\alpha_4\beta_1$ as the receptor responsible for leukocyte adhesion to OPN. Affinity chromatography was used to show that the $\alpha_4\beta_1$ integrin from HL-60 and Ramos cells bound OPN-Sepharose. An isolated N-terminal thrombin fragment of OPN promoted adhesion of leukocytes in an $\alpha_4\beta_1$-dependent manner. These data collectively show that the $\alpha_4\beta_1$ integrin recognizes OPN and that at least one binding site for the integrin is present within the N-terminal region of the protein.
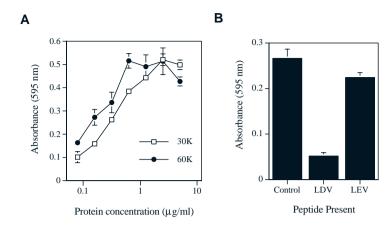
The $\alpha_4\beta_1$ integrin promotes leukocyte adhesion to osteopontin

In our system, leukocyte adhesion to OPN required cell activation. This was accomplished by the addition of TPA and the β_1-activating antibody, 8A2 for HL-60 cells while only 8A2 was necessary for the activation of Ramos cells. These findings are consistent with previous work showing that the activation state of $\alpha_4\beta_1$ is variable among different leukocyte cell lines and that different degrees of activating stimuli are necessary to induce $\alpha_4\beta_1$-mediated adhesive events (Masumoto and Hemler, 1993). Activation of leukocytes in the presence of Mg^{2+} and Ca^{2+} resulted in significant leukocyte adhesion to OPN as compared to non-activated cells. Leukocyte adhesion occurred in various combinations of Ca^{2+} and Mg^{2+} including physiological concentrations (2 mM Ca^{2+}, 1 mM Mg^{2+}).

Antibody and peptide inhibition experiments were performed to provide evidence that the $\alpha_4\beta_1$ integrin was responsible for leukocyte adhesion to OPN. Consistent with the participation of $\alpha_4\beta_1$, the addition of α_4 or β_1 blocking antibodies significantly inhibited HL-60 and Ramos cell adhesion to OPN. In peptide inhibition experiments, the $\alpha_4\beta_1$-specific peptide, LDV, completely abolished cell attachment compared to control for both cell types, while LEV, the control peptide, did not block adhesion. In addition, our affinity chromatography data show that OPN-Sepharose bound the

Fig. 8. The 30 kDa fragment of OPN promotes dose- and LDV-dependent adhesion of leukocytes. (A) Comparison of the ability of intact and 30 kDa OPN fragment to promote HL-60 cell adhesion. Concentrations of the 30 kDa fragment and intact OPN were determined using spectral analysis based on a method by Pace et al. (1995). Wells were coated with 5 µg/ml of each protein and serially diluted twofold. Activated cells were added in the presence of physiological cations and allowed to adhere for one hour. The data shown are from a representative experiment performed in triplicate wells and values are shown as mean absorbance (± s.e.m.). (B) Blockade of HL-60 adhesion to the 30 kDa fragment of OPN with the LDV peptide. Triplicate wells were coated with 5 µg/ml of the 30 kDa fragment and blocked with BSA. HL-60 cells were activated and incubated with the 20 µg/ml of the LDV and LEV peptides or nothing for 15 minutes prior to plating. Cells were allowed to adhere for one hour and quantitated as described in Materials and Methods. The data shown are the average of two experiments performed in triplicate wells. Values represent mean absorbance (± s.e.m.).

$\alpha_4\beta_1$ integrin from both HL-60 and Ramos cell lysates. These data suggest that $\alpha_4\beta_1$ binds OPN and mediates leukocyte adhesion to OPN.

Thrombin fragments of OPN were generated to investigate the location of $\alpha_4\beta_1$ binding sites in OPN. These fragments were loaded onto an FPLC ion-exchange column and one major peak eluting at 0.5 M NaCl was recovered. Copper staining of the elution profile revealed a single 30 kDa band corresponding to the major elution peak. Amino-terminal sequence analysis confirmed its identity as an NH_2-terminal fragment of OPN. Our data are consistent with the characteristics of the thrombin-generated N-terminal fragment of human OPN previously reported (Senger et al., 1996) in that staining was only accomplished using the copper staining technique and the fragment promoted endothelial cell adhesion. Here, we have shown that this OPN fragment promoted dose-dependent adhesion of leukocytes through $\alpha_4\beta_1$. Although we were successful in isolating an N-terminal OPN fragment, we were unable to isolate sufficient amounts of defined C-terminal fragments. We believe that thrombin digestion resulted in heterogeneous fragmentation of the C-terminus of OPN. While we cannot rule out the possibility that sequences within the C-terminal domain of OPN may facilitate leukocyte adhesion through $\alpha_4\beta_1$, the N-terminal domain promoted adhesion in an essentially identical manner to that of intact OPN, suggesting it contains an $\alpha_4\beta_1$ binding site. An important point is that there are no LDV, IDS or EDV sequences within this domain which are known $\alpha_4\beta_1$-binding sites from either fibronectin or VCAM-1 (Springer, 1994; Carlos and Harlan, 1994; Lobb and Hemler, 1994). Our peptide inhibition experiments with LDV and RGD peptides indicate the RGD site in OPN probably does not play a major role in the $\alpha_4\beta_1$-OPN interaction. Thus, the $\alpha_4\beta_1$-binding site within OPN will be a novel binding site, possibly related to LDV or IDS, and further work will be necessary to identify this site.

Relevance to vascular injury and inflammation

Ample evidence in the literature exists showing the expression of OPN following many types of injury. In granulation tissue and necrotic myocardium derived from either rat or human myocardial infarction, OPN mRNA and protein were found to be expressed at high levels (Murry et al., 1994). OPN was also found to be upregulated in cases of tubulointerstitial injury (Eddy et al., 1995; Giachelli et al., 1994) and glomerulonephritis (Pichler et al., 1994). OPN has been found to be heavily expressed in human atherosclerotic plaques (Giachelli et al., 1993; Hirota et al., 1993; Ikeda et al., 1993; O'Brien et al., 1994) and in neointimal cells of arteries injured by balloon angioplasty (Giachelli et al., 1991; Liaw et al., 1995a). As a result of these data, it has been proposed that OPN is a general marker for injury (Murry et al., 1994).

Recent work also indicates an intriguing overlap of OPN expression and the $\alpha_4\beta_1$ integrin following vascular injury. An increase in the expression of the $\alpha_4\beta_1$ integrin was observed in both smooth muscle cells of human atherosclerotic plaques and dedifferentiated VSMCs in vitro (Duplaá et al., 1997). OPN expression has been shown to be induced under similar conditions (Giachelli et al., 1991, 1993; Hirota et al., 1993; Ikeda et al., 1993; O'Brien et al., 1994). Thus, the present findings showing the interaction between the $\alpha_4\beta_1$ integrin and OPN may have functional relevance for a variety of cell types in vascular injury or disease.

The function of OPN in pathophysiological states remains to be determined. The induction of OPN may serve as a recruiting stimulus for macrophages and monocytes to the injured area since subcutaneous injection of OPN resulted in a substantial increase in leukocytes within the injection site (Singh et al., 1990; Nasu et al., 1995). Several studies have demonstrated that OPN expression precedes monocyte/macrophage accumulation (Giachelli et al., 1994; Murry et al., 1994; Pichler et al., 1994; Eddy et al., 1995; Wiener et al., 1996). In addition, OPN may be an adhesive or immobilizing substrate for monocytes/macrophages based on evidence that OPN promotes adhesion of macrophages (Singh et al., 1990). OPN may also stimulate other macrophage functions such as phagocytosis or production of cytokines and proteolytic enzymes which are involved in wound repair responses.

In the work presented here, we provide evidence that leukocytes can utilize the $\alpha_4\beta_1$ integrin as a receptor for OPN. This $\alpha_4\beta_1$-OPN interaction may be relevant in a variety of cellular responses during tissue injury. This finding adds further support to the general concept that induction of OPN

following injury may be an important signal for cells involved in the modulation of inflammatory and tissue injury responses.

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