0026-895X/96/030529-07$3.00/0

MOLECULAR PHARMACOLOGY, 50:529–537 (1996).

Studies on $\alpha_v\beta_3$/Ligand Interactions Using a [^{3}H]SK&F-107260 Binding Assay

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Received March 14, 1996; Accepted May 23, 1996

SUMMARY

The vitronectin receptor ($\alpha_v\beta_3$) is a member of the integrin superfamily that mediates cell attachment on arginine-glycine-aspartic acid (RGD)-containing adhesive proteins. A solid-phase microtiter assay was developed to investigate the binding properties of purified $\alpha_v\beta_3$, using tritiated [^{3}H]SK&F-107260 as the radiolabeled ligand. $\alpha_v\beta_3$, purified from human platelets, human placenta, and chicken osteoclasts, bound [^{3}H]SK&F-107260 saturably and specifically. Saturation binding studies using platelet $\alpha_v\beta_3$ revealed a single class of high affinity binding sites, exhibiting a K_d of 1.44 nM and B_{max} of 0.20 mol of [^{3}H]SK&F-107260/mol of $\alpha_v\beta_3$. [^{3}H]SK&F-107260 binding was inhibited by a variety of RGD-containing peptides and by the snake venom protein echistatin, whereas an RGE-containing peptide and four nonpeptide fibrinogen receptor ($\alpha_{IIb}\beta_3$) antagonists failed to do so. This study shows that $\alpha_v\beta_3$ exhibits distinct ligand specificity from the structurally homologous fibrinogen receptor, $\alpha_{IIb}\beta_3$. The relative potencies of the RGD-containing peptides in inhibiting [^{3}H]SK&F-107260 binding to $\alpha_v\beta_3$ were the same as their relative potencies in inhibiting biotinylated-fibrinogen binding to the receptor. $\alpha_v\beta_3$ purified from chicken osteoclasts and human placenta bound [^{3}H]SK&F-107260 with similar affinities and displayed the same pharmacological profile as the platelet vitronectin receptor. The $\alpha_v\beta_3$ antagonists inhibited the attachment of MG63 human osteosarcoma cells or rat osteoclasts to recombinant rat osteopontin. The rank order of potency of the antagonists in the cell adhesion assays was similar to that of the receptor binding assay, suggesting that the purified $\alpha_v\beta_3$-[^{3}H]SK&F-107260 binding assay is a valid reflection of the ligand binding to $\alpha_v\beta_3$ on cell systems.

$\alpha_v\beta_3$ is the most promiscuous member of the integrin family, allowing cells to interact with a wide spectrum of extracellular matrix proteins (1). $\alpha_v\beta_3$ is expressed on a variety of cell types, including osteoclasts, platelets, endothelial cells, and smooth muscle cells (for a review, see Ref. 2), and plays important roles in bone resorption, angiogenesis, and neointima formation (3–5). By inhibiting these processes, $\alpha_v\beta_3$ antagonists may be useful for the prevention and treatment of osteoporosis, restenosis, and cancer (6, 7).

Small proteins or peptides containing the RGD sequence have been demonstrated to block extracellular matrix binding to $\alpha_v\beta_3$ and thereby inhibit the $\alpha_v\beta_3$-mediated cell functions (1, 6–8). Considerable effort has been made to design RGD peptides or their equivalent that bind $\alpha_v\beta_3$ with a high affinity. In addition to potency, integrin selectivity is an important factor to consider because the RGD-binding integrin receptors are widely distributed and participate in various physiological and pathological processes. Of particular concern is the fibrinogen receptor, $\alpha_{IIb}\beta_3$, which has the identical β subunit and a highly homologous α subunit (9). $\alpha_{IIb}\beta_3$ plays a pivotal role in hemostasis by mediating platelet aggregation, and inhibition of this receptor may cause unwanted bleeding (10).

In the course of searching for potent and selective $\alpha_v\beta_3$ antagonists, we established a radioligand binding assay for purified $\alpha_v\beta_3$, using tritiated [^{3}H]SK&F-107260 [cyclo-(*S*,*S*)-N^α-2-mercaptobenzoyl-N^α-methylarginyl-glycyl-aspartyl-2-mercaptophenyl-amide] as the radioligand. [^{3}H]SK&F-107260 is a cyclic, RGD-containing pentapeptide that has been shown to bind $\alpha_{IIb}\beta_3$ with high affinity.[2] Using this assay, we demonstrated that $\alpha_v\beta_3$ purified from three tissue sources, including the chicken osteoclast, human platelet, and human placenta, exhibited similar ligand binding properties. The $\alpha_v\beta_3$ antagonists, identified by the [^{3}H]SK&F-107260 binding assay, were assayed for their abilities to

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[2] A. Wong, S. M. Hwang, K. Johanson, J. Samanen, D. Bennett, S. W. Landvatter, R. Heys, F. Ali, T. W. Ku, W. Bondinell, D. A. Powers, and J. M. Stadel. A cyclic RGD peptide [^{3}H]-SK&F-107260, and a nonpeptide [^{3}H]-SB-214857 bind to the same site on $\alpha_{IIb}\beta_3$. Submitted for publication.

ABBREVIATIONS: $\alpha_v\beta_3$, vitronectin receptor(s); $\alpha_{IIb}\beta_3$, fibrinogen receptor(s); RGD, arginyl-glycinyl-aspartic acid; ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RPMI, Roswell Park Memorial Institute.

inhibit biotinylated-fibrinogen binding to purified $\alpha_v\beta_3$ and for their abilities to prevent rat osteoclast and MG63 human osteosarcoma cell adhesion on osteopontin. Results show that these compounds display the same rank order of potency in the biotinylated-fibrinogen binding assay and in the cell adhesion assays. Therefore, the [^{3}H]SK&F-107260 binding assay is a valid reflection of the binding of $\alpha_v\beta_3$ to its natural ligand, fibrinogen, and that the $\alpha_v\beta_3$ expressed on osteoclast and MG63 cell membranes display similar pharmacological profile as the purified receptor.

Experimental Procedures

Materials

[^{3}H]SK&F-107260 (65–85 Ci/mmol) was synthesized and obtained from the Department of Synthetic Chemistry, Radiochemistry Section, SmithKline Beecham (King of Prussia, PA).[1] SK&F-107260, VRGD-dF, Ac-C(NMe)RGDPen-NH_2 [N^α-acetyl-cyclo-(*S*,*S*)-cysteinyl-N^α-methylarginyl-glycyl-aspartyl-penicillamine-amide], PRGDG-dP {cyclo-(1,6)-prolinyl-arginyl-glycyl-aspartyl-glycyl-D-proline}, and nonpeptides **1-4** {**1**, *N*-[*m*-(*p*-amidinobenzamido)]benzoyl-β-alanine; **2**, [[4-(4-aminoiminomethyl-*N*-methylbenzamido)-acetyl]*o*-phenylene]dioxy]diacetic acid; **3** (SB-207448), [8-[[4-(aminoiminomethyl)phenyl]amino]carbonyl]4-(2-phenylethyl)-1,3,4,5-tetrahydro-3-oxo-1,4-benzodiazepine-2-acetic acid; **4** (MK383), *N*-(butylsulfonyl)-*o*-[4,(4-piperidinyl)butyl]L-tyrosine} (Fig. 1) were synthesized and obtained from the Department of Medicinal Chemistry, SmithKline Beecham. GPenGRGDSPCA [glycyl-cyclo-(*S*,*S*)-penicillamine-glycyl-arginyl-glycyl-aspartyl-serinyl-prolinyl-cysteinyl-alanine], RGDS, GRGDS, GRGDSP, GRGDSPC, and GRGESP were purchased from GIBCO BRL (Gaithersburg, MD). Echistatin was purchased from Bachem California (Torrance, CA).

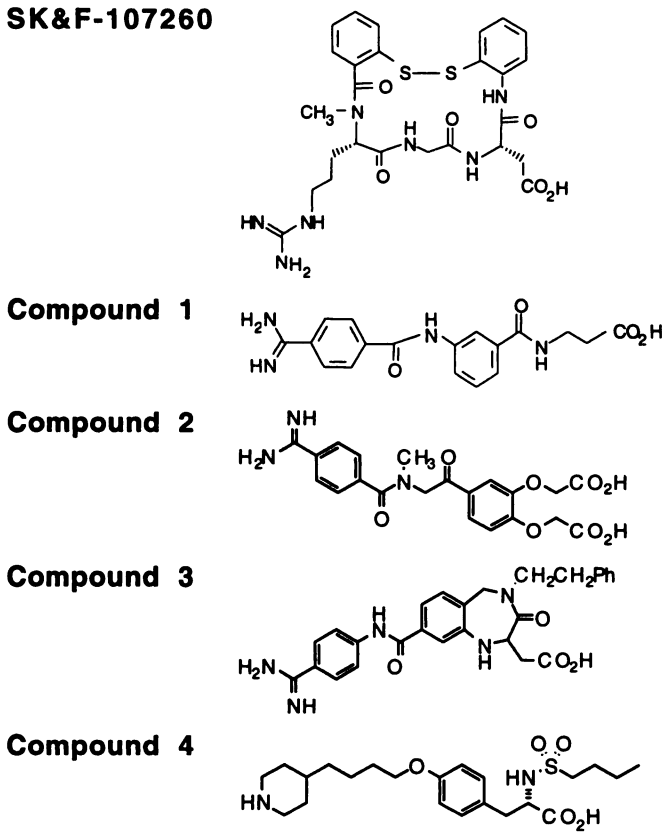

Fig. 1. Structures of inhibitors.

The human osteosarcoma cell line MG63 was purchased from American Type Culture (Rockville, MD). The OPTI-MEM I and the RPMI media were obtained from GIBCO (Grand Island, NY). LM609-producing mouse ascites (1, 11) were obtained from the Department of Immunology, Research Institute of Scripps Clinic (La Jolla, CA). Hybridoma cells secreting 23C6 (12) and F11 (13) were obtained from Imperial Cancer Institute (London, UK). The mouse anti-human $\alpha_v\beta_5$ (P1F6) and anti-human β_1 (JB1a) monoclonal antibodies were obtained from Chemicon (Temecula, CA). Lentil lectin Sepharose 4B column was purchased from E. Y. Labs (San Mateo, CA). Human fibrinogen and Aquaside III were purchased from Calbiochem (San Diego, CA). Sulfo-NHS-biotin was purchased from Pierce Chemical (Rockford, IL). *p*-Nitrophenylphosphate, Protein A-Sepharose, and Protein MAPS II buffer kit were purchased from BioRad (Richmond, CA). Phosphatidylcholine and phosphatidylserine were obtained from Avanti Polar Lipids (Birmingham, AL). Ready Safe was purchased from Beckman Instruments (Palo Alto, CA). Alkaline phosphatase-conjugated anti-biotin antibody, Lipifree, cyanogen bromide-activated Sepharose 4B, poly-L-lysine, and all other chemicals unless specified were purchased from Sigma Chemical (St. Louis, MO). All reagents for protein sequencing were purchased from Applied Biosystems (Norwalk, CT).

Purification of 23C6 and F11

Hybridoma tissue culture supernatant containing 23C6 or F11 and 16% bovine fetal calf serum (350 ml) was applied to 15 ml of Protein G-Sepharose Fast Flow column (2.5 × 3 cm), which was previously equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The column was washed with the same buffer, and the bound antibody was subsequently eluted with 0.5 M acetic acid buffer, pH 3.0. The pooled fractions were quickly dialyzed against 20 mM phosphate buffer, pH 7.0. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis indicated that the antibody sample was contaminated with bovine IgG at 10–20%. To remove bovine IgG, the sample was applied to 10 ml of Protein A-Sepharose Fast Flow column (2.5 × 2 cm), which was previously equilibrated with 50 mM sodium phosphate buffer, pH 7. The column was eluted with 200 ml of a linear pH gradient of 50 mM phosphate buffer, pH 7.0, to 50 mM citric acid, pH 3.0. The antibody containing fractions, which was eluted at pH 5.0, were pooled and dialyzed against phosphate-buffered saline. The total yield was 20 mg.

Preparation of LM609-Affinity Column

Monoclonal antibody LM609 was purified from mouse ascites by using Protein A-Sepharose and Protein MAPS II buffer kit. The mouse ascites were treated with an equal volume of Lipifree. The top layer was removed and filtered through a 0.2-μm filter. The filtrate was then mixed with an equal volume of Protein MAPS II binding buffer and applied to a Protein A column at pH 8.9. LM609 retained in the column was eluted with buffer at pH 5.9. The fractions containing LM609 were pooled and dialyzed against phosphate-buffered saline. The antibody was immobilized on a cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions at 1 mg of IgG/ml of resin.

Purification of $\alpha_v\beta_3$

From human platelets. One hundred units of outdated platelets (unfrozen) was washed once with 50 mM Tris·HCl, pH 7.4, 100 mM NaCl (buffer A) containing 1 mM phenylmethylsulfonyl fluoride. The washed platelets were solubilized by gentle overnight stirring at 4° in buffer A containing 2 mM $CaCl_2$ and 3% octylglucoside. The solubilized platelets were centrifuged at 100,000 × *g* for 1 hr, and the clear supernatant (~250 ml) was collected. The supernatant was applied to a 25-ml LM609 affinity column pre-equilibrated with buffer A containing 2 mM $CaCl_2$ and 1% octylglucoside. Bound $\alpha_v\beta_3$ was eluted with 0.1 M glycine acetate buffer, pH 3.0. The eluted fractions were collected in tubes containing 1 M Tris, pH 8.0. The

pooled fractions were concentrated against Aquaside III using a 10-kDa cutoff membrane and dialyzed against buffer A containing 2 mM $CaCl_2$ and 1% octylglucoside. The purified $\alpha_v\beta_3$ was stored at −70° until use.

From human placenta. Placenta that had been were frozen immediately after delivery were cut into small pieces and washed with buffer A containing 0.05% digitonin, 2 mM $CaCl_2$, and 2 mM phenylmethylsulfonyl fluoride. $\alpha_v\beta_3$ was extracted from the pieces of placenta and immunopurified using the method described above.

From chicken osteoclasts. Osteoclasts were isolated from laying hens (30 weeks old) that had been maintained for 10 days on a calcium-deficient diet, as described previously (14). Briefly, a group of 20 hens were killed, and their femurs and tibia were split longitudinally. The soft spongy materials, enriched with osteoclasts, were obtained by scraping the bones with a curette. The cell solution obtained was resuspended in a hypotonic buffer for the lysis of red blood cells. The osteoclast-containing solution was then resuspended in phosphate-buffered saline (15 ml) and overlaid onto 30 ml of fetal calf serum. After an 80-min incubation on ice, the top layer was aspirated off, and the osteoclasts were pelleted. The cells were resuspended and overlaid onto fetal calf serum for a second time (45 min). The top layer was aspirated, and the osteoclasts were pelleted (900 × *g* for 10 min). The yield was ~8 × 10^6 osteoclasts/hen at a purity of 1:10 (osteoclast/contaminating cell). Because osteoclasts were much larger than the contaminating mononuclear cells, the preparation was estimated to contain 80–90% osteoclast-derived membranes (15). $\alpha_v\beta_3$ was extracted from the osteoclast enriched preparations and immunopurified using the method described previously. We used 200 × 10^6 osteoclasts in each $\alpha_v\beta_3$ purification.

Amino-Terminal Sequence Analyses

Sequence analyses were performed on an Applied Biosystems 470A gas-phase protein sequencer equipped with a Beckman 126/166 system for on-line PTH analysis. Data were acquired using System Gold chromatography software. Samples were either spotted directly onto Polybrene-coated GF/C filter (ABI) or electroblotted onto polyvinylidene difluoride type supports, and standard ABI sequencing cycles were used.

Solid-Phase [^{3}H]SK&F-107260 Binding to $\alpha_v\beta_3$

Human placenta or human platelet $\alpha_v\beta_3$ (0.1–0.3 mg/ml in buffer A containing 2 mM $CaCl_2$ and 1% octylglucoside) was diluted with buffer A containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$ (buffer B), and 0.05% NaN_3, and then immediately added to 96-well ELISA plates (Corning, New York, NY) at 0.1 ml/well; 0.1–0.2 μg of $\alpha_v\beta_3$ was added to each well. The plates were incubated overnight at 4°. At the time of the experiment, the wells were washed once with buffer B and incubated with 0.1 ml of 3.5% bovine serum albumin in the same buffer for 1 hr at room temperature. After incubation, the wells were aspirated completely and washed twice with 0.2 ml of buffer B. Aliquots (0.1 ml) of [^{3}H]SK&F-107260 (0.25–10 nM in buffer B) were added to the wells in triplicate. The plates were incubated for 1 hr at room temperature. After incubation, the wells were aspirated completely and washed once with 0.2 ml of ice-cold buffer B in a well-to-well manner. The receptors were solubilized with 0.1 ml of 1% sodium dodecyl sulfate, and the bound [^{3}H]SK&F-107260 was determined by liquid scintillation counting with the addition of 3 ml of Ready Safe in a Beckman LS 6800 Liquid Scintillation Counter, with 40% efficiency. Nonspecific binding of [^{3}H]SK&F-107260 was determined in the presence of 2 μM SK&F-107260 and was consistently <1% of total radioligand input. The radiolabeled and unlabeled SK&F-107260 were added to the wells simultaneously. The data presented are from single experiments, representative of three separate experiments. All data points are the mean of triplicate determinations. Standard deviations of the triplicates were always <5% of the mean value.

In a [^{3}H]SK&F-107260 competition binding assay, various concentrations of unlabeled antagonists (0.001–100 μM) were added to the wells, followed by the addition of 5.0 nM [^{3}H]SK&F-107260. The IC_{50} (concentration of the antagonist to inhibit 50% binding of [^{3}H]SK&F-107260) was determined by a nonlinear, least-squares curve-fitting routine, which was modified from the LUNDON-2 program (15). The K_i (dissociation constant of the antagonist) was calculated according to the equation of Cheng and Prusoff (16): $K_i = IC_{50}/(1 + L/K_d)$, where L and K_d are the concentration and the dissociation constant of [^{3}H]SK&F-107260, respectively.

Biotinylated-Fibrinogen Competition Binding Assay

Binding of biotinylated-fibrinogen to $\alpha_v\beta_3$ was performed essentially as described previously.[1]

MG63 Human Osteosarcoma Cell Attachment Assays

MG63 cells were maintained as monolayer cultures in 75- or 150-cm^2 flasks in OPTI-MEM I medium supplemented with 5% fetal calf serum. Corning 96-well ELISA plates were precoated overnight at 4° with 0.1 ml of rat osteopontin (1 μg/ml in RPMI medium). Rat osteopontin was purified to homogeneity from the cultured medium of Chinese hamster ovary cells transfected with the rat osteopontin gene. At the time of the experiment, the plates were washed once with RPMI medium and blocked with 3.5% bovine serum albumin in RPMI medium for 1 hr at room temperature. Before the adhesion assay, MG63 cells were rinsed with RPMI medium and trypsinized until the cells dislodged. Cells were washed once with RPMI medium, supplemented with 20 mM HEPES and 0.1% bovine serum albumin, and resuspended in the same medium at a concentration of 0.5 × 10^6 cell/ml. Then, 0.1 ml of the cell suspension was added to each well and incubated for 1 hr at 37° in the presence or absence of various $\alpha_v\beta_3$ antagonists. After the incubation, 0.025 ml of a 10% formaldehyde solution, pH 7.4, was added, and the cells were fixed at room temperature for 10 min. The plates were washed three times with 0.2 ml of RPMI medium, and the adherent cells were stained with 0.1 ml of 0.5% toluidine blue for 20 min at room temperature. Excess stain was removed by extensive washing with distilled water. The toluidine blue incorporated into cells was eluted by the addition of 0.1 ml of 50% ethanol and 50 mM HCl. Cell adhesion was quantified at an adsorbance of 600 nm on a microtiter plate reader (Titertek Multiskan MC, Sterling, VA).

Rat Osteoclast Adhesion Assay

Osteoclasts were isolated from long bones of the 1–2-day-old neonatal rats as described previously (17). Briefly, femora, tibiae, and humeri were dissected, split, and scraped into Medium 199, pH 7.4, supplemented with 10% fetal calf serum. Cell solution was filtered through nylon mesh to remove debris. Cells were pelleted, resuspended in 3 ml of the same buffer, and overlaid onto 6 ml of fetal calf serum. After incubation on ice for 30 min, the top layer was aspirated, and osteoclasts were obtained by centrifugation at 500 × *g* for 6 min. Cells were resuspended in RPMI medium and supplemented with 20 mM HEPES, pH 7.4, and 0.1% bovine serum albumin, in the presence or absence of $\alpha_v\beta_3$ antagonists. Aliquots (0.5 ml) of osteoclast suspension (each obtained from bones of two pups) were plated onto each well, which had been coated with 0.1 μg of recombinant rat osteopontin (Nunc Lab-Tek Chamber Slide, Naperville, IL). The cells were incubated for 45 min at 37°. This yielded a mixed population of adherent cells, which contained 50–80 multinucleate, tartrate-resistant acid phosphatase-positive osteoclasts/well in control samples. Nonadherent cells were washed twice with 0.5 ml of the adhesion buffer, and the attached cells were fixed with glutaraldehyde. Osteoclasts were visualized by staining for tartrate-resistant acid phosphatase (17).

Results

Purification of $\alpha_v\beta_3$ and amino-terminal sequencing. $\alpha_v\beta_3$ was purified from human platelets, human placenta,

and chicken osteoclasts using LM609 affinity chromatography. Fig. 2 shows the sodium dodecyl sulfate-polyacrylamide gel analysis of the purified materials under nonreducing and reducing conditions; also shown is $\alpha_{IIb}\beta_3$ purified from human platelets. Two polypeptides of nonreduced molecular mass of 150 and 90 kDa, which correspond to the molecular mass of the reported α_v and β_3 chains, respectively, were obtained in all preparations. Densitometric scans of the silver-stained proteins showed that the two polypeptides represented >90% of the protein. Under reducing conditions, the two polypeptides migrated with molecular mass of 125 and 90 kDa. The 20-kDa polypeptide that is known to be released from the α_v subunit to yield the reduced 125-kDa polypeptide migrated in the dye front. The amino-terminal sequence of the first 15 amino acids of the 90-kDa polypeptide purified from platelet and placenta (data not shown) was identical to the sequence of the β_3 subunit obtained from endothelial cells (18) and from human erythroleukemia cells (19). The amino-terminal sequence of the first 15 amino acids of the 150-kDa polypeptide was identical to the sequence of the α_v subunit obtained from endothelial cells and from fibroblasts (20–22). These results show that the two polypeptides we obtained from human platelet and placenta preparations were α_v and β_3 subunits. The yield of $\alpha_v\beta_3$ was 0.5 mg from one placenta and 1.0 mg from 100 units of human platelet.

Fig. 3 shows amino-terminal amino acid sequence data of the 125-, 20-, and 90-kDa polypeptides obtained from chicken osteoclast preparation under reducing conditions. The 125- and 20-kDa polypeptides sequence were found to be identical to the α_v previously reported from chicken fibroblast (23). Sequencing of 11 amino acids was achieved for the 90-kDa polypeptide, and the sequences were highly homologous to those of the reported human β_3, which made it likely that the purified materials was $\alpha_v\beta_3$. The yield of $\alpha_v\beta_3$ was 60–100 μg from 200×10^6 osteoclasts. This number was calculated to be $0.6–1 \times 10^6$ $\alpha_v\beta_3$ molecules isolated from one osteoclast.

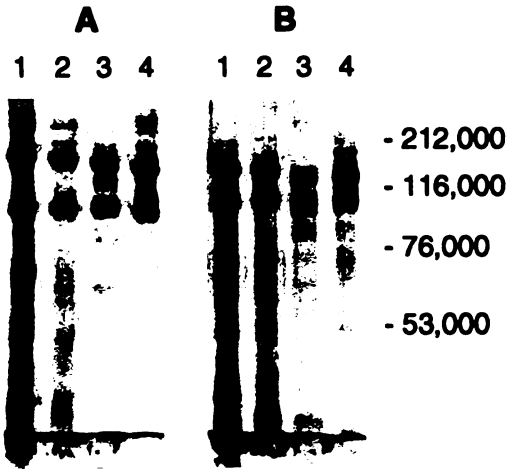

Fig. 2. Affinity purification of $\alpha_v\beta_3$. An octylglucoside extract of human platelets, human placenta, or chicken osteoclast preparation was applied to a LM609 affinity column. Bound $\alpha_v\beta_3$ was eluted with a glycine acetate buffer. A sample of the purified material was analyzed on a 7.5% polyacrylamide gel under nonreducing (A) and reducing (B) conditions. After electrophoresis, the gel was stained with silver. *Lane 1*, platelet $\alpha_v\beta_3$ (50 ng). *Lane 2*, placenta $\alpha_v\beta_3$ (50 ng). *Lane 3*, chicken osteoclast $\alpha_v\beta_3$ (15 ng). *Lane 4*, lentil lectin purified $\alpha_{IIb}\beta_3$ (50 ng). *Right*, mobility of the molecular mass standards.

Saturation of [^{3}H]SK&F-107260 binding to $\alpha_v\beta_3$. Incubation of the platelet, placental, or osteoclast $\alpha_v\beta_3$ with increasing concentrations of [^{3}H]SK&F-107260 resulted in saturable binding. A typical binding curve for the platelet $\alpha_v\beta_3$ is shown in Fig. 4A. Scatchard analysis of the binding data gave a linear fit (Fig. 4B), with a K_d of 1.44 ± 0.08 nM and a B_{max} of 0.20 ± 0.02 mol/mol $\alpha_v\beta_3$ [mean ± standard error from three $\alpha_v\beta_3$ preparations, as determined by the LIGAND computer program (24)]. The K_d of [^{3}H]SK&F-107260 for binding placental $\alpha_v\beta_3$ and osteoclast $\alpha_v\beta_3$ was 1.72 ± 0.34 and 1.98 ± 0.40 nM, respectively, each determined from three preparations.

Inhibition of biotinylated-fibrinogen and [^{3}H]SK&F-107260 binding to $\alpha_v\beta_3$ by specific monoclonal antibodies. We examined the effects of two $\alpha_v\beta_3$-specific monoclonal antibodies, LM609 and 23C6, on inhibition of the binding of biotinylated-fibrinogen and [^{3}H]SK&F-107260 to human platelet $\alpha_v\beta_3$. LM609 was generated against the $\alpha_v\beta_3$ complex on M21 human melanoma cell and was capable of inhibiting the purified $\alpha_v\beta_3$ binding to vitronectin and M21 cell adhesion on vitronectin (1, 11). 23C6 was generated against the human osteoclastoma cells and later shown to recognize $\alpha_v\beta_3$ on osteoclast membranes (3, 12). 23C6 prevented osteoclast adhesion and inhibited the resorption of bone slices in culture (8). Fig. 5A shows that both LM609 and 23C6 dose-dependently inhibited the binding of 10 nM biotinylated-fibrinogen to $\alpha_v\beta_3$. Half-maximal inhibition was obtained at 0.1 and 0.7 μg/ml concentrations of LM609 and 23C6, respectively. The two antibodies were much less effective in the [^{3}H]SK&F-107260 binding assay. LM609 produced a maximal of 40% inhibition at 1 μg/ml (Fig. 5B). An increase in the antibody concentrations to 300 μg/ml did not result in further inhibition. 23C6 at 30 μg/ml (highest concentration examined) produced only a 15% inhibition of [^{3}H]SK&F-107260 binding. These results suggest that the binding epitopes of both LM609 and 23C6 on $\alpha_v\beta_3$ are not directly involved in the interaction with the RGD motif and may inhibit fibrinogen binding by steric hindrance.

$\alpha_v\beta_3$ purified from human placenta and chicken osteoclast also bind biotinylated-fibrinogen, and the binding can be inhibited by both LM609 and 23C6. The IC_{50} values of the two monoclonal antibodies were similar to those of the platelet $\alpha_v\beta_3$ (IC_{50} of LM609 and 23C6 were 0.1–0.2 and 0.7–1 μg/ml, respectively). The $\alpha_v\beta_5$- and the β_1-specific monoclonal antibodies were inactive in blocking biotinylated-fibrinogen binding to $\alpha_v\beta_3$ purified from platelets, placenta, or chicken osteoclasts (data not shown).

[^{3}H]SK&F-107260 competition binding assay. Fifteen compounds were examined for their abilities to compete for the binding of [^{3}H]SK&F-107260 to $\alpha_v\beta_3$ purified from platelets, placenta, and osteoclasts. Results showed that these compounds exhibit similar affinities (K_i) in binding to the three $\alpha_v\beta_3$ preparations and, therefore, only the K_i values of the platelet $\alpha_v\beta_3$ are shown (Table 1). The K_i of SK&F-107260 was determined to be 0.0026 ± 0.0003 μM (three measurements), which agreed with the K_d value calculated from saturation analyses (Fig. 4). Echistatin, an RGD-containing snake venom protein that potently inhibits bone resorption *in vivo* (6), competed for [^{3}H]SK&F-107260 binding with a K_i of 0.001 μM. Four cyclic RGD peptides, GPenGRGDSPCA, VRGD-dF, Ac-C(NMe)RGDPen-NH_2, and PRGDG-dP, also inhibited [^{3}H]SK&F-107260 binding to $\alpha_v\beta_3$. Linear

Amino Acids	1-	2-	3-	4-	5-	6-	7-	8-	9-	10-	11-	12-	13-	14-	15
125 kDa	X-	X-	Leu-	X-	Ala-	Glu-	X-	Pro-	Ala-	Val-	Tyr-	Ser-	Gly-	Ala-	Glu
Chicken α_v (1)	Phe-	Asn-	Leu-	Asp-	Ala-	Glu-	Arg-	Pro-	Ala-	Val-	Tyr-	Ser-	Gly-	Ala-	Glu
20 kDa (2)	X-	X-	Thr-	Ala-	Ile-	Glu-	Gly-	Asp-	Val-	Gln-	Thr-	Leu-	Gly-	X-	Gly
Chicken α_v (2)	Asp-	Leu-	Thr-	Ala-	Ile-	Glu-	Gly-	Asp-	Val-	Gln-	Thr-	Leu-	Gly-	Cys-	Gly
90 kDa	Pro-	Asn-	Ile-	X-	Ala-	Thr-	Arg-	Gly-	Val-	Thr-	Ser				
Human β_3	Pro-	Asn-	Ile-	Cys-	Thr-	Thr-	Arg-	Gly-	Val-	Ser-	Ser				

Fig. 3. Amino-terminal amino acid sequence of the 125-, 20-, and 90-kDa polypeptides isolated from chicken osteoclast preparations. ×, Unassigned amino acid at the position indicated. Cysteine residues were not alkylated before sequence analysis and appeared as a "hole" in the sequence data generated.

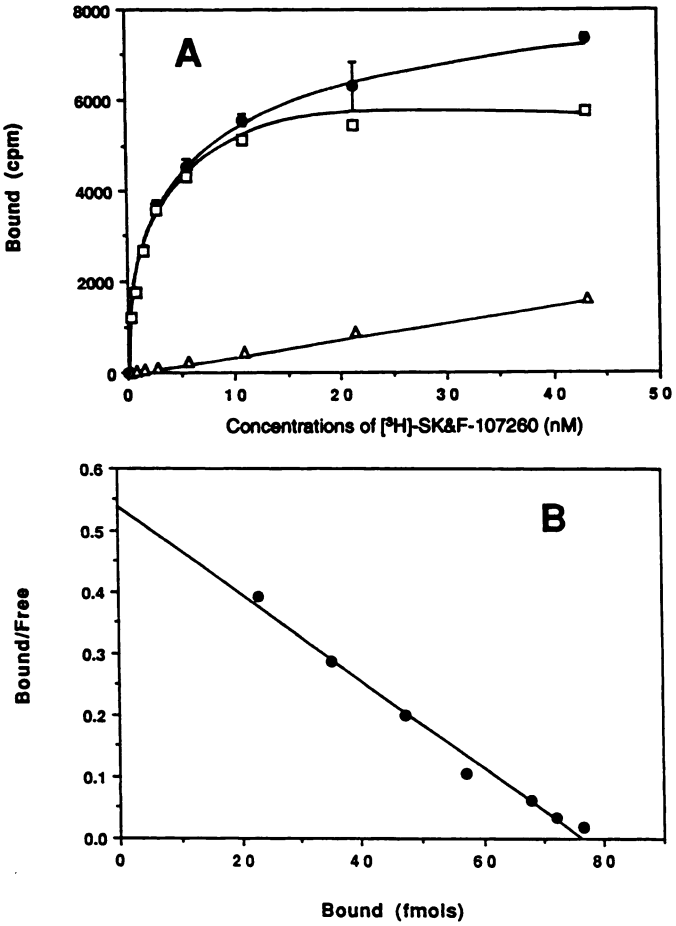

Fig. 4. Saturation curve and Scatchard analysis of [^{3}H]SK&F-107260 binding to purified platelet-$\alpha_v\beta_3$. A, $\alpha_v\beta_3$ were immobilized overnight on 96-well microtiter plates at 0.15 μg/well. At the time of experiment, $\alpha_v\beta_3$ were incubated with various concentrations of [^{3}H]SK&F-107260 for 1 hr at room temperature. The amount of [^{3}H]SK&F-107260 bound was assayed as described in Experimental Procedures. Nonspecific binding was determined in the presence of 2 μM unlabeled SK&F-107260: ●, total [^{3}H]SK&F-107260 bound; □, specific binding; and △, nonspecific binding. Each point is the mean ± standard error of triplicate determinations. Similar results have been obtained using three different $\alpha_v\beta_3$ preparations. B, The same data are plotted according to the method of Scatchard.

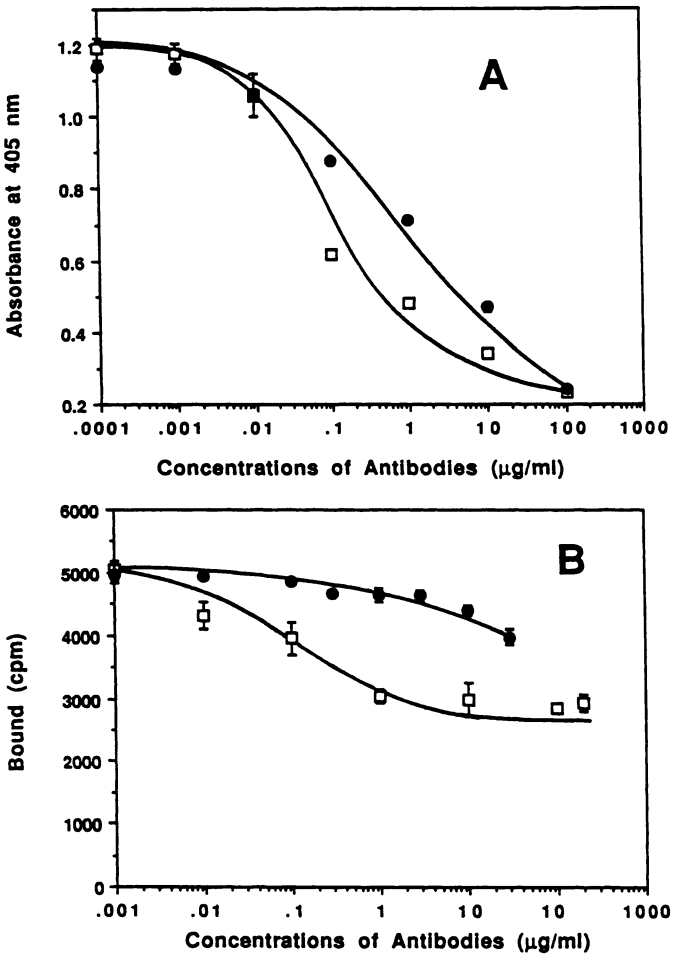

Fig. 5. Effects of LM609 and 23C6 on the binding of biotinylated-fibrinogen and [^{3}H]SK&F-107260 to platelet $\alpha_v\beta_3$. $\alpha_v\beta_3$ was incubated with 10 nM biotinylated-fibrinogen (A) or 5 nM [^{3}H]SK&F-107260 (B) in the presence of various concentrations of monoclonal antibodies: □, LM609; ●, 23C6. *Points*, mean ± standard error of triplicate determinations. Similar results were obtained in three experiments.

RGD peptides bound $\alpha_v\beta_3$ with high affinity. GRGDSPC, the sequence of which was taken from fibronectin, exhibited a K_i of ~0.022 μM. Other linear RGD peptides (GRGDSP, GRGDS, and RGDS) exhibited K_i values comparable to those of the prototypic peptide GRGDSPC. A control peptide, GRGESP, inhibited 20% of [^{3}H]SK&F-107260 binding at 100 μM, the highest concentration used. Four potent $\alpha_{IIb}\beta_3$ nonpeptide antagonists, including **1** (see Fig. 1 for structure), an aminoethyl-phenoxyacetic acid analog (25); **2**, an aminobenzoic acid analog (25); **3**, an 1,4-benzodiazepine analog (26); and **4**, an o-alkylated-L-tyrosine analog (27), at concentrations of 0.001–100 μM, exhibited little effect on inhibiting [^{3}H]SK&F-107260 binding to $\alpha_v\beta_3$.

Previous studies have shown that the lipid environment around $\alpha_v\beta_3$ modulates the ligand specificity of $\alpha_v\beta_3$ (28). We therefore incorporated the platelet $\alpha_v\beta_3$ into mixed liposomes containing phosphatidylcholine and phosphatidylserine and compared its ligand binding properties with those of the $\alpha_v\beta_3$ immobilized on microtiter plates. Results show that the two assays gave nearly identical K_d values for [^{3}H]SK&F-107260 and similar K_i values for the $\alpha_v\beta_3$ antagonists (data not shown), suggesting that the coating of the receptor on the microtiter plate wells did not change the conformation of the receptor or denature the receptor.

TABLE 1

Effects of various compounds in inhibiting the binding of biotinylated-fibrinogen and [^{3}H]SK&F-107260 to platelet $\alpha_v\beta_3$ and the binding of [^{3}H]SK&F-107260 to platelet $\alpha_{IIb}\beta_3$

Purified human platelet $\alpha_v\beta_3$ was immobilized on 96-well microtiter plates; 0.15 μg/well and 0.2 μg/well of receptors were used for assay of [^{3}H]SK&F-107260 and biotinylated-fibrinogen binding, respectively. For the lentil lectin purified $\alpha_{IIb}\beta_3$, 0.5 μg was immobilized on each well. Various concentrations of the competing ligands were added to the wells, followed by the addition of 5 nM [^{3}H]SK&F-107260 or 10 nM biotinylated-fibrinogen. The samples were incubated at room temperature for 2 hr.

Competing ligand	biotin-Fg/$\alpha_v\beta_3$	[^{3}H]107260/$\alpha_v\beta_3$	[^{3}H]107260/$\alpha_{IIb}\beta_3$
		K_i μM[a]	
Echistatin	0.00145	0.0015 ± 0.0002	0.0022 ± 0.0003
SK&F-107260	0.00052	0.0026 ± 0.0003	0.0026 ± 0.0006
VRGDdF	0.0010	0.010 ± 0.001	41.8 ± 3.2
GPenGRGDSPCA	0.0015	0.010 ± 0.001	4.12 ± 0.23
GRGDS	0.003	0.015 ± 0.002	10.15 ± 0.42
GRGDSPC	0.005	0.022 ± 0.004	>50
GRGDSP	0.008	0.026 ± 0.004	27.5 ± 1.4
RGDS	0.008	0.027 ± 0.004	18.4 ± 0.8
Ac-C(NMe)RGDPen-NH_2	0.010	0.123 ± 0.010	0.102 ± 0.018
PRGDG-dP	0.021	0.151 ± 0.003	1.90 ± 0.4
1	>10	>50	0.023 ± 0.004
2	>10	>50	0.015 ± 0.003
3	>10	>50	0.0023 ± 0.0001
4	>10	65.0 ± 5.0	0.0028 ± 0.0003
GRGESP	>10	>50	>50

[a] The K_i of the competing ligands were calculated according to the Cheng and Prusoff equation: $K_i = IC_{50}/(1 + L/K_d)$, where L and K_d are the concentration and the dissociation constant of the labeled ligand, respectively. The K_d of [^{3}H]SK&F-107260 bindings to $\alpha_v\beta_3$ and to $\alpha_{IIb}\beta_3$ were both determined as 1.5 nM. The apparent K_d of biotinylated-fibrinogen binding to $\alpha_v\beta_3$ was 5.0 nM. K_i values of the $\alpha_v\beta_3$ binding experiments were mean ± standard error from three experiments, and those of the $\alpha_{II}\beta_3$ binding were mean ± standard error from four experiments.

In parallel experiments, we determined the K_i values of the $\alpha_v\beta_3$ antagonists in inhibition of the binding of biotinylated-fibrinogen to $\alpha_v\beta_3$. Table 1 shows that the same rank order of potency is obtained from the two competition binding assays, but the K_i of the antagonists determined by the biotinylated-fibrinogen binding assay is 10–100-fold lower than values obtained from the [^{3}H]SK&F-107260 binding assay. In these two binding assays, both the coating conditions and the surface densities of $\alpha_v\beta_3$ were kept constant; the only difference was the labeled ligand that was used. Because an antibiotin antibody was used to quantify the binding of biotinylated-fibrinogen, this caused nonlinearity of the detection signals and a 10–100-fold decrease in K_i values. Similar findings were observed in a study of the radioligand versus biotinylated-fibrinogen binding to $\alpha_{IIb}\beta_3$.[1]

Integrin specificity of the competing ligands. Because [^{3}H]SK&F-107260 binds $\alpha_{IIb}\beta_3$[1] with affinities similar to its binding to $\alpha_v\beta_3$, we used the [^{3}H]SK&F-107260 competition binding assay to examine the integrin selectivity of the 15 ligands. Table 1 shows that echistatin and two cyclic peptides (SK&F-107260 and Ac-C(NMe)RGDPen-NH_2) are nonselective ligands, each exhibiting similar binding affinities to the two β_3 integrins. On the other hand, three cyclic peptides (VRGDdF, GPenGRGDSPA, and PRGDG-dP) displayed 4000-, 400-, and 10-fold, respectively, higher affinity in binding to $\alpha_v\beta_3$. The linear peptides GRGDSPC, GRGDS, GRGDSP, and RGDS were 1000-fold more active in binding to $\alpha_v\beta_3$. Four nonpeptides (**1-4**) were highly selective $\alpha_{IIb}\beta_3$ antagonists, whereas GRGESP was a poor inhibitor for both receptors.

Inhibition of MG63 human osteosarcoma cell adhesion on rat osteopontin by $\alpha_v\beta_3$ antagonists. The 16 compounds were assayed for their interactions with $\alpha_v\beta_3$ in a cell system. Adhesion of MG63 cells on recombinant rat osteopontin could be completely blocked by the addition of either LM609 or 23C6 but not by the $\alpha_v\beta_5$- or the β_1-specific monoclonal antibodies (data not shown), suggesting that this process is mediated primarily by $\alpha_v\beta_3$. Half-maximal inhibition of cell adhesion was obtained at 0.15 and 1 μg/ml concentration of LM609 and 23C6, respectively. Table 2 shows that echistatin was highly potent in the assay, exhibiting an IC_{50} of 0.0008 μM, which was similar to its binding affinity to the purified receptor. In contrast, the IC_{50} of the cyclic RGD peptides, including SK&F-107260, VRGDdF, GPenGRGDSPA, PRGDG-dP, and Ac-C(NMe)RGDPen-NH_2, were 10–60-fold higher in value than their K_i. The difference between IC_{50} and K_i values was even more pronounced for the linear peptides. GRGDS, RGDS, GRGDSP, and GRGDSPC exhibited IC_{50}/K_i ratios of 100–170. Nonpeptides **1-4** and GRGESP failed to inhibit MG63 cell adhesion at concentrations of ≤100 μM. Despite the difference in the IC_{50}/K_i ratio of these compounds, their rank order of potency in the cell adhesion assay correlates with that obtained in the receptor binding assay.

Inhibition of rat osteoclast adhesion on osteopontin by $\alpha_v\beta_3$ antagonists. To examine whether the $\alpha_v\beta_3$ on the osteoclast surface display similar ligand specificity as those on MG63 cell, we assayed the abilities of 13 $\alpha_v\beta_3$ antagonists in inhibiting rat osteoclast adhesion on rat osteopontin. Rat osteoclasts adhered and spread well on osteopontin-coated slides. F11, a mouse anti-rat β_3 specific monoclonal antibody (13), inhibited osteoclast adhesion on osteopontin in a dose-dependent manner, exhibiting an IC_{50} of 0.3 μg/ml (Table 2). These results are consistent with a previous report that adhesion of rat osteoclasts on osteopontin is an β_3 integrin-mediated process (17). The $\alpha_v\beta_3$ antagonists displayed comparable IC_{50} values in inhibiting rat osteoclast and MG63 cell adhesion, suggesting that $\alpha_v\beta_3$ retained the same ligand specificity when expressed on distinct cell types from different species.

TABLE 2

Correlation of the binding affinities of 15 compounds and their abilities to inhibit MG63 cell and rat osteoclast adhesion on osteopontin

Compound	[^{3}H]107260/$\alpha_v\beta_3$	MG63 cells/OP IC_{50}[b]	IC_{50}/K_i[c]	Osteoclast/OP IC_{50}[d]
	μM			μM
Echistatin	0.0022	0.0003 ± 0.0001	0.14	0.0025
SK&F-107260	0.0026	0.03 ± 0.005	12	0.2
VRGDdF	0.010	0.4 ± 0.1	40	0.25
GPenGRGDSPCA	0.010	0.6 ± 0.1	60	1.0
GRGDS	0.015	1.5 ± 0.12	100	2.1
GRGDSPC	0.022	2.0 ± 0.15	167	5.0
GRGDSP	0.026	2.8 ± 0.10	108	3.0
RGDS	0.027	3.0 ± 0.20	111	2.1
Ac-C(NMe)RGD-Pen-NH_2	0.123	3.0 ± 0.20	24	5.0
PRGDG-dP	0.151	4.0 ± 0.30	27	15.0
1	>50	>100		>100
2	>50	>100		>100
3	>50	>100		>100
4	>50	>100		>100
GRGESP	>50	>100		>100
F11	>50 μg/ml	>30 μg/ml		0.03 μg/ml

[a] K_i values of the compounds were determined in a [^{3}H]SK&F-107260-$\alpha_v\beta_3$ competition binding assay. The same set of data were described in Table 1.
[b] Corning 96-well ELISA plates were coated overnight with 0.1 ml of rat osteopontin (1 μg/ml). MG63 cells were added to the wells at 0.05 × 10^6 cells/well. Cells were incubated at 37°c for 1 hr. The adherent cells were stained with 0.5% toluidine blue and quantified at an adsorbance of 600 nm on a microtiter plate reader.
[c] Ratio of the IC_{50} values obtained in the cell adhesion assay to the K_i values obtained the receptor binding assay.
[d] Nunc chamber slides were coated with 0.1 μg of recombinant rat osteopontin. Rat osteoclast suspensions were plated onto the wells. Cells were incubated at 37° for 45 min. Attached cells were fixed with glutaraldehyde and visualized by staining for tartrate-resistant acid phosphatase.
The K_i and IC_{50} values of the compounds were the mean from two or three experiments. The standard error values were <20% of the mean values.

Discussion

The major findings of the current study were as follows. First, we established a quantitative and reproducible solid-phase radioligand binding assay for $\alpha_v\beta_3$, using [^{3}H]SK&F-107260 as the radioligand. The rank order of potency of 15 compounds in inhibiting [^{3}H]SK&F-107260 binding to $\alpha_v\beta_3$ is identical to that in inhibiting biotinylated-fibrinogen binding, suggesting that the [^{3}H]SK&F-107260/$\alpha_v\beta_3$ binding assay is a valid reflection of the binding of adhesive proteins. Second, $\alpha_v\beta_3$ purified from three different sources, chicken osteoclasts, human platelets, and human placenta, exhibit similar ligand binding characteristics, suggesting that no appreciable species or tissue specificity for $\alpha_v\beta_3$/ligand binding could be detected. Third, binding affinities of the $\alpha_v\beta_3$ antagonists correlate with their abilities to inhibit MG63 osteosarcoma cell and rat osteoclast adhesion on rat osteopontin, suggesting that the purified receptor binding assay reflects $\alpha_v\beta_3$ binding on cell surfaces. Fourth, $\alpha_{IIb}\beta_3$, an integrin that contains the identical β subunit and a highly homologous α subunit compared with $\alpha_v\beta_3$, exhibits distinct ligand specificity from $\alpha_v\beta_3$.

Two recent studies reported the binding of RGD-containing peptides to purified $\alpha_v\beta_3$ (29, 30). One study used ^{125}I-vitronectin as the labeled ligand, whereas the other used unlabeled vitronectin, followed by an antivitronectin antibody in an ELISA. To compare the binding affinity obtained from the [^{3}H]SK&F-107260 assay with those from the previous studies, we adopted their assay conditions. In all three assays, purified $\alpha_v\beta_3$ were immobilized on plastic, and similar coating conditions and surface densities (0.1–0.2 μg/well) were applied. The experimental conditions, which include the buffer system, incubation time, and temperature, were also similar. Binding affinities (K_i) of the linear RGD peptides obtained from the [^{3}H]SK&F-107260 assay (0.015–0.03 μM) corresponded more closely to the IC_{50} values (0.03 μM) obtained from the ^{125}I-vitronectin assay (29) than did those from the ELISA assay (IC_{50} = 0.32 μM) (30). In the latter assay, the IC_{50} value of the cyclic peptide VRGD-dF was determined as 0.05 μM compared with the value of 0.02 μM obtained from the [^{3}H]SK&F-107260 assay. Because both [^{3}H]SK&F-107260 and ^{125}I-vitronectin assays involve only a single step, it is not surprising that they give similar binding affinity values. The ELISA assay, on the other hand, can be considered a three-step assay, and the nonlinearity of the detection signal may contribute to the differences in IC_{50} values of the RGD peptides (31).

One of the major goals of our study was to identify potent antagonists for the osteoclast $\alpha_v\beta_3$. Several lines of evidence suggest that there may be functional and epitopic heterogeneity in $\alpha_v\beta_3$ obtained from different tissues. Human placental tissue expresses a variant β_3 messenger RNA, which differs with the platelet β_3 sequence in the cytoplasmic region (32). In addition, an $\alpha_v\beta_3$ monoclonal antibody, 10C4.1.3, specifically recognizes $\alpha_v\beta_3$ on the osteoclasts (33). Furthermore, it has been shown that the $\alpha_v\beta_3$ antagonists exhibit different potencies in inhibiting bone resorption and endothelial cell adhesion (34). In view of these observations, we decided to compare the ligand binding properties of the osteoclast $\alpha_v\beta_3$ with those on human platelet and placenta. We used an avian osteoclast isolation protocol, which yielded large numbers of osteoclasts from chick hatchlings maintained on a low-calcium diet (14). $\alpha_v\beta_3$ was purified from these cells using LM609 immunoaffinity chromatography. As occurs with many other osteoclast isolation protocols, our population was not totally pure and contained various types of bone marrow cells. Because LM609 is specific for $\alpha_v\beta_3$ and, in bone, $\alpha_v\beta_3$ is restricted to its expression on osteoclasts (8, 35, 36), it is reasonable to assume that the presence of other cell types in the osteoclast preparation would not affect the quality of the purified receptor. Our data reveal that oste-

oclast $\alpha_v\beta_3$ exhibits similar binding affinities for RGD peptides and monoclonal antibodies to the human platelet and placental receptors. Therefore, the platelet or placental $\alpha_v\beta_3$ can substitute for the osteoclast $\alpha_v\beta_3$ in antagonist screening. Our data, however, also suggest that $\alpha_v\beta_3$ antagonists could act nondiscriminatingly on various tissues. Immunohistological study has shown that $\alpha_v\beta_3$ has a relatively restricted distribution *in vivo*, exhibiting high expression on selective tissues and cell types, including osteoclast, placenta, some epithelial tissues, vascular smooth muscle, and endothelium (2). Therefore, it is important to examine the effects of $\alpha_v\beta_3$ antagonists on these tissues in an intact organism.

$\alpha_v\beta_3$ exhibits monophasic binding isotherm in binding to [^{3}H]SK&F-107260, indicating a single class of noncooperative binding site is involved. The B_{max} and K_d values for [^{3}H]SK&F-107260 obtained in $\alpha_v\beta_3$ binding are very similar to those obtained in $\alpha_{IIb}\beta_3$ binding.[1] Because of the quantitative, noncooperative, and nonselective nature of the [^{3}H]SK&F-107260 binding to the two β_3 integrins, this ligand is particularly useful for assaying the integrin selectivity of the competing ligands. Results show that echistatin, SK&F-107260, and Ac-C(NMe)RGDPen-NH_2, are nonselective to the two receptors. Three cyclic peptides (VRGDdF, GPenGRGDSPCA, and PRGDG-dP) and the prototypic linear RGD peptides display affinities in binding to $\alpha_v\beta_3$ that are 2–3 orders of magnitude higher than those to $\alpha_{IIb}\beta_3$. In contrast, four nonpeptides (**1-4**) bind exclusively to $\alpha_{IIb}\beta_3$. Therefore, despite the remarkable structural similarities and overlapped matrix ligands between the two β_3 integrins, they exhibit distinct specificity in interacting with small RGD peptides and their equivalent.

Previous studies have shown that the ligand/$\alpha_v\beta_3$ interactions could be modulated by the cell environment; these include the membrane lipid composition (37, 38), divalent cations (39), and specific membrane proteins surrounding $\alpha_v\beta_3$ (40, 41). Our data demonstrate that the $\alpha_v\beta_3$ antagonists exhibit the same rank order of potency in inhibiting cell adhesion (IC_{50}) and binding to the purified receptor (K_i), suggesting that the ligand specificity of $\alpha_v\beta_3$ remains unchanged when expressed on the rat osteoclast and MG63 cell membranes. The IC_{50}/K_i ratio, however, varies among different groups of antagonists. Echistatin was extremely active in the cell adhesion assay. In contrast, the cyclic and linear RGD peptides lost their activities by 1–2 orders of magnitude. It is not known at the present time what causes the difference in IC_{50}/K_i ratios. It could be due to a decrease in binding affinities to cell surface receptors. Alternatively, these $\alpha_v\beta_3$ antagonists may elicit different postreceptor binding events and therefore vary in their efficacies in inhibiting cell adhesion.

In summary, we established a radiolabeled binding assay for $\alpha_v\beta_3$. Using this assay, we demonstrated that $\alpha_v\beta_3$, purified from different tissue sources, exhibit similar ligand binding properties. The [^{3}H]SK&F-107260 binding assay is a valid reflection of the binding of a native ligand, fibrinogen. Furthermore, $\alpha_v\beta_3$ antagonists identified in the purified binding assay inhibit MG63 cell and rat osteoclast adhesion on rat osteopontin. Therefore, the radioligand binding assay provides a useful primary screening assay for $\alpha_v\beta_3$ antagonists. $\alpha_v\beta_3$ and the highly homologous $\alpha_{IIb}\beta_3$ exhibit distinct binding specificity toward RGD peptides and nonpeptides. Further studies on the structural requirements of the RGD peptide and its equivalent in binding to the two integrin receptors may lead to a better design of potent and selective antagonists for different therapeutic uses.

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