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Osteopontin adhesion receptors on gingival fibroblasts

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Osteopontin (OPN) promotes attachment and spreading of cells in an RGD dependent fashion, suggesting that OPN interacts with integrins on cell surfaces. Here in, we show that LM-609, a monoclonal antibody to the $\alpha_v\beta_3$ integrin (a vitronectin receptor), inhibited OPN-mediated attachment of gingival fibroblasts. To characterize the cell surface receptors responsible for this interaction, we performed OPN-sepharose affinity chromatography using detergent extracts of ^{35}S-methionine or ^{125}I-surface labeled gingival fibroblasts. Proteins bound to the OPN-matrix were eluted with EDTA and subjected to SDS-PAGE under reducing conditions. EDTA eluates from both ^{125}I-surface labeled and ^{35}S-methionine labeled extracts demonstrated prominent bands in the 90kDa and 50kDa regions, by both autoradiography and fluorography, respectively. These studies suggest that OPN is associated with other cell surface molecules in addition to $\alpha_v\beta_3$. Furthermore, these as yet to be characterized proteins, may prove to have a stronger affinity for OPN than $\alpha_v\beta_3$.

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Cell-cell and cell-substrata interactions are important to many physiological and pathological events including cell differentiation, platelet aggregation, wound healing and malignant invasion and metastasis (1–6). In wound healing research particular emphasis has been placed on understanding the roles of extracellular matrix adhesion molecules and their associated cell surface receptors in controlling regeneration of hard and soft tissues (1–6). Particularly for restoring periodontal tissues, research has been directed toward understanding the specific cells and growth factors required to stimulate formation of new bone, cementum and periodontal ligament attachment tissue. An initial event required for tissue regeneration is the migration and subsequent attachment of appropriate cells at the healing site. We reported previously that osteopontin (OPN), a bone-associated attachment protein, promotes the attachment of both gingival fibroblasts and periodontal ligament cells, as well as other bone-like cells *in vitro* (7–11). However, the greatest OPN-mediated cell attachment was observed routinely with gingival fibroblasts (9). Hence, we have limited our studies to cell surface receptors on gingival fibroblasts that bind OPN.

OPN is a phosphorylated glycoprotein, rich in aspartic acid, sialic acid and glutamic acid (12, 13). cDNA sequencing studies reveal an arginine-glycine-aspartic acid (RGD) cell attachment sequence (14) similar to that in other adhesion molecules, including fibronectin, bone sialoprotein (BSP), vitronectin and collagen (1, 2). In mineralized tissues, OPN is expressed prior to mineralization, is regulated by osteotropic hormones, binds to hydroxyapatite, and enhaces osteoclast and osteoblast adhesion (12, 15, 16). OPN is also associated with kidney, placenta, neurosensory cells of the ear, macrophages, smooth muscle, urine, blood and human milk (17–24). In addition, OPN is expressed by many transformed fibroblasts and epithelial cells *in vitro* (25, 26) and is found at elevated levels in plasma of patients with metastatic carcinoma (26, 27).

Integrins, a family of cell adhesion receptors, play a major role in controlling interactions between cells and attachment proteins containing RGD domains (1–6, 28). Structurally, the integrin

family of receptors are similar, containing heterodimeric complexes of noncovalently associated α and β subunits. For example, the β_1 subunit is capable of associating with a minimum of six related α subunits, each having a common β_1 chain, but a distinct α chain. Each combination promotes different ligand binding specificity. This group includes receptors that bind fibronectin, laminin and collagens. Receptors containing the β_3 subunit include the $\alpha_v\beta_3$ receptor expressed by several cell types and having several binding ligands (29, 30) and the IIb/IIIa complex expressed by platelets (30). The adhesion molecules associated with $\alpha_v\beta_3$ include thrombospondin, vitronectin, fibronectin, von Willebrand factor, fibrinogen, OPN and bone sialoprotein (BSP) (1, 2, 16, 29, 31, 33). In addition to β_3, the α_v subunit complexes with other β subunits including β_1 (34), β_5 (35), β_6 (36) and β_8 (37).

Because of the complexity of the integrin family, it is important to establish definitively whether OPN interacts with known integrin receptors, as well as with any other novel cell surface receptors. Studies to date have shown only indirectly that OPN interacts with the $\alpha_v\beta_3$ cell surface receptor on gingival fibroblasts (8) and osteoclasts (16, 31–33, 38, 39). Sauk et al. (40) reported that an OPN peptide (GRGDSL(K)) sepharose affinity matrix binds the $\alpha_v\beta_3$ integrin on gingival fibroblasts. The observed differential binding between the RGD domain of a protein and the complete protein, e.g. fibronectin (41), prompted us to prepare an OPN affinity matrix to determine whether or not OPN was associated directly with the $\alpha_v\beta_3$ integrin. We show here that OPN associates with several, as yet uncharacterized, cell surface molecules. In addition, $\alpha_v\beta_3$ integrin bound to the OPN-affinity matrix to a limited extent.

Material and methods

Cells

Human gingival fibroblasts, cultured from explants, were used in these studies. They were grown in Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS) and antibiotics. Cells were used between the second to ninth passage (42).

OPN Affinity Chromatography

Cell surface receptors for OPN were isolated by affinity chromatography following classical procedures used for selecting receptors for fibronectin and vitronectin (28, 30, 43). The OPN-sepharose was prepared by coupling 20 mg bovine OPN with 2 ml of CNBr-activated sepharose (Pharmacia LKB Biotechnology, Inc.). The OPN peptide column was prepared by coupling 20 mg GRGDSL(K) peptide with 2 ml of activated CNBr-Sepharose (Pharmacia LKB Biotechnology, Inc.). The RGD peptide was prepared by the American Red Cross, Rockville MD. Bovine OPN was prepared as described by Prince et al. (12).

One hundred fifty mm^2 tissue culture flasks (Corning) were seeded with approximately 5×10^6 cells. When cells reached 80% confluency, usually 2 days, cultures were metabolically radiolabeled with 20μCi/ml of ^{35}S-methionine in methionine-free medium for 24 h at 37°C (30). Cell surface labeling was performed as described by Ginsberg & Jaques (44) or with Iodogen (Pierce). Radioactively labeled cells were scraped from the flasks, the solution collected and centrifuged at room temperature for 3 min. Cell pellets were washed extensively with phosphate-buffered saline and lysed by adding extraction buffer (50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1 m*M* $CaCl_2$ containing 25 m*M* n-octyl-β-D glucopyranoside (octylglucoside) and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)). Lysed cells were incubated at 4°C for 10 min and then centrifuged at 17000×g for 30 min to remove insoluble material. Octylglucoside extracts were then applied to a sepharose CL-4B column (Pharmacia) and the flow through applied to OPN-sepharose. The column was then washed with extraction buffer to remove any unbound proteins. These unbound proteins were applied to an OPN-peptide (GRGDSL(K)) sepharose column and eluted as described below for OPN-sepharose.

Since integrin-ligand binding has been shown to require divalent cations (45), putative integrins were eluted with ethylenediaminetetraacetic acid (EDTA). Two bed volumes (4 ml) of 20 m*M* EDTA containing 50 m*M* Tris-HCl, pH 7.5, 25 m*M* octylglucoside, 150 m*M* NaCl and 1 m*M* PMSF were collected in eight 500 μl fractions. Next, to remove additional molecules bound to the column, two bed volumes (4 ml) of 8 *M* urea, in 50 m*M* Tris-HCl, pH 7.5 with 1 m*M* PMSF were collected in eight 500 μl fractions.

Eluted fractions were analyzed on 7.5% polyacrylamide SDS gels (SDS-PAGE) directly or after immunoprecipitation, followed by fluorography or autoradiography (46).

Immunoprecipitations

For immunoprecipitation assays, EDTA eluted fractions were dialyzed against water and lyophilized. Samples were then dissolved in 120 m*M* NaCl, 1.5 *M* EDTA, 0.3% Triton X-100, 0.006% NaN_3, 15 m*M* Tris-HCl, 2 m*M* levamisole, 4 m*M* PMSF and 40 m*M* 6-aminocaproic acid.

Aliquots of these samples were exposed to pre-

immune rabbit serum-absorbed protein A-sepharose (Sigma) at 4°C for 2 h to remove any non-specific binding. The Sepharose was pelleted and affinity purified rabbit anti-$\alpha_v\beta_3$ antibody was added to the supernatant. Following an overnight incubation at 4°C, fresh non-absorbed protein A-sepharose was added and the protein A-sepharose immune complexes washed extensively. The immune complexes were released by heating to 90°C for 5 min in SDS-PAGE sample buffer (50 m*M* Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue and 10% glycerol) containing 5% 2-mercaptoethanol. These samples were subjected to SDS-PAGE and visualized by fluorography. The $\alpha_v\beta_3$ antibody was a gift from W. Scott Argraves, American Red Cross, Rockville, MD. This antibody, a rabbit antiserum against the human $\alpha_v\beta_3$ receptor, was shown to be specific to the α_v and β_3 subunits (43).

Gel electrophoresis

For gel electrophoresis, samples (reduced with 5% 2-mercaptoethanol) were analyzed by 7.5% SDS-PAGE with a 4% stacking gel, according to the method of Laemmli (47). Molecular weight standards (Amersham or Biorad) were used. Radioactive gels were fixed, dried and analyzed for ^{35}S-methionine labeled proteins by fluorography and for ^{125}I-labeled proteins by autoradiographed.

Attachment assay

The assay used to determine the ability of OPN to promote attachment of gingival fibroblasts was a modification of the Klebe method (48). Uncoated 24 well bacteriological plates (Corning 25820) were precoated with 400 μl of water containing 10 μg/ml of OPN (230 pM) or 10 μg/ml fibronectin (50 pM). Both coated and uncoated (control) dishes were preincubated for 1 h at 37°C in 400 μl DMEM containing 1 mg/ml bovine serum albumin (BSA) alone or with a specific agent at 100 μg/ml, i.e. anti-fibronectin antibody, GRGDSP, GRGESP, LM-609. After the preincubation period, cells were detached from flasks with 0.008% trypsin containing 0.04% EDTA and centrifuged. Cell pellets were rinsed twice and resuspended in DMEM/BSA. Cells were added at a concentration of $2\text{-}4\times10^4$ cells/well in 100 μl DMEM/BSA for 1.5 h. Following incubation, wells were rinsed with 500 μl Hank's buffered salt solution to remove unattached cells. Remaining cells were removed enzymatically and counted by Coulter Counter. All agents were evaluated in triplicate. The affinity purified monoclonal antibody, LM-609, was provided by Dr. David Cheresh, Scripps Clinic, LaJolla CA. LM-609 was generated to a functional site on an intact RGD-adhesion receptor complex of M21 human melanoma cells and prevents M21 cell attachment to vitronectin, von Willebrand factor, fibrinogen and an RGD-peptide, but not to fibronectin (29). The GRGDSP and GRGESP peptides were prepared by the American Red Cross, Rockville, MD and goat anti-human fibronectin antibody was purchased from Jackson Immunoresearch Laboratories, Inc., Avondale, PA.

Results

The rationale for determining whether or not OPN bound directly to the $\alpha_v\beta_3$ receptor was based on studies which provided indirect evidence that OPN mediates adhesion of osteoclasts via the $\alpha_v\beta_3$ receptor (16, 31-33, 38, 39). Also, we had shown previously that an OPN-peptide affinity column binds to the $\alpha_v\beta_3$ integrin on gingival fibroblasts (40). Additional indirect evidence that OPN binds to the $\alpha_v\beta_3$ receptor is shown in Fig. 1. The results demonstrated that LM-609, an antibody to $\alpha_v\beta_3$ receptor, blocked OPN-mediated cell attachment

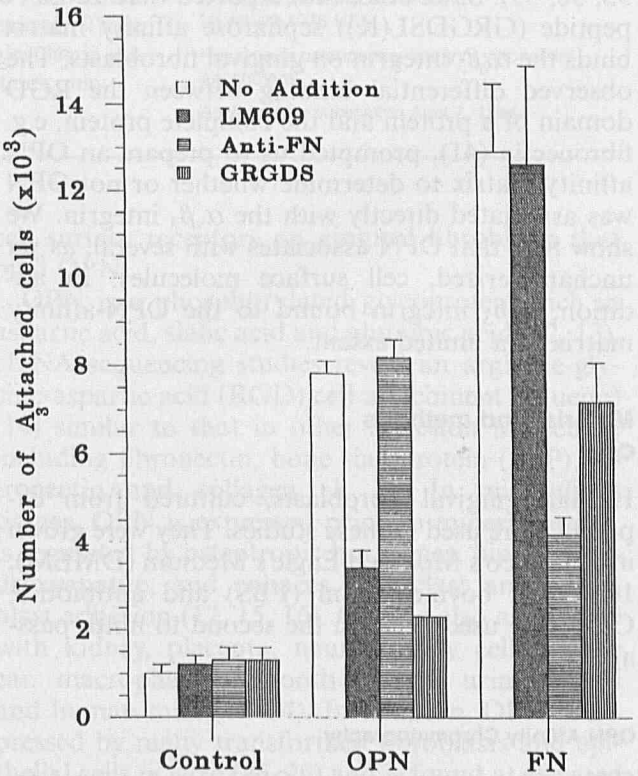

Fig. 1. Attachment assays showing OPN/cell-receptor interactions. Dishes were dry coated with OPN (4 μg) or fibronectin (FN) (4 μg) and incubated for 1 h in DMEM containing BSA (1 mg/ml) alone or with LM-609, anti-fibronectin antibody or GRGDS-peptide. Cells (2.3×10^4 cells/well) were allowed to attach for 1.5 h and attached cells quantitated by Coulter Counter. All agents were evaluated in triplicate. Data expressed as total number of attached cells ($\times10^3$)±standard deviation.

but not fibronectin-mediated cell attachment. Fibronectin antibodies blocked fibronectin-mediated cell attachment, but had no effect on OPN-mediated cell attachment. The GRGDSP-peptide blocked both OPN and fibronectin mediated attachment (Fig. 1), while the variant peptide, GRGESP, had no effect (data not shown). Controls included evaluation of GRGDSP and GRGESP peptides for cell attachment. These peptides had no effect on cell attachment beyond that observed with the uncoated control dishes (data not shown).

Next, to further characterize the interactions of OPN with cell surface receptors, an OPN affinity column was used. Detergent extracts of ^{35}S-methionine labeled gingival fibroblasts were applied to the column. A chelating buffer containing 20 m*M* EDTA was used to elute OPN associated proteins. Then, an 8*M* urea buffer was applied to the column to remove remaining proteins. SDS-PAGE of fractions under reducing conditions and then visualization by fluorography revealed several bands that eluted with EDTA (Fig. 2). Proteins eluted with EDTA can be seen as prominent bands around 90 kDa, 70 kDa and below 50 kDa. Several bands were also seen eluting with urea, including those above the 200 kDa marker. Proteins from the urea eluate are currently under investigation.

To establish that detergent extracts of gingival fibroblasts included $\alpha_v\beta_3$ integrin, the flow through from the OPN column (Fig. 2) was applied to an OPN-peptide column (Fig. 3). Proteins bound to this matrix were eluted with buffer containing 20 m*M* EDTA, followed by buffer containing 8*M* urea. The EDTA and urea fractions were analyzed by SDS-PAGE under reducing conditions and visualized by fluorography. The results in Fig. 3 imply that gingival fibroblasts contain significant amounts of the $\alpha_v\beta_3$ integrin, based on the migration patterns. Furthermore, a clear difference

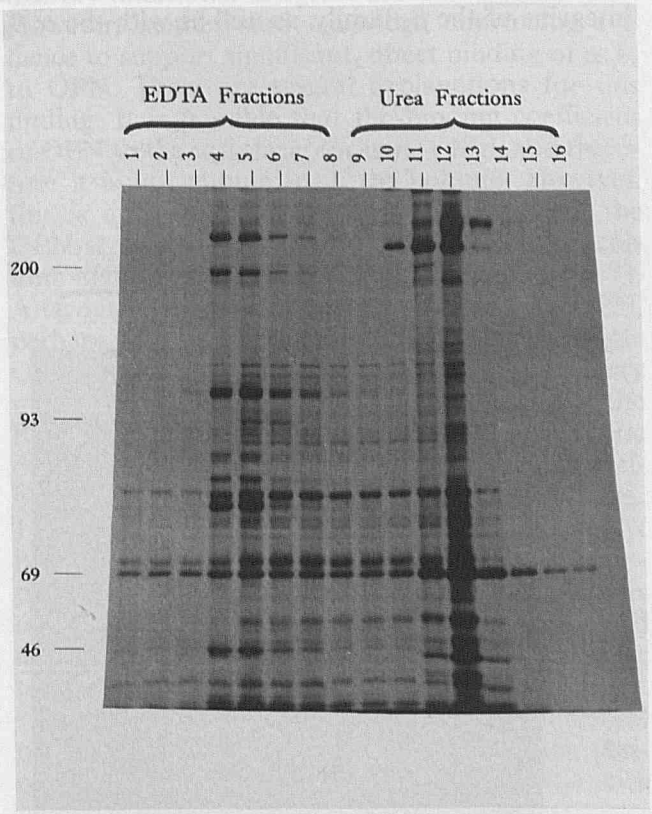

Fig. 2. Fluorograph of cell fractions (^{35}S-methionine labeled) eluted from OPN affinity matrix. Octylglucoside extracts prepared from ^{35}S-methionine labeled fibroblasts were allowed to bind to OPN-Sepharose, the column was washed and then eluted with 20 m*M* EDTA, followed by 8*M* urea, as described in Material and methods. 50 μl samples from each of 16 fractions, eluting with EDTA (fractions 1–8) or urea (fraction 9–16) were subjected to SDS-PAGE under reducing conditions, dried and bands visualized by fluorography (three-week exposure).

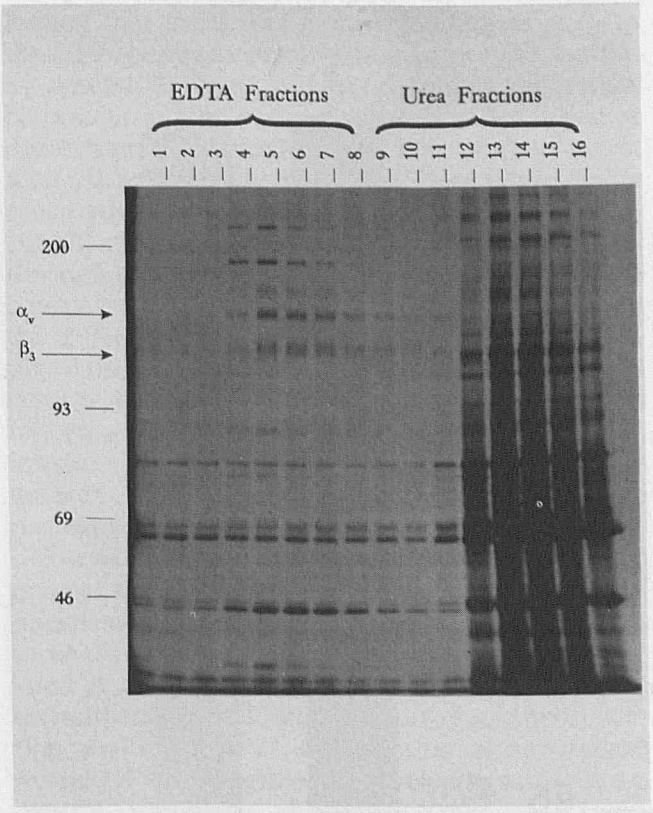

Fig. 3. Fluorograph of cell fractions (^{35}S-methionine labeled) eluted from OPN peptide (GRGDSL(K)) affinity matrix. Octylglucoside extracts prepared from ^{35}S-methionine labeled fibroblasts were allowed to bind to (GRGDSL(K))-Sepharose, the column was washed and then eluted with 20 m*M* EDTA, followed by 8*M* urea, as described in Material and methods. 50 μl samples from each of sixteen fractions, eluting with EDTA (fractions 1–8) or urea (fraction 9–16) were subjected to SDS-PAGE under reducing conditions, dried and bands visualized by fluorography.

was noted in the banding pattern between the OPN-matrix and the OPN-peptide column, Figs. 2 and 3 respectively, indicating a lack of $\alpha_v\beta_3$ binding to the OPN-matrix.

Next to confirm that proteins eluting from the OPN-peptide column included $\alpha_v\beta_3$, immunoprecipitation assays were performed. Furthermore, immunoprecipitation assays were also performed on similar eluates from the OPN-matrix to determine whether any proteins eluting with EDTA corresponded to $\alpha_v\beta_3$. EDTA pooled fractions, 4–6, from the OPN and OPN-peptide columns, respectively, were immunoprecipitated with affinity purified $\alpha_v\beta_3$ antibody. Proteins from these fractions immunoprecipitated with the $\alpha_v\beta_3$ antibody, thus verifying that α_v and β_3 were binding to the OPN-peptide column (Fig. 4). In addition, these results indicated that $\alpha_v\beta_3$ did bind to the OPN-matrix, but not to the same extent when compared to the binding of $\alpha_v\beta_3$ to the OPN-peptide column.

To specifically identify and characterize cell surface proteins bound to the OPN-matrix, cells were surface-labeled with ^{125}I. As seen in Fig. 5, detergent extracts of ^{125}I labeled cells, chromatographed on OPN-sepharose and electrophoresed under reducing conditions, yielded several distinct bands around 90 kDa, 50 kDa and 30 kDa. The flow through from the OPN-matrix of the ^{125}I surface labeled proteins were also run over the OPN-peptide column and $\alpha_v\beta_3$ was seen almost exclusively (data not shown).

Discussion

Our blocking studies demonstrate that OPN-mediated attachment of gingival fibroblasts is controlled, at least in part, by the $\alpha_v\beta_3$ receptor on these cells. However, the OPN-affinity chromatography results suggest that other cell surface molecules, as yet to be characterized, are associated with OPN to a much more significant extent than $\alpha_v\beta_3$ (Figs. 2, 5). This result is not entirely surprising since RGD containing adhesion molecules are capable of binding to several different integrins. For example, fibronectin is associated with several integrins of the β_1 family, as well as with the $\alpha_v\beta_3$

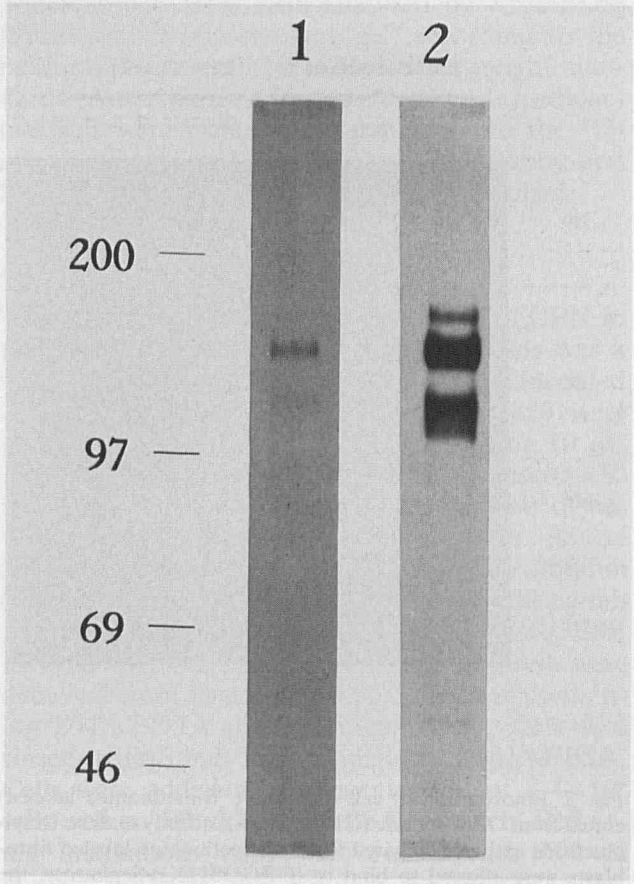

Fig. 4. Immunoprecipitation of EDTA eluted fractions from the OPN-affinity column (1) and the OPN-peptide column (2). Fractions 4–6 were pooled, lyophilized and processed for immunoprecipitation with affinity purified $\alpha_v\beta_3$ antibody and reduced samples run on SDS-PAGE and visualized by fluorography.

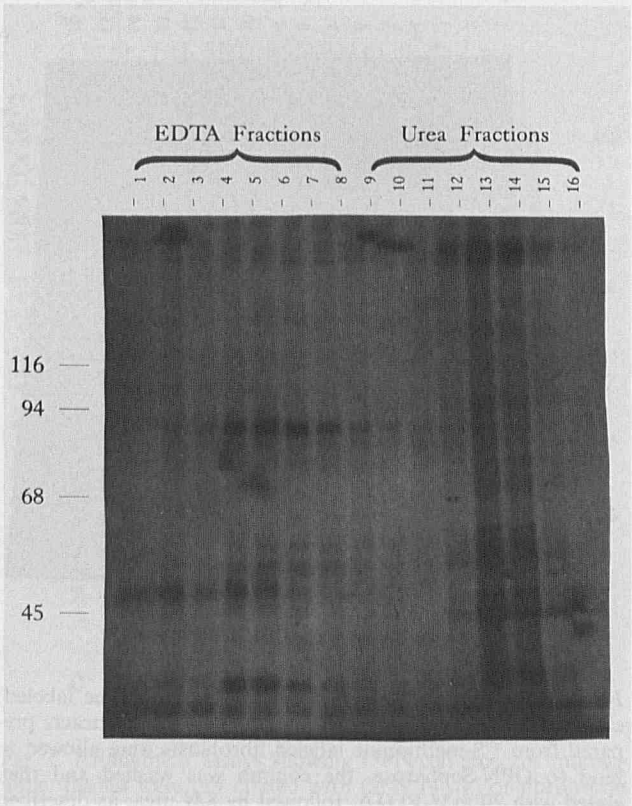

Fig. 5. Autoradiograph of cell fractions (^{125}I-surface labeled) eluted from OPN-affinity matrix. Fifty μl samples from each of 16 fractions, eluting with EDTA (fractions 1–8) or urea (fractions 9–16) were applied to SDS-PAGE under reducing conditions and visualized by autoradiography.

receptor (36). However the $\alpha_5\beta_1$ receptor appears to be selective for fibronectin only (1, 2, 30). Nevertheless, these results apparently conflict our blocking studies here (Fig. 1) and suggest that OPN may bind directly to receptors other than $\alpha_v\beta_3$. Due to similarities within integrin subunits, it is possible that the antibody LM-609 used in our studies may interact with cell surface receptors other than $\alpha_v\beta_3$, for example with the β_5 subunit, which may explain this contradiction.

To our knowledge, only one other study has attempted to identify directly cell surface receptor(s) interacting with OPN. Also using OPN-affinity chromatography, Oldberg *et al.* (49) attempted to identify cell surface receptors for OPN and bone sialoprotein (BSP). Detergent extracts of ROS 17/2.8 osteosarcoma cells were used. They reported that $\alpha_v\beta_3$ bound to the BSP affinity matrix. In contrast to BSP, in their discussion they stated that no cell surface proteins bound to OPN-sepharose. Both of our studies were designed to prove that $\alpha_v\beta_3$ binds directly to OPN and/or to identify additional integrins associated with OPN. However, neither study provided convincing evidence to support significant, direct binding of $\alpha_v\beta_3$ to OPN. There are several explanations for this finding. It is possible that the binding coefficient of OPN to the $\alpha_v\beta_3$ receptor is very high and therefore it is not eluting from the column. However, this is unlikely, since the flow through from the OPN-sepharose, applied to an OPN-peptide column and eluted with EDTA, yielded $\alpha_v\beta_3$ (Fig. 3). Alternatively, conformational changes in OPN, perhaps related to the procedures used to couple OPN to CNBr sepharose and produce the affinity matrix, may have resulted in inaccessibility of the RGD region. When we coated OPN on plastic dishes, we were able to demonstrate that antibodies to $\alpha_v\beta_3$ blocked cell binding to OPN (Fig. 1), thus providing indirect evidence that OPN binds to the $\alpha_v\beta_3$ receptor. The coating of the plastic dishes may have immobilized linear molecules of OPN to the plastic. Alternatively, as discussed above, the LM-609 antibody may not be specific to $\alpha_v\beta_3$.

The $\alpha_v\beta_3$ receptor is also associated with several other cell types, including fibroblasts of the periodontal ligament and other periodontal cells both *in vitro* (40) and *in vivo* (50). Thus the importance of this receptor and its interaction with adhesion molecules linked to mineralized tissues needs to be clarified. OPN is one of at least six RGD containing molecules associated with mineralized tissues including: tenascin, bone sialoprotein (BSP), fibronectin, thrombospondin and collagens. Interestingly, four of these proteins, BSP, fibronectin, thrombospondin and OPN are associated with the $\alpha_v\beta_3$ cell surface receptor. *In situ* hybridization studies demonstrate selective expression of $\alpha_v\beta_3$ on osteoclasts (50–55). Importantly, both OPN and BSP mediate attachment of oseoclasts *in vitro* and this attachment is blocked by antibodies to $\alpha_v\beta_3$. Such studies support a role for $\alpha_v\beta_3$ in regulating osteoclast attachment to a mineralized surface, prior to resorptive activity.

A major difference between OPN and fibronectin or BSP, is that it has the unique property of promoting persistent attachment. Fibroblasts seeded on OPN coated dishes remain attached, spread and viable for at least seven days in serumless media (11). Furthermore, this long-term spreading appears to be associated with protection from heat stress (11), such as that associated with inflammatory and traumatic conditions (56). An adhesion molecule that has the properties of maintaining cell attachment in stressed situations would likely be important to the regulation and maintenance of macrophages/osteoclasts at the site of wound healing. This proposed function of OPN may provide some physiological importance to our finding that there was greater binding of $\alpha_v\beta_3$ to the OPN-peptide column, than to the OPN-affinity matrix. In areas of wound healing OPN may be present in high concentration in a denatured or degradated form due to interactions with products secreted by inflammatory cells. Thus, while highly speculative, cells, such as osteoclasts, may preferentially bind to the denatured form of OPN through $\alpha_v\beta_3$, a cell surface receptor found in high concentrations on osteoclasts. This may explain the differences between the affinity chromatography data presented here and prior immunological studies. In support of this hypothesis, Davis (56) reported much higher yields of Mac-1 and p150,95 integrins from neutrophil extracts applied to a denatured protein-sepharose column versus native protein-sepharose.

The exact role for adhesion molecules during the process of tissue regeneration has yet to be determined and is continuing to evolve. OPN appears to be expressed in mineralized tissues and is regulated by osteotropic hormones *in vitro* (57, 58). Immunohistochemical analyses and *in situ* hybridization studies of OPN indicate that in mineralized tissues OPN is expressed in developing teeth, bone, osteoid, osteoblasts, and putative precursors to osteoblasts and/or cells of hemopoietic origin (19, 50–53). A key to understanding the function of OPN is identifying cell surface receptors associated with this protein. Establishing the interaction of OPN with cell surface receptors will provide new clinical information on factors required for maintaining and regenerating soft and hard tissues, including tissues of periodontium.

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