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The Integrin Specificity of Human Recombinant Osteopontin

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ABSTRACT. The ability of full-length human recombinant osteopontin (OPN) to support the adhesion of various α_V integrin-expressing cell lines was determined in order to characterize its integrin selectivity. The identity of this protein was assessed by cDNA sequence and mass spectroscopic analysis, and confirmed as full-length OPN. Neither the human embryonic kidney 293 cell line, which expresses the $\alpha_V\beta_1$ integrin, nor the human colonic adenocarcinoma HT-29 cell line, which expresses the $\alpha_V\beta_5$ integrin, were able to adhere to OPN; both of these cell lines are deficient in the β_3 subunit. In contrast, an $\alpha_V\beta_3$ integrin-expressing cell line, SK-MEL-24, was able to adhere to OPN in an arginine-glycine-aspartic acid dependent manner. In addition, this OPN-mediated cellular adhesion was completely blocked with an anti-$\alpha_V\beta_3$ integrin antibody (LM609), confirming that only the $\alpha_V\beta_3$ integrin mediated this cellular adhesion. These data demonstrate that, at least among the α_V integrins, only the $\alpha_V\beta_3$ is able to support cellular adhesion to osteopontin. This finding may have implications for the design of therapeutics targeting OPN–integrin interactions. BIOCHEM PHARMACOL **58**;10: 1567–1578, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cellular adhesion; osteopontin; RGD; $\alpha_V\beta_3$; $\alpha_V\beta_1$; $\alpha_V\beta_5$

OPN§ is a 34 kDa extracellular matrix protein that contains the RGD integrin recognition motif [1]. Although this acidic, secreted phosphoprotein had originally been identified as a major component of the non-collagenous organic bone matrix [2], its expression has been shown in a variety of tissues, cell types, and body fluids [reviewed in 3], suggesting a broad physiological relevance. Functionally, OPN has been shown to support integrin-dependent cellular adhesion, ostensibly through the RGD motif, as shown through site-directed mutagenesis of both mouse and human recombinant proteins [4, 5], although examples of RGD-independent adhesion have been described [6]. In addition, a relatively high sialic acid content and enrichment of acidic amino acids (25–30% aspartic and glutamic acid residues) predicts a calcium-binding capacity for this protein; indeed, OPN has been shown to bind calcium ions (approximately 50 atoms/molecule) [7], calcium oxalate crystals in urine [8], and hydroxyapatite crystals [9]. These functional properties appear to be important to the hypothesized roles of OPN in bone homeostasis [reviewed in 10], cardiovascular diseases [11, 12], renal disorders [13], and tumorigenesis and metastasis [14, 15].

With respect to the functioning of OPN in integrin-dependent cellular adhesion, interaction with the vitronectin receptor (the $\alpha_V\beta_3$ integrin) is well established. Recent evidence suggests that, in addition to the $\alpha_V\beta_3$ integrin, other integrins are also capable of ligating OPN. Hu *et al.* [16, 17] reported that human, recombinant OPN, expressed as a truncated GST-fusion protein, was able to promote the adhesion of human cell lines to non-adhesive substrates in an RGD-dependent manner through either the $\alpha_V\beta_1$ or $\alpha_V\beta_5$ heterodimers, in addition to the $\alpha_V\beta_3$ integrin. The ability of these integrins to ligate the OPN utilized in their studies was confirmed by solid-phase radioligand binding experiments [16]. Liaw *et al.* [18] described the ability of OPN, purified from the conditioned media of primary cultures of rat smooth muscle cells, to stimulate migration and adhesion of $\alpha_V\beta_5$-expressing human smooth muscle cells, while $\alpha_V\beta_3$-deficient cells adhered, but did not migrate, on OPN-coated substrates: RGD peptides completely blocked these activities. The authors concluded that, in addition to β_3 integrins, β_1 and β_5 integrins were also capable of ligating OPN.

The purpose of the studies described in this report was to follow up on the previously reported specificity of integrin-mediated OPN-dependent cellular adhesion. Utilizing human cell lines which express wild-type $\alpha_V\beta_1$, $\alpha_V\beta_3$, or $\alpha_V\beta_5$ integrins, we assessed the ability of full-length human

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§ *Abbreviations:* OPN, osteopontin; RGD, arginine-glycine-aspartic acid; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; D-PBS, Dulbecco's phosphate-buffered saline; PBS-T, phosphate-buffered saline and 0.1% Tween-20; FACS, fluorescence-activated cell sorting; and mAbs, monoclonal antibodies.

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recombinant osteopontin to promote cellular adhesion. We show that only the $\alpha_V\beta_3$ integrin was able to mediate cellular adhesion to this extracellular matrix protein. The determination of this selectivity may have important implications for the design of therapeutics targeting OPN–integrin interactions.

MATERIALS AND METHODS

ECM Proteins, Peptides, and Antibodies

The ECM proteins vitronectin (VN), type I collagen (CN), human plasma fibronectin (FN), the fibronectin fragments FN 120K and FN 40K, and the synthetic peptide GPenGRGDSPCA were purchased from GIBCO, while FN 45K was from Sigma. Monoclonal antibodies utilized in these studies included P1F6 (anti-$\alpha_V\beta_5$), LM609 (anti-$\alpha_V\beta_3$), P3G8 (anti-$\alpha_V\beta_1$), MAB 1980 (anti-α_V), MOPC-21 (mouse IgG_1 [immunoglobulin isotype G_1], κ, isotype control), mAb 13 (anti-β_1), integrin β_3, mAb (CD61; anti-β_3), and an FITC-labeled goat anti-mouse IgG. P1F6, LM609, P3G3, and MAB 1980 were purchased from Chemicon, MOPC-21 was from PharMingen and the FITC-labeled mAb from Southern Biotechnology Associates, Inc., while mAb 13 and integrin β_3, mAb (CD61) were purchased from Becton Dickinson.

Cell Lines

The SK-MEL-24 cell line (human malignant melanoma, metastasis to node), 293 cell line (transformed human primary embryonal kidney), and HT-29 (adenocarcinoma, colon, moderately well-differentiated grade II, human) were obtained from the American Tissue Type Collection (ATCC), and were maintained according to ATCC recommendations.

Recombinant Osteopontin Expression and Purification

In these studies, human recombinant osteopontin with an amino-terminal histidine tag was utilized as an extracellular matrix protein. A human cDNA library (monocyte THP-1 cell line; Clontech HL1021a) was used as the source of the osteopontin gene. Two polymerase chain reaction (PCR) primers were designed as follows and used for amplification: 5′GGTCTAGAGGATCCATACCAGTTAAACAGGCTGATTCTGG3′, and 5′GGGGATCCGTCGACTTAATTGACCTCAGAAGATGCAC-3′. The resulting PCR product (about 1 kb) was cleaved exhaustively with *Bam*H1 (GIBCO) before being ligated with *Bam*H1-cleaved pQE9 (Qiagen). The ligation product was used to transform *Escherichia coli* M15 cells. The transformants were selected at 37° on LB agar plates supplemented with 100 μg/mL ampicillin. Plasmid DNA was isolated from individual ampicillin-resistant colonies and analyzed by restriction endonuclease digestion to identify the osteopontin-producing cells. The sequence of the inserts generated from these clones was confirmed to be human osteopontin. Osteopontin-expressing *E. coli* cells were grown in shake flasks at 37° to an $O.D._{600}$ (optical density at 600 nm) of approximately 0.6 in 2 L of LB broth with 100 μg/mL ampicillin. Isopropyl-β-D-thiogalactopyranoside was added to 0.4 mM, and, 5 hr later, the cells were recovered by centrifugation and frozen at −70°. *E. coli* cells were resuspended in buffer A (6 M guanidine–HCl, 0.1 M sodium phosphate, 0.01 M Tris–HCl, pH 8.0) at 10 mL/*g* of cell pellet. The suspension was stirred for 1 hr at room temperature, then was subjected to centrifugation at 10,000 *g* for 20 min at 4°. The supernatant was collected and added to a slurry of Ni-NTA (nickel-*N*-(5-amino-1-carboxypentyl)iminodiacetic acid) resin previously equilibrated in buffer A. The mixture was gently shaken for 2 hr at 4°, the resin was centrifuged at 3000 *g* for 5 min, and washed sequentially with buffer A, buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris–HCl, pH 8.0), and PBS. In each wash, the resin was gently shaken and re-collected by centrifugation. The resin was then loaded onto a column and washed thoroughly with PBS. The column was washed with 3 volumes of 40 mM imidazole in PBS. Finally, the osteopontin was eluted with 250 mM imidazole in PBS.

FACS

Cells were harvested with 0.05% trypsin/0.53 mM EDTA (GIBCO) and washed twice in cold wash medium (unsupplemented growth media with 5% fetal bovine serum). The cells (10^6) and primary antibodies (2 μg) were resuspended in the same media in a total volume of 100 μL and incubated on ice for 1 hr. Following the incubation period, the cells were washed twice with cold wash medium, resuspended with 12 μg of the FITC-labeled antibody (volume = 200 μL), and incubated on ice, in the dark, for 45 min. The cells were then washed three times in cold wash medium, resuspended in 1 mL of D-PBS, and relative fluorescence intensity was determined in a Becton Dickinson FACSort.

Cell Adhesion

Cells were harvested as described above. The cells were pelleted by low-speed centrifugation and washed three times with 0.5 mg/mL trypsin inhibitor (Sigma) in D-PBS. The final cell pellet was resuspended at a concentration of 10^6 cells/mL in growth media without serum supplementation (cell suspension medium). ECM proteins were diluted in D-PBS, and 100 μL of this solution was added to each test well of PRO-BIND assay plates (Falcon 3915) and incubated for 2 hr at 37°. The wells were aspirated and washed once with D-PBS. Wells were blocked with 100 μL of 1% BSA in cell suspension medium for 1 hr at 37° and washed once with D-PBS. For peptide inhibition studies, 100 μL of various concentrations of GPenGRGDSPCA was added to ECM-coated wells (in triplicate) followed by 100 μL of cell suspension. In separate experiments to determine the IC_{50} (concentration of peptide which inhibited adhesion by 50%), full dose–inhibition curves were generated, and this parameter was determined using a

non-linear regression program (NONLIN). In blocking antibody studies, various dilutions of antibodies were incubated with the cells for 1 hr at room temperature, centrifuged briefly, and the resulting cell pellet was resuspended at 10^6 cells/mL in cell suspension medium. One hundred microliters of the cell suspension was added to ECM-coated wells containing 100 μL of cell suspension medium supplemented with 0.2% BSA; background attachment was determined in uncoated wells. The plate was incubated in a humidified atmosphere at 25° for 90 min. Following the incubation period, the wells were gently aspirated and washed twice with D-PBS. The number of cells attached was determined by a hexosaminidase reaction [19]. Fifty microliters of hexosaminidase substrate (*p*-nitrophenol-*N*-acetyl-β-D-glucosaminide at 7.5 mM in 0.1 M citrate buffer, pH 5 mixed with an equal volume of 0.5% Triton X-100 in water) was added to each well. The plate was incubated for 1 hr at 25° in a humidified atmosphere followed by the addition of 100 μL stopping solution (50 mM glycine buffer, pH 10.4, containing 5 mM EDTA). The O.D. of the wells was measured at a test wavelength of 405 nm with a reference measurement (background) taken simultaneously at 630 nm.

Western Blot Analysis

Plasma membrane preparations were generated by homogenizing the cells at 10% (w/v) in homogenization buffer (25 mM Tris–HCl, pH = 7.4; 250 mM sucrose) with 2 × 30-sec bursts of a polytron homogenizer. The homogenate was centrifuged at 3000 *g* for 10 min at 4° to remove debris. The salt concentrations of the supernatant were adjusted to 100 mM in NaCl and 0.2 mM in $MgSO_4$, and centrifuged at 22,000 *g* for 20 min at 4°. The pellet was washed by recentrifugation in membrane buffer (25 mM Tris–HCl, pH = 7.4; 100 mM NaCl; 2 mM $MgCl_2$). Proteins were electrophoresed on 10% SDS-PAGE gels (Bio-Rad) and transferred to nitrocellulose membranes (Amersham). Membranes were blocked in 5% non-fat dried milk/PBS-T for 1 hr at room temperature. Anti-integrin antibodies were added at 1:1000 in PBS-T and incubated for 1 hr at room temperature. Membranes were washed three times in PBS-T, followed by incubation with a biotinylated secondary antibody (Amersham) in PBS-T for 1 hr at room temperature. Membranes were again washed in PBS-T, and streptavidin–horseradish peroxidase (HRP; Amersham) in PBS-T was added for 1 hr at room temperature. Membranes were washed and protein was visualized using the enhanced chemiluminescence Western blot reagent (Amersham) with exposure to Hyperfilm-ECL (Amersham).

RESULTS

Cell Surface Integrin Expression of HT-29, 293, and SK-MEL-24 Cell Lines

In order to identify models of cellular adhesion, the cell surface integrin expression of the HT-29, 293, and SK-MEL-24 cell lines was determined by FACS and Western blot analysis. The human colon adenocarcinoma had previously been shown to express the $\alpha_V\beta_5$ heterodimer [20], while the 293 expresses functional $\alpha_V\beta_1$ as a vitronectin [21] or fibronectin [22] receptor. The human malignant melanoma cell line, SK-MEL-24, which was derived from a metastasis to a lymph node, was evaluated as a source of $\alpha_V\beta_3$ since this integrin appears to play a role in melanoma metastasis to the lymph node [23]. All of the cell lines appeared to express cell surface α_V and β_1 integrin subunits as evidenced both by FACS (Fig. 1A) and Western blot analysis (Fig. 2, A and B). Additionally, with respect to the presence of the $\alpha_V\beta_5$ heterodimer, only the HT-29 adenocarcinoma cell line appeared, by FACS analysis, to express amounts of this integrin (Fig. 1B). Finally, only the SK-MEL-24 melanoma cell line appeared to express heterodimeric $\alpha_V\beta_3$: by FACS analysis only this cell line demonstrated a peak (Fig. 1B), while by Western blot analysis of plasma membranes from these cell lines, a protein was recognized by an anti-β_3 antibody only from the SK-MEL-24 cell line (Fig. 2). Thus, based on the expression of cell surface integrins, the 293 cells appeared to have utility as a model of $\alpha_V\beta_1$-dependent cellular adhesion, the HT-29 a model of $\alpha_V\beta_5$-dependent adhesion, and the SK-MEL-24 cells an $\alpha_V\beta_3$-dependent model.

Extracellular Matrix-Dependent Cellular Adhesion of HT-29, 293, and SK-MEL-24 Cell Lines

To permit the assessment of the functioning of the integrins expressed on the HT-29, 293, and SK-MEL-24 cell lines, the adhesion of these cell lines to a panel of ECM proteins was determined (Fig. 3). The adhesion of cells to human plasma fibronectin (220K molecular mass) was also compared with various chymotryptic fragments, specifically, the 40K fragment (heparin-binding domain), the 45K fragment (gelatin-binding domain), and the 120K fragment (cell attachment domain) [24]. Additionally, the RGD-containing protein vitronectin [25] and type I collagen, which contains a cryptic RGD-binding site [26], were also utilized.

All three cell lines adhered well to human plasma fibronectin over the concentration range tested (3 to 30 μg/mL), with adhesion appearing to be maximal at the highest concentration tested (30 μg/mL). Comparison of adhesion of cells to native fibronectin to the chymotryptic fragments revealed qualitative differences: For the HT-29 cell line, a marked reduction in adhesion to the 120K fragment at all concentrations, as well as a reduction in adhesion to the 40K fragment at 10 μg/mL, was observed. The 293 cells showed a reduction of approximately 50% in adhesion to the 40K and 120K fragments as compared to the full-length fibronectin. Finally, the SK-MEL-24 cells appeared to adhere equally well to the native fibronectin and the 120K and 40K fragments. None of the cell lines adhered to any extent to the 45K fragment.

All cell lines adhered, at varying degrees, to both vitronectin and type I collagen over the concentration

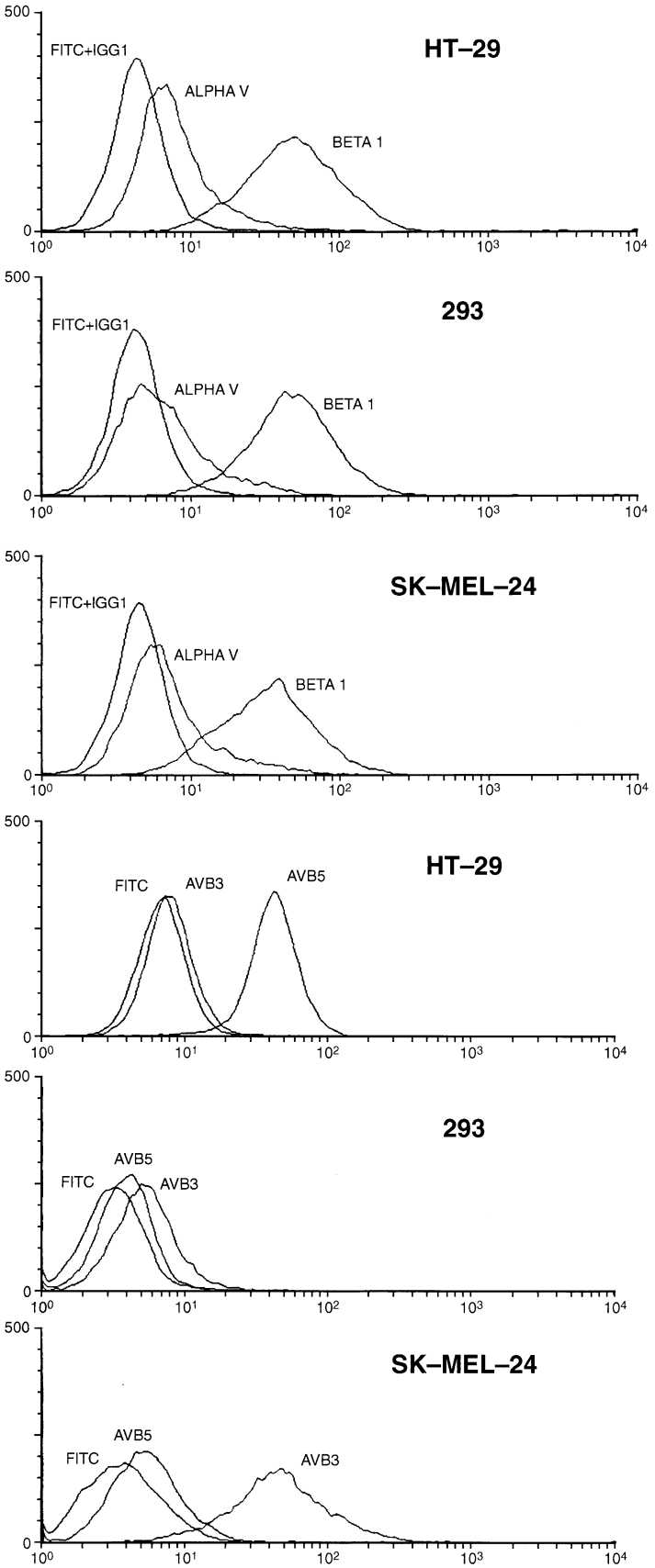

FIG. 1. FACS analysis of integrin expression on cell lines utilized in these studies. Monoclonal antibodies against human α_V, β_1 (Panel A), $\alpha_V\beta_3$, or $\alpha_V\beta_5$ (Panel B) were used to assess the cell surface expression of various integrins on the surface of the cell lines. The relative expression level of the integrins is determined by the mean fluorescence intensity (x-axis).

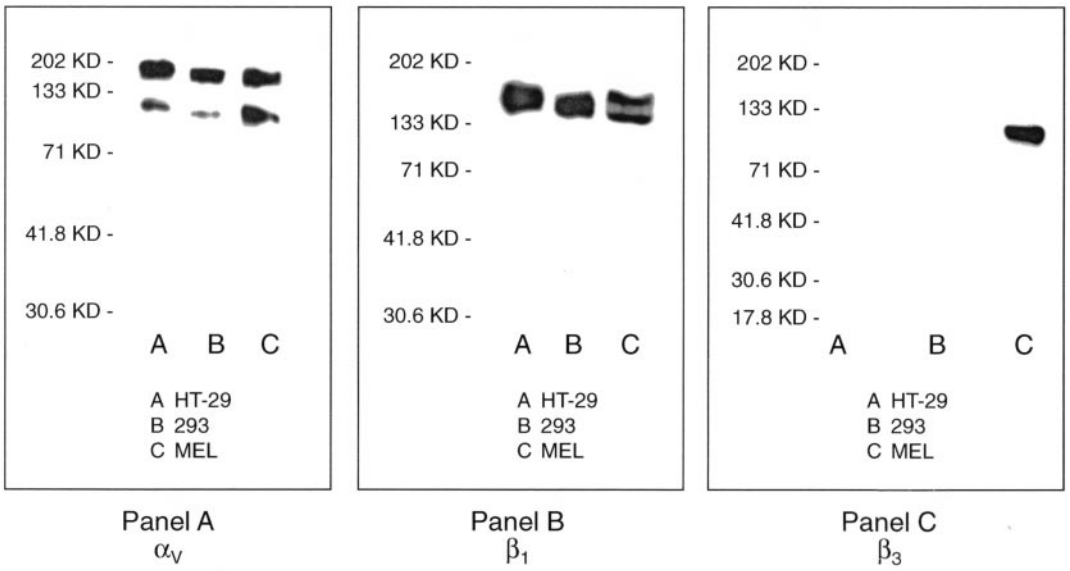

FIG. 2. Western blot analysis of cell surface integrin expression. Plasma membrane proteins from the three cell lines were electrophoresed on 10% SDS-PAGE gels, the proteins were transferred to nitrocellulose, and the resulting blot probed with various primary anti-integrin antibodies. Panel A, anti-α_V; Panel B, anti-β_1; Panel C, anti-β_3.

range tested. With respect to the human recombinant osteopontin, only the SK-MEL-24 cell line showed any appreciable adhesion at concentrations up to 30 μg/mL. A dose–response relationship for the adhesion of the SK-MEL-24 cell line to osteopontin was generated over the concentration range of 0.03 to 100 μg/mL (Fig. 4). Adhesion was observed to increase in a dose-dependent fashion, with half-maximal (EC_{50}) attachment observed at 1.8 μg/mL. From these data, additional adhesion studies were performed with wells coated with proteins at 10 μg/mL.

The Role of the RGD Recognition Motif in Integrin-Dependent Cellular Adhesion

Osteopontin contains the three amino acid integrin recognition motif, arginine-glycine-aspartic acid (RGD), and this site appears to mediate α_V-dependent cellular adhesion exclusively, since mutation of the aspartic acid to glutamic acid destroys adhesive and migration functions of this protein [5, 6]. Therefore, in order to focus on adhesion mediated by the α_V-integrins, the ability of an RGD-containing peptide, GPenGRGDSPCA, to inhibit cellular adhesion was determined at 30 and 100 μM (Fig. 5).

Adhesion of the 293 and SK-MEL-24 cell lines to FN 40K was not significantly affected by GPenGRGDSPCA: significant inhibition of the HT-29 cell line was observed at both 30 and 100 μM concentrations, although greater than 50% inhibition was only observed at 100 μM, a concentration which is well above the affinity for the $\alpha_V\beta_3$ integrin [27]. Therefore, it is likely that the RGD motif mediates only a fractional component of the adhesion of the HT-29 cell line to FN 40K; adhesion of the 293 and SK-MEL-24 was essentially unaltered. With respect to FN 120K adhesion, no inhibition of the 293 cell line was observed up to 100 μM, whereas both the HT-29 and SK-MEL-29 cell lines were inhibited in a dose-dependent fashion. Determination of the relative half-maximal inhibitory concentration (IC_{50}) yielded values of 1.68 and 0.72 μM for the HT-29 and SK-MEL-24 cell lines, respectively. Adhesion of all three cell lines to vitronectin was inhibited in a similar fashion by GPenGRGDSPCA: comparable IC_{50} values were generated, from 0.15 μM for 293 cells to 2.0 μM for the HT-29 cell line (Table 1). Since only the SK-MEL-24 cell line adhered to osteopontin, inhibition of cellular adhesion by GPenGRGDSPCA was only determined in this cell line. Similar to the inhibition of cellular adhesion to FN 120K and vitronectin, GPenGRGDSPCA dose dependently inhibited SK-MEL-24 cellular adhesion to osteopontin with an IC_{50} value of 0.42 μM (Table 1). Finally, cellular adhesion of all three cell lines to type I collagen was relatively resistant to GPenGRGDSPCA inhibition as expected, based on the fact that the cryptic RGD sequence does not play a role in cellular adhesion in the natural protein [26].

Characterization of Functional Integrin Expression

The ability of various integrins to mediate cellular adhesion was assessed through the use of blocking antibodies. The effect of an antibody against the α_V subunit on cellular adhesion to vitronectin and collagen for all three cell lines, and for adhesion to osteopontin for the SK-MEL-24 cell line, was determined (Fig. 6). This antibody partially inhibited adhesion (approximately 50% of maximal) of the 293 and SK-MEL-24 cell lines to vitronectin, markedly inhibited adhesion of the SK-MEL-24 cell line to osteopontin, and appeared to enhance adhesion of the HT-29 cell

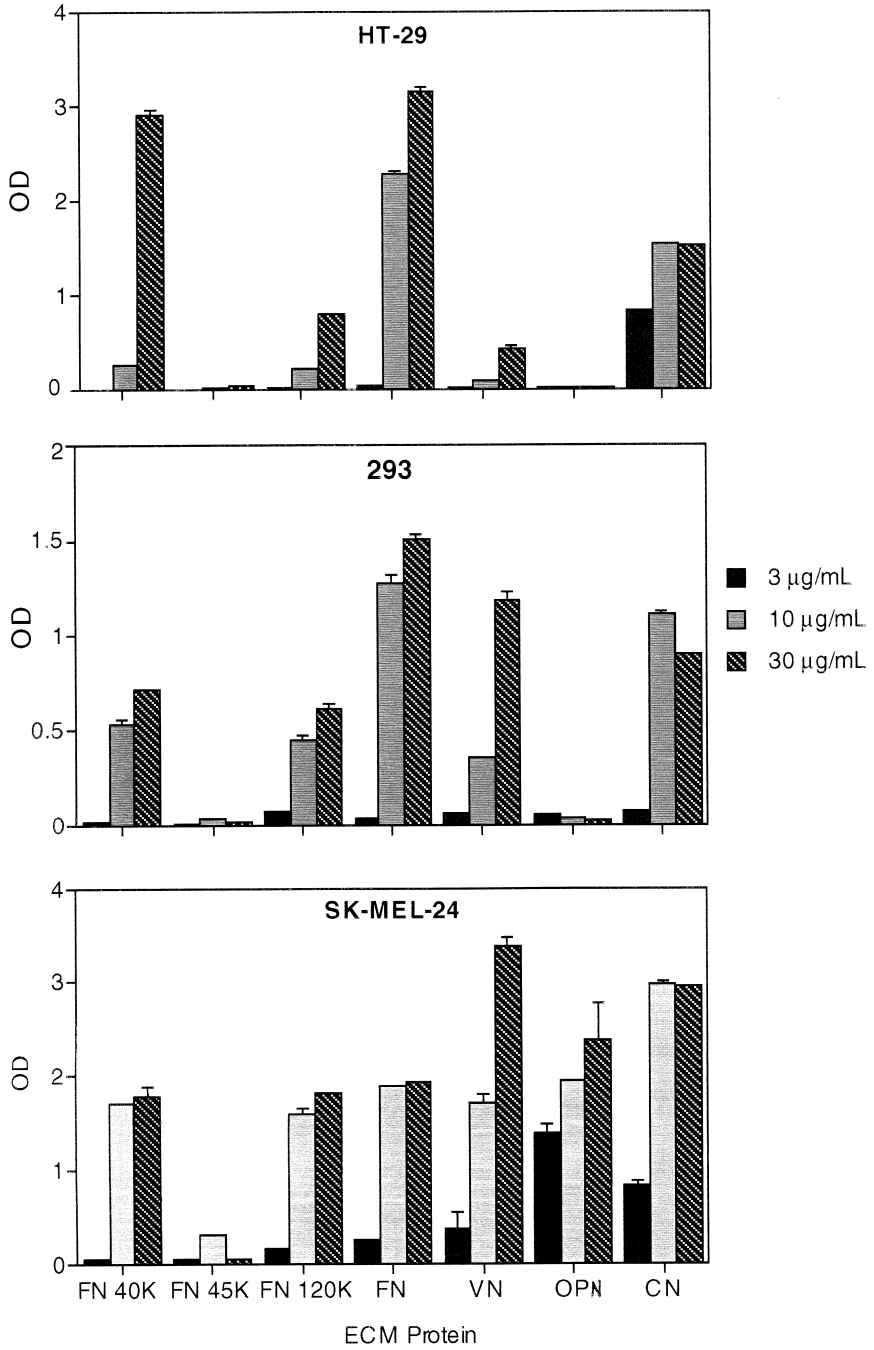

FIG. 3. Adhesion of cell lines to various extracellular matrix proteins. The adhesion of the HT-29 (upper panel), 293 (middle panel), and SK-MEL-24 (lower panel) to 3, 10, or 30 μg/mL of various extracellular matrix proteins was determined. Each bar represents the mean ± SEM of triplicate determinations; each experiment was repeated three times, and a representative experiment is shown. FN, fibronectin; VN, vitronectin; CN, collagen.

line to vitronectin; the effect of this antibody on cellular adhesion to collagen was not determined. Previous reports describe the ability of certain polyclonal antibodies to enhance integrin-dependent cellular adhesion [28]. An antibody against the β_1 integrin subunit partially inhibited adhesion of all three cell lines (approximately 50% of maximal) to vitronectin, and essentially completely inhibited adhesion of these cell lines to collagen. Adhesion of SK-MEL-24 cells to osteopontin was, for the most part, unaffected. These data would suggest that, for the 293 and SK-MEL-24 cell lines, functional α_V and β_1 integrin subunits are expressed on the cell surface; these subunits likely form a functional $\alpha_V\beta_1$ heterodimer, which has been shown to mediate cellular adhesion to vitronectin [21] and fibronectin [22]. Additionally, it is well appreciated that β_1 integrins mediate adhesion to collagen; in these studies, collagen-mediated adhesion appeared to be completely dependent on this integrin in all three cell lines. Finally,

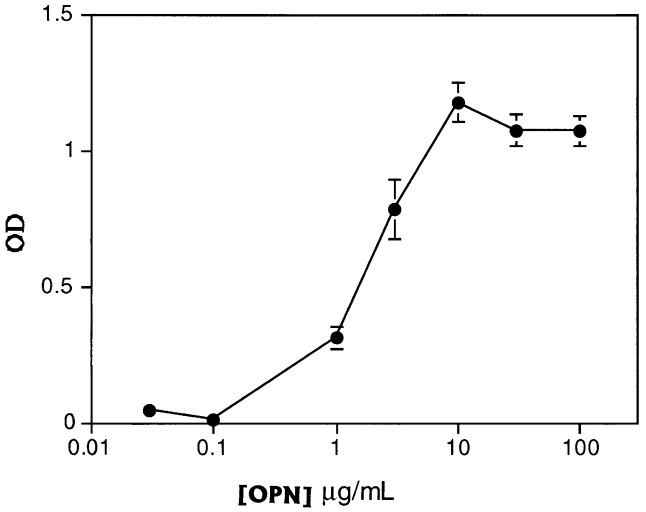

FIG. 4. Dose–adhesion relationship for SK-MEL-24 cell adhesion to recombinant human osteopontin. The adhesion of SK-MEL-24 cells to increasing concentrations of osteopontin was determined, and the concentration yielding half-maximal (EC_{50}) adhesion estimated from non-linear regression analysis. Each point represents the mean ± SEM of triplicate determinations from three separate experiments.

the α_V, but not the β_1 integrin, appeared to mediate cellular adhesion of the SK-MEL-24 cell line to osteopontin.

In addition to blocking antibodies against single integrin subunits, the ability of antibodies against the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ heterodimers to inhibit adhesion was assessed. An antibody against the $\alpha_V\beta_3$ heterodimer was relatively ineffective in inhibiting HT-29 or 293 cellular adhesion to either vitronectin or collagen; this result agrees with the FACS and Western blot data, which showed that these cell lines do not express the β_3 integrin subunit. In contrast, this antibody completely inhibited adhesion of the SK-MEL-24 cell line to osteopontin, suggesting that, at least for this cell line, adhesion to this extracellular matrix protein is completely dependent upon $\alpha_V\beta_3$ expression. Finally, an antibody against the $\alpha_V\beta_5$ heterodimer completely inhibited HT-29 adhesion and partially inhibited 293 and SK-MEL-24 cellular adhesion to vitronectin, while adhesion to collagen for all three cell lines was unaffected. This result agrees well with respect to FACS analysis of HT-29 cells, which showed high levels of $\alpha_V\beta_3$ heterodimer expression, although, by FACS analysis, neither the 293 nor SK-MEL-24 cell line appeared to express appreciable amounts of the $\alpha_V\beta_5$ heterodimer. It is possible that, under these conditions, cellular adhesion is able to identify low-level $\alpha_V\beta_5$ heterodimer expression. With respect to SK-MEL-24 adhesion, this antibody was unable to appreciably affect cellular adhesion to osteopontin.

DISCUSSION

In this report, we describe the ability of human recombinant full-length osteopontin to support cellular adhesion of the SK-MEL-24 cell line, but not of the HT-29 nor 293 cell lines. In addition, we detected the expression of the α_V and β_1 integrin subunits in all three cell lines by FACS and Western blot analysis, but only observed expression of the β_3 subunit in the SK-MEL-24 cell line. Furthermore, blocking antibody studies demonstrated the presence of functional (i.e. able to support cellular adhesion) α_V, β_1 (presumably forming an $\alpha_V\beta_1$ heterodimer), $\alpha_V\beta_3$, and $\alpha_V\beta_5$ integrins in these cell lines. Finally, since only an $\alpha_V\beta_3$-, and neither an $\alpha_V\beta_1$- nor an $\alpha_V\beta_5$-expressing cell line adhered to osteopontin, these data suggest that, at least among the α_V integrins, only the $\alpha_V\beta_3$ heterodimer is able to support cellular adhesion to osteopontin.

Several recent reports describe the ability of $\alpha_V\beta_1$ and $\alpha_V\beta_5$ integrin heterodimers, in addition to $\alpha_V\beta_3$, to bind to osteopontin and mediate cellular adhesion [16–18]. In this report, we describe the inability of these integrins to mediate cellular attachment to osteopontin. The reason for this discrepancy is not clear, although the source of the osteopontin in our studies and those reported by Hu *et al.* [17] and Liaw *et al.* [18] were all different. In the studies reported by Hu *et al.* [17], a fragment of 228 amino acids of human osteopontin was used as a GST-fusion protein, whereas in the studies reported here, the full-length human osteopontin (314 amino acids) was utilized; the size of the protein in both studies was confirmed by mass spectroscopic analysis. Why the deletion of the carboxy-terminal 86 amino acids would affect cellular adhesion by the RGD sequence is not known; the RGD sequence, which appeared to mediate cellular adhesion in the studies reported by Hu *et al.* [17] and our studies, is located in exon 5 (amino acids 159, 160, 161), which is well removed from the carboxyl-terminal portion of osteopontin. A possible explanation is that these deleted amino acids affect the tertiary conformation of the RGD sequence, and, therefore, the integrin selectivity. It has been shown that osteopontin contains an internal thrombin cleavage site which is within six amino acids of the RGD recognition motif [29, 30], and that cleavage at this site regulates the adhesive properties of osteopontin [4]. Therefore, there is a likelihood that the use of a truncated protein led to the difference in results.

Liaw *et al.* [18] also reported that the $\alpha_V\beta_1$ and $\alpha_V\beta_5$ integrins were capable of mediating cellular adhesion to OPN. In that study, the source of the osteopontin was conditioned media from rat aortic smooth muscle cells. Comparison of amino acid sequences around the RGD in human and rat osteopontins reveals that, whereas the amino-terminal sequence of the RGD site is identical (DG), the carboxyl-terminal sequence varies (VVY in human, LAY in rat). It is well appreciated that, although the RGD recognition motif is common to many integrin ligands, the amino acids surrounding this sequence can markedly affect affinity. For example, the snake venom protein echistatin possesses a 1000-fold higher affinity for the $\alpha_V\beta_3$ integrin than the leach protein decorsin, although both contain the RGD sequence [31]. As described for these snake venoms, the amino acid sequence differ-

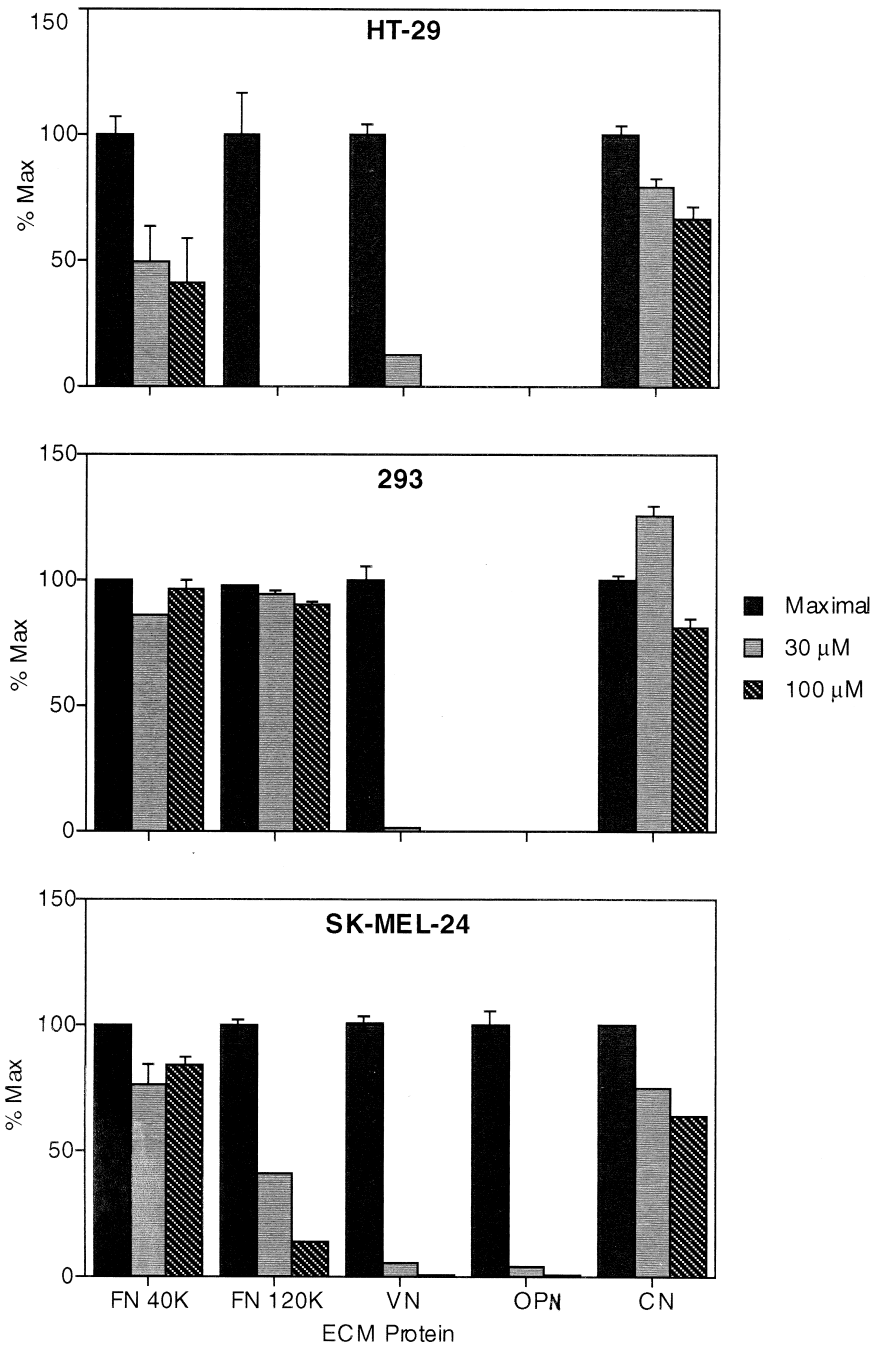

FIG. 5. RGD dependence of cellular adhesion to various ECM proteins. The ability of a synthetic RGD-containing peptide (GPenGRGDSPCA), at 30 and 100 μM, to inhibit cellular adhesion of the HT-29 (upper panel), 293 (middle panel), and SK-MEL-24 (lower panel) to various ECM proteins was determined. Each bar is the mean ± SEM of triplicate determinations from a representative experiment. FN, fibronectin; VN, vitronectin; CN, collagen.

ences noted between rat and human osteopontin around the RGD-binding motif may alter the affinity, and thus the selectivity, of OPN for various integrins. This may explain the difference in the observed integrin selectivity.

Another possibility is that the cellular models of integrin-dependent adhesion used in our studies affected the integrin selectivity. Examples of cell-specific adhesion are available, albeit relatively uncommon. With respect to the current studies, we used the same cell line (kidney 293) expressing the native $\alpha_V\beta_1$ integrin as was used in a report by Hu *et al.* [17] under the same assay conditions used by these authors to demonstrate adhesion to osteopontin. We were not able to observe any adhesion at osteopontin concentrations as high as 30 μg/mL, a concentration where, in our studies, maximal adhesion to vitronectin was observed. In addition, we observed that both the 293 and HT-29 cell lines (the latter expressing the $\alpha_V\beta_5$ integrin) adhere to vitronectin in an RGD-dependent manner. This

TABLE 1. Relative inhibition constants (IC_{50} values) for GPenGRGDSPCA inhibition of cellular adhesion

ECM Protein	HT-29	293	SK-MEL-24
VN	2.03 μM (1.27–3.03)	0.151 μM (0.113–0.201)	0.765 μM (0.624–0.907)
OPN	No Adhesion	No Adhesion	0.418 μM (0.324–0.512)
FN120	ND	No Inhibition	1.68 μM (1.08–2.33)

Dose–inhibition relationships were generated from cellular adhesion assay data, and the concentration of GPenGRGDSPCA inhibiting adhesion by 50% was estimated by non-linear regression analysis. Numbers in parentheses are the 95% confidence interval. ND: Not determined.

adhesion of the 293 cell line was shown to be attributable, at least partially, to a β_1 integrin by the use of a blocking antibody and, with respect to the HT-29, wholly through the $\alpha_V\beta_5$ integrin. This would clearly demonstrate the fully functional nature of both the $\alpha_V\beta_1$ and $\alpha_V\beta_5$ integrins in these cell lines. Furthermore, although it has been shown that the $\alpha_V\beta_3$ integrin can be activated [32], no such demonstration exists for either the $\alpha_V\beta_1$ or $\alpha_V\beta_5$ integrin; this demonstration of activation is more relevant to the cellular source of the $\alpha_V\beta_3$ integrin (platelets), which are well known to be activated, than to any of the cell lines used in the studies reported here. Therefore, the use of these cell lines is relevant to the demonstration of $\alpha_V\beta_1$ and $\alpha_V\beta_5$ integrin-ligand specificity.

Divalent cation regulation of adhesion has been demonstrated in a few recent publications. The most relevant to the work described in this report come from Hu *et al.* [16, 17]. In the first of these studies, the authors show that Ca^{2+} *suppresses* adhesion of the $\alpha_V\beta_3$ integrin to osteopontin while in the second, Ca^{2+} was shown to be ineffective in supporting either $\alpha_V\beta_1$- or $\alpha_V\beta_5$-mediated adhesion to osteopontin. Thus, since in our studies concentrations of calcium were utilized which were not in the range where suppression of $\alpha_V\beta_3$-dependent adhesion had previously been reported, Ca^{2+} concentration cannot explain our lack of adhesion of the $\alpha_V\beta_1$- or $\alpha_V\beta_5$-expressing cell lines to osteopontin. However, in the second report, there were effects by both Mn^{2+} and Mg^{2+} on the adhesion of both the $\alpha_V\beta_1$- and $\alpha_V\beta_5$-expressing cell lines. In this study, the concentrations of these cations were either 0.2 mM for Mn^{2+} or 2 mM for Mg^{2+}. With respect to the cation concentrations used in the cell adhesion studies described in our studies, Mg^{2+} concentrations were in the order of 0.5 to 1 mM. Although these concentrations are below those observed for maximal adhesion, it is very likely that they were adequate for detecting any ability of the $\alpha_V\beta_1$ or $\alpha_V\beta_5$ integrins to mediate adhesion. In our studies, no adhesion was observed, even at high coating concentrations of osteopontin (30 μg/mL). Thus, differing cation concentrations do not explain the differences in integrin selectivity.

Characterization of the cell surface integrin expression of human osteoclasts has shown the presence of the α_V, $\alpha_V\beta_3$, β_3, β_1, α_2, and α_4 subunits/heterodimers; based on this profile, it is presumed that the repertoire of heterodimers would include the $\alpha_V\beta_1$, $\alpha_4\beta_1$, and $\alpha_2\beta_1$ integrins, in addition to the $\alpha_V\beta_3$ [reviewed in 33]. Although it has not been demonstrated that the only extracellular matrix protein which mediates cellular adhesion of the osteoclast to bone is OPN, indirect evidence, obtained by immunolocalization studies, demonstrates a role for OPN as the ECM ligand of the osteoclast $\alpha_V\beta_3$ integrin [34]. Further, it has been shown *in vitro* that an antibody to the $\alpha_V\beta_3$ integrin (23C6) inhibited the ability of isolated osteoclasts to resorb dentin slices to the same extent (76% inhibition) as an RGD peptide (GRGDSP, 78%) [35]. Taken together, these observations strongly suggest a critical role for OPN–$\alpha_V\beta_3$ integrin ligation in osteoclast function, an interaction which is not served through the $\alpha_V\beta_1$ integrin. Therefore, at least in osteoclasts, it would appear that the $\alpha_V\beta_3$, and not the $\alpha_V\beta_1$ integrin, can ligate OPN.

Osteopontin is rich in sialic acid residues, and this property appears to play a role in the calcium-binding properties of this protein. Recent evidence suggests that this posttranslational modification may also affect cell adhesion activity; a decrease in the degree of sialyation of OPN, which has been demonstrated in oncogenically transformed cells, has been shown to decrease the ability of this protein to bind to cell surface $\alpha_V\beta_3$ receptors [36]. There is thus the possibility that altered posttranslational modification of the recombinant osteopontin used in our studies explains our results. However, reports show that the alteration in OPN sialyation affects $\alpha_V\beta_3$ binding, whereas in the studies reported here full cell adhesion activity mediated by this integrin was retained. This is an interesting result, since it is known that protein sequence alone (RGD) has integrin-binding activity. Thus, the possibility exists that the posttranslational sialyation of OPN alters protein tertiary structure. This could be an explanation for the disparity between our selectivity results and those of Liaw *et al.*, who used protein purified from conditioned media [18], although Hu *et al.* [16, 17] also used a bacterially expressed recombinant protein and observed a different integrin selectivity. Therefore, posttranslational modifications alone do not explain differences in integrin selectivity.

OPN expression is not limited to bone, but is found in a variety of tissues, cell types, and body fluids [reviewed in 3], suggesting a role in diverse processes. Expression has been shown to increase during embryonic bone development [37], following arterial wall injury [38], and, in some cases, as a result of neoplastic transformation [14, 15]. Based on the results described in this paper, this would suggest that, in physiological or pathophysiological processes in which OPN functions as an adhesion molecule, the vitronectin receptor would be expected to play a fundamental role. Therefore, the potential for therapeutic intervention in these processes by vitronectin receptor modulators or antagonists exists: This potential has already been demonstrated in prevention of osteoclast-mediated osteolysis [reviewed in 10].

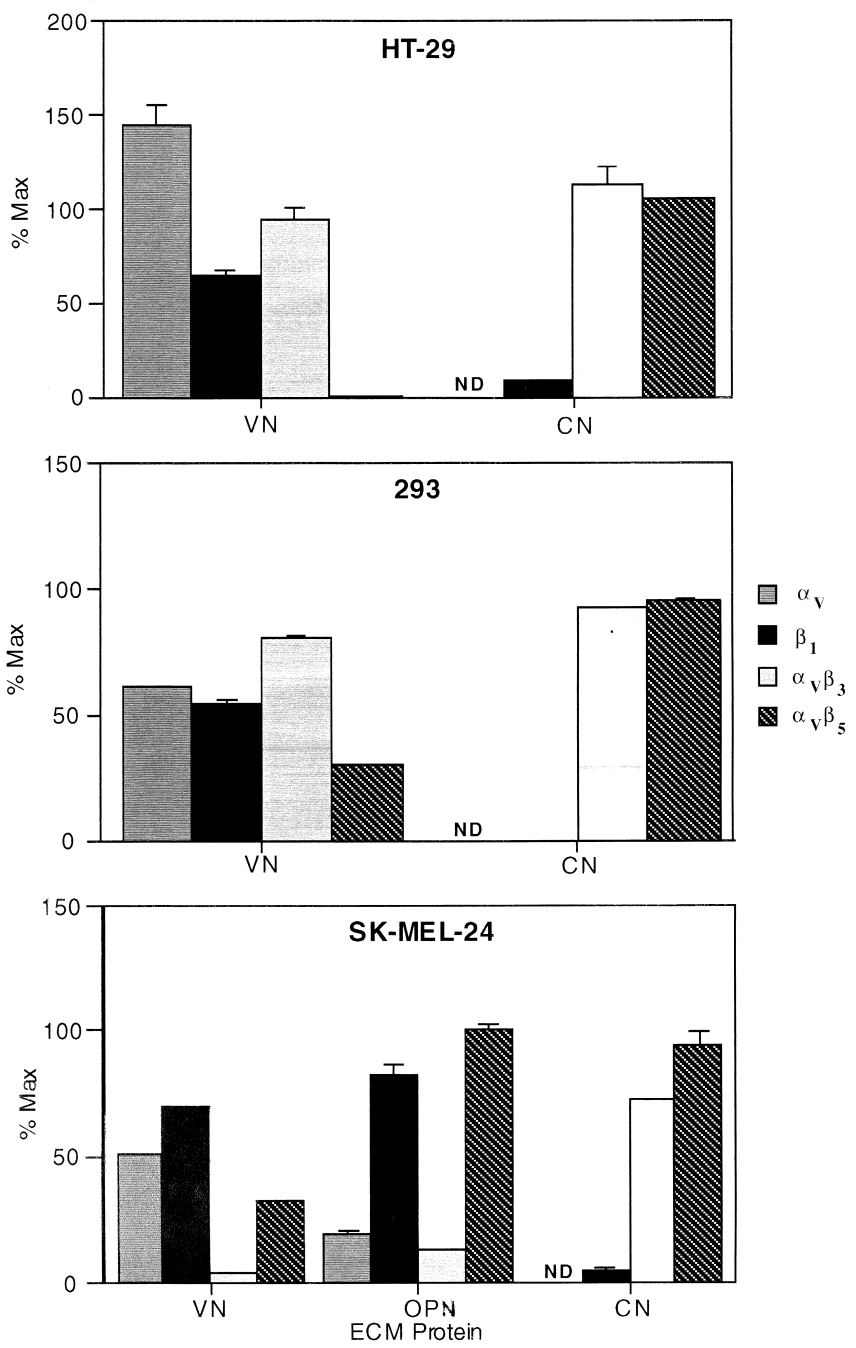

FIG. 6. Effect of various anti-integrin antibodies on cellular adhesion to various ECM proteins. The ability of various blocking anti-integrin monoclonal antibodies to inhibit cellular adhesion was determined. Each bar is the mean ± SEM of triplicate determinations from a representative experiment. ND, not determined; VN, vitronectin; CN, collagen.

In conclusion, we have demonstrated that full-length human recombinant osteopontin expresses a unique monospecific integrin ligand specificity for the vitronectin receptor. This observation is particularly intriguing since, among the RGD-dependent integrins, the vitronectin receptor shows the most diverse ligand selectivity, with at least 10 proteins identified as ligands to date. Although this would suggest that there would likely exist marked redundancy in the functioning of the vitronectin receptor, there would appear to be a fundamental role played by this integrin with respect to processes which are mediated by the adhesive functioning of OPN.

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