An All- d Amino Acid Peptide Model of α1(IV)531−543 from Type IV Collagen Binds the α 3 β 1 Integrin and Mediates Tumor Cell Adhesion, Spreading, and Motility †

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An All-D Amino Acid Peptide Model of α1(IV)531−543 from Type IV Collagen Binds the $\alpha_3\beta_1$ Integrin and Mediates Tumor Cell Adhesion, Spreading, and Motility†

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ABSTRACT: Type IV collagen promotes integrin-mediated cell adhesion, spreading, and motility. Several regions within the triple-helical domain of type IV collagen have been identified as tumor cellular recognition sites. Among these regions, the α1(IV)531−543 sequence, designated L-Hep-III, promotes integrin-mediated tumor cell adhesion and directly binds to the $\alpha_3\beta_1$ integrin [Miles, A. J., et al. (1994) *J. Biol. Chem. 269*, 30939−30945; Miles, A. J., et al. (1995) *J. Biol. Chem. 270*, 29047−29050]. We have presently compared the activities of the all-D enantiomeric peptide model of α1(IV)531−543, designated D-Hep-III, with L-Hep-III, for promoting the adhesion, spreading, and motility of metastatic melanoma and breast carcinoma cells. D-Hep-III was found to support melanoma and breast carcinoma cell adhesion, spreading, and motility in a dose-dependent fashion similar to that of L-Hep-III. The adhesions of melanoma and breast carcinoma cells to both type IV collagen and fibronectin were effectively inhibited by L-Hep-III and D-Hep-III. Melanoma cell invasion of the basement membrane was also inhibited by D-Hep-III. Characterization of the cell surface receptor for D-Hep-III was acheived via cell adhesion assays and affinity chromatography using monoclonal antibodies against integrin subunits. Immunoprecipitation analysis following EDTA elution from a D-Hep-III affinity column indicated that D-Hep-III binds to the $\alpha_3\beta_1$ integrin but not to the α_2 or α_6 integrin subunits. In summary, these studies demonstrate that an all-D model of the α1(IV)531−543 sequence mimics the biological activities of the all-L peptide. D-Hep-III is the first all-D peptide that has been shown to promote tumor cell adhesion, spreading, and migration, inhibit tumor cell adhesion and migration on type IV collagen and invasion of the basement membrane, and bind directly to an integrin. Due to the resistance to proteolysis, all-D receptor-binding peptides such as D-Hep-III have great potential for *in vivo* studies and as therapeutic agents.

Type IV collagen is the major collagenous component of the basement membrane, and both provides the structural framework of all basement membranes and interacts with cell surface biomolecules such as integrins and proteoglycans. Type IV collagen and synthetic peptides derived from type IV collagen have been shown to promote cell adhesion, spreading, and migration (*1*−*5*). The sequence [α1(IV)414−452]$_2$α2(IV)432−469 has been reported to be an $\alpha_1\beta_1$-mediated cell adhesion site of fibrosarcoma cells (*6*). A peptide model of the α1(IV)1263−1277 sequence promotes the adhesion, spreading, and motility of the highly metastatic melanoma cells (*2*,*3*) and the adhesion and aggregation of platelets (*7*). Peptide models of the α1(IV)531−543[1] sequence promote $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrin-mediated corneal epithelial cell adhesion (*8*) and $\alpha_2\beta_1$ integrin-mediated keratinocyte motility (*9*). Furthermore, α1(IV)531−543 binds directly to the $\alpha_3\beta_1$ integrin and supports melanoma and ovarian carcinoma cell adhesion (*10*). While the [α1(IV)414−452]$_2$α2(IV)432−469 and α1(IV)1263−1277 sites appear to be dependent upon the triple-helical conformation for optimal activity, triple-helicity does not significantly increase the adhesion-promoting activity of α1(IV)531−543 (*5*).

One of the most intriguing features of the α1(IV)531−543 sequence is that promotion of tumor cell adhesion and inhibition of cell adhesion to type IV collagen occur regardless of whether the all-L or all-D amino acid-containing peptide is used (*5*). Activities of all-D amino acid peptides are of great interest due to the potential enhanced *in vivo* activity of such compounds, compared to those of all-L peptides. All-D peptides are resistant to proteolysis (*11*, *12*), and have been shown in some cases to have activities similar to those of all-L peptides. A laminin-derived synthetic peptide (A chain residues 2097−2108) and its all-D enantiomer had similar activities for promotion of cell attachment and support of tumor growth in mice (*13*). Calmodulin binds both the all-L and all-D forms of melittin with apparently

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[1] Abbreviations: α1(IV)531−543, Gly-Glu-Phe-Tyr-Phe-Asp-Leu-Arg-Leu-Lys-Gly-Asp-Lys; BSA, bovine serum albumin; CZE, capillary zone electrophoresis; DMEM, Dulbecco's Modified Eagle's Medium; FACS, fluorescence-activated cell sorter; D-Hep-III, Gly-D-Glu-D-Phe-D-Tyr-D-Phe-D-Asp-D-Leu-D-Arg-D-Leu-D-Lys-Gly-D-Asp-D-Lys-D-Tyr; L-Hep-III, Gly-Glu-Phe-Tyr-Phe-Asp-Leu-Arg-Leu-Lys-Gly-Asp-Lys-Tyr; mAb, monoclonal antibody; MEM, Minimum Essential Medium; PBS, phosphate-buffered saline solution.

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similar affinities (*14*). To investigate the potential application of α1(IV)531−543 *in vivo* and for drug development studies, the α1(IV)531−543 sequence was synthesized in both all-L (designated L-Hep-III) and all-D (designated D-Hep-III) amino acid forms. We have compared the activities of L-Hep-III and D-Hep-III for promoting human melanoma and breast carcinoma cell adhesion, spreading, and motility. We have also studied the efficacy of L-Hep-III and D-Hep-III for inhibiting the adhesion of melanoma and breast carcinoma cells to type IV collagen and fibronectin and for inhibiting melanoma cell invasion through Matrigel. Finally, we have characterized the receptor that binds to D-Hep-III via cell adhesion assays and affinity chromatography using monoclonal antibodies (mAbs) against integrin subunits.

EXPERIMENTAL PROCEDURES

Materials. All standard peptide synthesis chemicals were analytical reagent grade or better and were purchased from Applied Biosystems, Inc. (Foster City, CA) or Fisher (Pittsburgh, PA). The two peptides used in this study, L-Hep-III and D-Hep-III, were synthesized as described (*5*). Peptide concentrations were determined spectrophotometrically (*5*). Intact type IV collagen was isolated from mouse Engelbreth−Holm−Swarm tumor as described (*15*, *16*). mAb P5D2 was prepared against the β_1 integrin subunit using methods described previously (*17*). mAbs prepared against integrin subunits α_2 (P1E6), α_3 (P1B5), and α_6 (GoH3) were purchased from Chemicon International (Temecula, CA), Life Technologies (Grand Island, NY), and Immunotech (Westbrook, ME), respectively.

Cells. M14 clone 5 human melanoma cells were propagated as described previously (*5*, *10*). Briefly, melanoma cells were cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine sera, 1 mM sodium pyruvate, 0.1 mg/mL gentamicin (Boehringer Mannheim, Indianapolis, IN), 50 units/mL penicillin, and 0.05 mg/mL streptomycin. Cells were passaged eight times and then replaced from frozen stocks of early passage cells to minimize phenotypic drift. All cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. All media reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Hs578T human breast carcinoma cells were obtained from American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine sera, 4.5 g/L glucose, and 10 μg/mL bovine insulin.

Cell Adhesion Assays. Melanoma and breast carcinoma cell adhesion assays were performed as described previously (*2*, *5*, *8*). Briefly, cells were radiolabled with [^{35}S]methionine, and 96-well Microlite 1 plates were coated with the proteins or peptides of desired concentrations in a 37 °C incubator overnight. The next day, nonspecific binding sites were blocked with 2 mg/mL BSA in phosphate-buffered saline solution (PBS) for 2 h at 37 °C. Cells were released from tissue culture flasks with trypsin−EDTA and washed with adhesion medium [DMEM or Minimum Essential Medium (MEM), 2 mg/mL bovine serum albumin (BSA), and 20 mM HEPES (pH 7.4)]. Cells (100 μL) were added into each well of the plate and incubated for ∼30 min at 37 °C. Nonadherent cells were removed by washing with PBS containing Ca^{2+} and Mg^{2+}, and then the plates were counted on a Top Count Microplate scintillation counter. Adhesion percentages were based on total counts of radioactivity added to each well.

Cell Spreading Assays. As for the adhesion assay, 96-well plates were coated with proteins or peptides, and then the next day, nonspecific binding sites were blocked. Cells (nonradiolabeled) were trypsinized and added into the wells. After cells had been incubated for 1 h at 37 °C, nonadherent cells were washed away gently with PBS. To each well was added 100 μL of 2% formaldehyde in PBS, and the plates were kept at room temperature for 30 min. Cells were stained with R-250 Coomassie Blue overnight. Cell spreading was evaluated by measuring cell area with the assistance of an image analysis system (Optomax) (*3*, *18*, *19*).

Cell Motility Assays. Modified Boyden microchambers were used to study cell motility (*2*, *20*, *21*). In the haptotaxis analysis, polyvinylpyrrolidone-free 8 μm pore size polycarbonate filters were coated with the ligands by floating the filter on PBS containing various concentrations of ligands overnight at 37 °C. Cells were trypsinized and washed with migration buffer [DMEM or MEM and 20 mM HEPES (pH 7.4)]. Approximately 28 μL of migration buffer was added into each well of the lower chamber. The cell suspensions were pipetted into the top chambers at 50 μL per well, and the chambers were assembled and incubated for 4−6 h at 37 °C. The filters were removed, and cells reaching the bottom surface of the membrane were fixed, stained, and counted using an image analysis system (Optomax) integrated with a Zeiss Universal Microscope and Apple Computer.

Inhibition of Cell Adhesion Assays. Cells were labeled with ^{35}S, and 96-well Microlite 1 plates were coated with peptides or proteins as in the adhesion assay. The cells were preincubated with various concentrations of mAbs or peptides for 30 min at 37 °C after they had been harvested. The cells were added to the wells to evaluate the adhesion to coated peptides or proteins in the continued presence of mAbs or peptides (*2*, *5*, *22*). Cells were allowed to adhere for 30 min at 37 °C, and cell adhesion was quantified as in the adhesion assay.

Cell Invasion Assay and Inhibition of Cell Invasion. The Matrigel invasion assay was performed in the matrix invasion chambers that contain Matrigel basement membrane (Collaborative Biomedical Products). MEM was added to each cell culture insert to equilibrate the Matrigel-coated filter for 90 min at 37 °C. MEM supplemented with 50 μg/mL fibronectin (200 μL/well) was added to the space below the inserts. Cells were released with trypsin−EDTA, washed, and resuspended. Cells were then mixed with peptides at desired peptide concentrations, and the mixture was added to each well (150 μL/well). The chamber was incubated overnight (18−24 h) at 37 °C. After incubation, the inserts were removed from the wells, fixed, and stained. The upper surface of each Matrigel-coated filter was wiped dry and then cut from the bottom of the insert. The filters were mounted on slides and quantitated as described for the cell motility assay.

Fluorescence-Activated Cell Sorter (FACS) Analysis. Cells were trypsinized and washed, and then incubated with normal goat IgG to block nonspecific binding sites on the cell surface. Cells were incubated with mouse anti-integrin subunit mAbs followed by a secondary antibody (fluorescein isothiocyanate goat anti-mouse IgG). Cells were fixed with

2% formaldehyde and then applied to a flow cytometer (Bacton Dickinson FACSTAR Plus).

Affinity Chromatography and Immunoprecipitation Analysis. D-Hep-III was coupled to activated CH-Sepharose according to the instructions of the supplier (Pharmacia Biotech). In addition, a mock-coupled column was made without the peptide. Melanoma cells were radiolabeled with $Na^{125}I$ and extracted in the lysis buffer [50 mM Tris-HCl, 15 mM NaCl (pH 7.2), 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM PMSF, 1 mM *N*-ethylmaleimide, 100 μg/mL soybean trypsin inhibitor, 100 μg/mL leupeptin, and 50 mM octyl β-glucoside] by shaking for 30 min at 4 °C. The lysates were cleared by centrifugation at 36 500 rpm for 60 min at 4 °C (*10*, *23*−*25*). Unbound peptide was removed by washing the beads with coupling buffer [15 mM Na_2CO_3 and 35 mM $NaHCO_3$ (pH 7.2)], and cleared radioactive cell lysates were shaken with the mock beads for 4 h at 4 °C. The unbound materials were collected and incubated with the peptide−Sepharose beads by rocking overnight at 4 °C. The mixture was then packed into a column, and D-Hep-III binding proteins were eluted with 20 mM EDTA in extraction buffer lacking cations followed by 1 M NaCl. The EDTA peak elution fractions were concentrated using Microsep Centrifugal Concentrators and neutralized with 5 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$. Eluates were incubated with anti-integrin subunit mAbs to immunoprecipitate specific integrins (*10*, *25*). Precipitated samples were electrophoresed by SDS−PAGE under nonreducing conditions. The prestain marker bands (Sigma) were visible, and the ^{125}I-labeled proteins were visualized by autoradiography.

Peptide Hydrolysis Assay. L- or D-Hep-III was dissolved in H_2O at a concentration of 0.5−1.0 mM and treated with 0.025% trypsin at room temperature. At various time points, samples were analyzed by capillary zone electrophoresis (CZE) on a Beckman PACE System 5000 apparatus in capillary cartridges (fused silica, 75 μm inside diameter × 50 cm length). Conditions were as follows: prewash for 10 min with running buffer, sodium tetraborate (50 mM, pH 9.0), equilibrate capillary for 2 min with running buffer, 5 s pressure injection, and separate for 15 min at 17 kV and 0.25 mA. Detection was at $\lambda = 214$ nm.

RESULTS

The promotion of human melanoma and breast carcinoma adhesion was compared for L-Hep-III and D-Hep-III over a peptide concentration range of 1.9−19.2 μM. Both peptides showed dose-dependent promotion of melanoma cell adhesion, with 50% cell adhesion occurring at ~7.7 μM for either L- or D-Hep-III (Figure 1A). There were very low levels of cell adhesion to control plates coated with bovine serum albumin (BSA) (data not shown). As was previously documented for A375SM melanoma cells (*5*), adhesion promotion of M14#5 melanoma cells was virtually identical for L- and D-Hep-III. Promotion of breast carcinoma cell adhesion was also equivalent for L- and D-Hep-III, with 50% cell adhesion occurring at ~7.7 μM (Figure 1B).

The ability of L- and D-Hep-III to promote cell spreading and migration was next studied. On the basis of the results from adhesion assays, a peptide concentration that promoted a high level of activity (19.2 μM) was used for spreading and migration assays. Melanoma cells spread equally well on L- and D-Hep-III (Figure 2). Melanoma cell migration

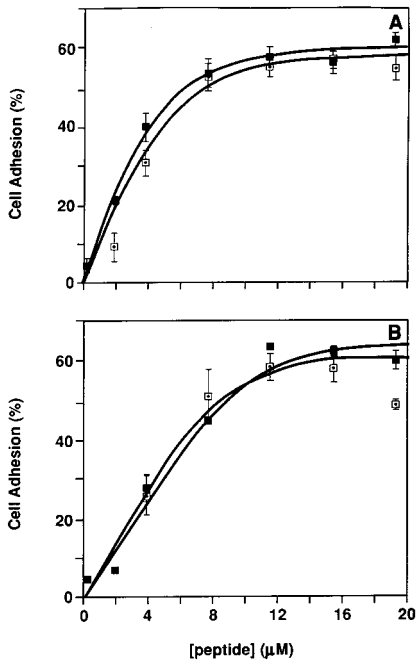

FIGURE 1: Adhesion of (A) M14#5 human melanoma cells or (B) Hs578T human breast carcinoma cells to L-Hep-III (□) or D-Hep-III (■). Cells were allowed to adhere to peptide-coated Microlite 1 plates for 30 min at 37 °C. BSA was coated as a negative control. The data represent the means of triplicate points plus or minus the standard errors of the means.

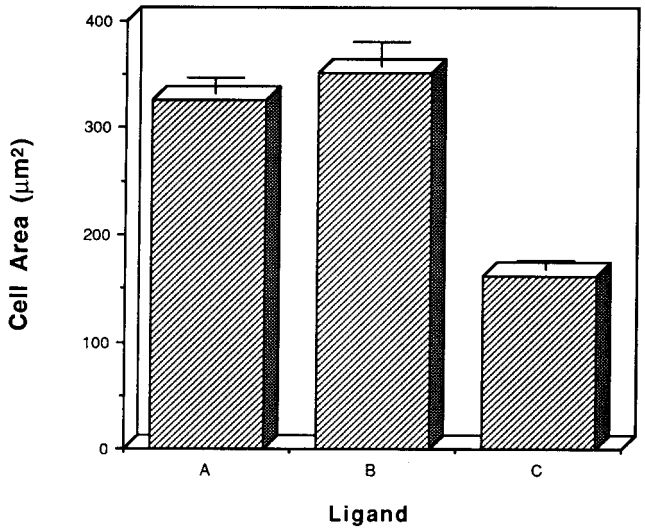

FIGURE 2: Spreading of M14#5 human melanoma cells on (A) 19.2 μM L-Hep-III, (B) 19.2 μM D-Hep-III, or (C) 100 μg/mL BSA. Cells were allowed to adhere to peptide- or BSA-coated Microlite 1 plates for 1 h at 37 °C. BSA was coated as a negative control. After gentle washing, cells were fixed and stained. The average area was calculated from counting 40 cells per well. The data represent the means of triplicate points plus or minus the standard errors of the means.

was also promoted equally well by L- and D-Hep-III (Figure 3). In a similar fashion, breast carcinoma cells spread on and migrated to both of these haptoattractants (data not shown).

Peptide competition assays were conducted to determine the inhibitory effects of L-Hep-III and D-Hep-III on melanoma and breast carcinoma cell adhesion to type IV collagen and fibronectin. The peptide concentration range was 1.9−38.4 μM. In the presence of L-Hep-III or D-Hep-III, melanoma cell adhesion to type IV collagen was inhibited

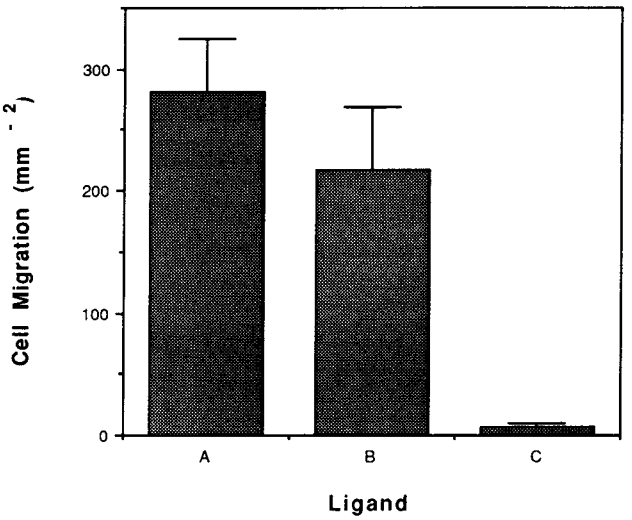

FIGURE 3: Migration of M14#5 human melanoma cells to (A) 19.2 μM L-Hep-III, (B) 19.2 μM D-Hep-III, or (C) 100 μg/mL BSA. Cells were pipetted into the top wells of a Boyden chamber that had filters precoated with L-Hep-III, D-Hep-III, or BSA. After incubation for 6 h at 37 °C, the filters were removed from the chambers and fixed and stained. The data represent the means of triplicate points plus or minus the standard errors of the means.

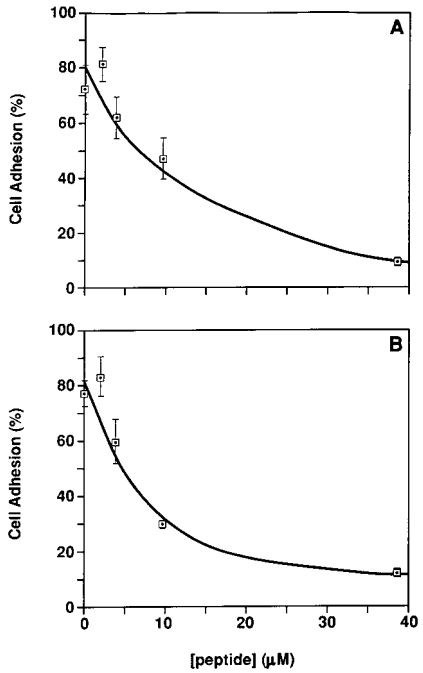

FIGURE 4: Inhibition of M14#5 human melanoma cell adhesion to 1 μg/mL type IV collagen by (A) L-Hep-III or (B) D-Hep-III. Cells were preincubated with the peptides for 30 min and then added to the wells in the presence of the peptides for a 30 min incubation at 37 °C. The data represent the means of triplicate points plus or minus the standard errors of the means.

(Figure 4). Inhibition was dose-dependent, with IC_{50} values of ~11.5 and 7.7 μM for L-Hep-III and D-Hep III, respectively. Both L-Hep-III and D-Hep-III also inhibited melanoma cell adhesion to fibronectin in dose-dependent fashions, with IC_{50} values of ~7.7 and 5.0 μM, respectively (Figure 5). Breast carcinoma cell adhesion to type IV collagen and fibronectin was inhibited similarly by L-Hep-III and D-Hep-III (data not shown).

Peptide competition studies were further extended to include invasion assays. Invasion of basement membrane (Matrigel) was examined in the absence and presence of

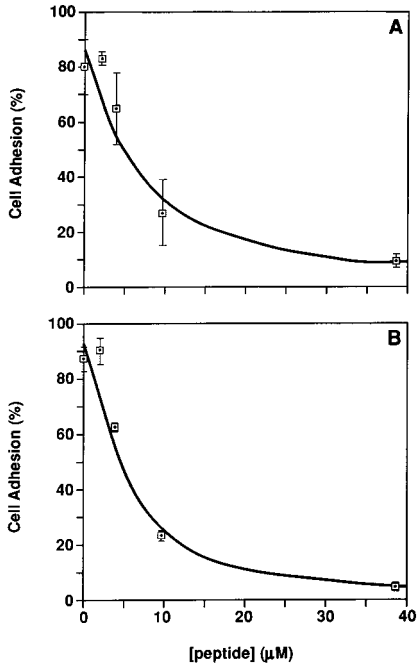

FIGURE 5: Inhibition of M14#5 human melanoma cell adhesion to 5 μg/mL fibronectin by (A) L-Hep-III or (B) D-Hep-III. Cells were preincubated with the peptides for 30 min and then added to the wells in the presence of the peptides for a 30 min incubation at 37 °C. The data represent the means of triplicate points plus or minus the standard errors of the means.

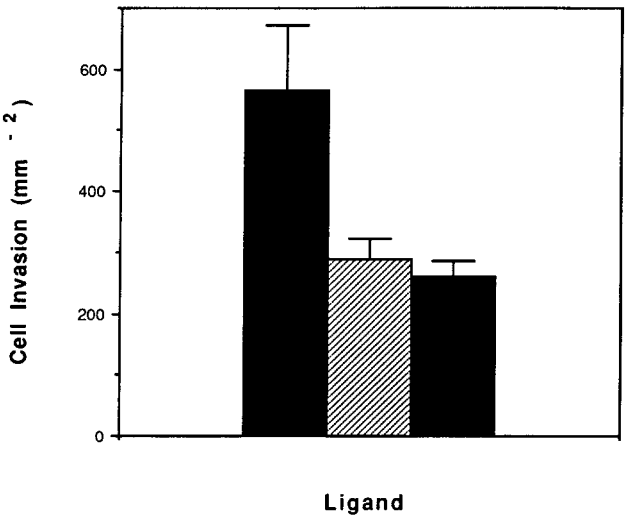

FIGURE 6: Inhibition of M14#5 human melanoma cell invasion through Matrigel by 9.6 μM D-Hep-III (middle bar), 19.2 μM D-Hep-III (right bar), or no peptide (left bar). Cells were mixed with D-Hep-III and then tested for their ability to invade through Matrigel. The data represent the means of triplicate points plus or minus the standard errors of the means.

D-Hep-III. Melanoma cell invasion of Matrigel was effectively inhibited (>50%) by D-Hep-III at concentrations of 9.6 and 19.2 μM (Figure 6).

The identification of the cell surface adhesion molecule responsible for binding to D-Hep-III was next pursued. FACS analysis was used to identify the integrin subunits expressed by M14#5 melanoma and Hs578T breast carcinoma cells. Integrin subunits α_2, α_3, α_5, α_6, and β_1 were detected on both melanoma and breast carcinoma cells (data not shown). To study whether an integrin is involved in mediating melanoma cell adhesion to L-Hep-III and/or D-Hep-III, anti-integrin mAbs were used as inhibitors of cell

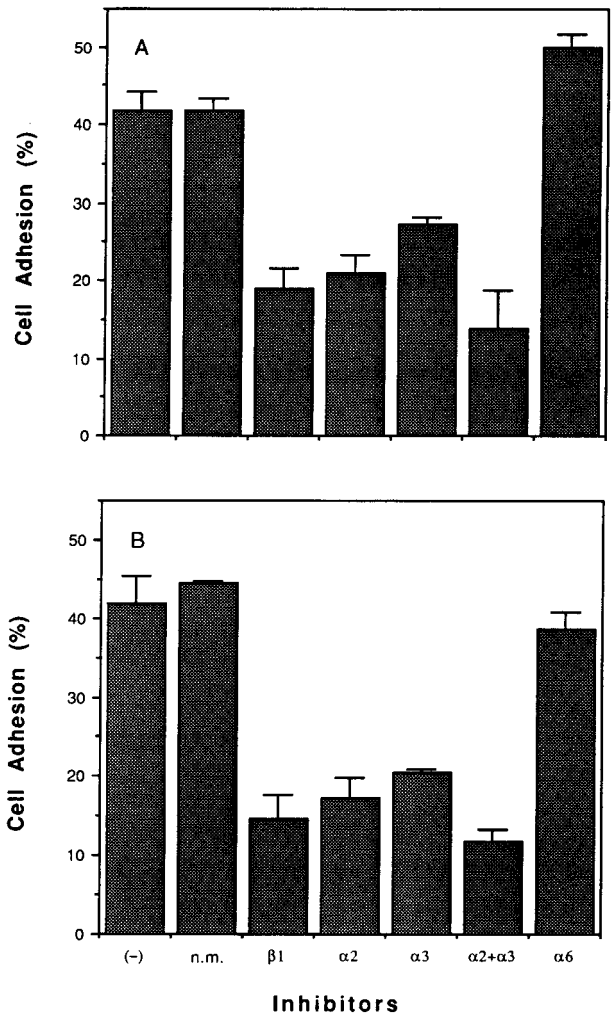

FIGURE 7: Inhibition of M14#5 human melanoma cell adhesion to 7.7 μM (A) L-Hep-III or (B) D-Hep-III by 10 μg/mL mAbs against integrin subunits β_1, α_2, α_3, α_2 plus α_3, or α_6. Control (−) was without any antibodies. Normal mouse (n.m.) IgG was used as a negative control. Cells were preincubated with the antibodies for 30 min and then added to the wells in the presence of the antibodies for a 30 min incubation at 37 °C. The data represent the means of triplicate points plus or minus the standard errors of the means.

adhesion. mAbs against the β_1, α_2, or α_3 integrin subunits affected both L- and D-Hep-III-induced melanoma cell adhesion (Figure 7). Inhibition was concentration-dependent (data not shown). The combination of the mAbs against the α_2 and α_3 integrin subunits gave slightly better inhibitory effects than either the anti-α_2 or -α_3 subunit mAb alone (Figure 7). The mAb against the α_6 integrin and normal mouse IgG did not inhibit cell adhesion (Figure 7). These results are consistent with prior studies on ovarian carcinoma and melanoma cell adhesion to L-Hep-III, where adhesion was inhibited by mAbs against the β_1, α_2, and α_3 integrin subunits (*10*).

Affinity chromatography was performed to further characterize the receptor for D-Hep-III. D-Hep-III was immobilized to CH-Sepharose, and an ^{125}I-labeled extract of human melanoma cells was added to the beads. Following application of cells, the column was first washed with extraction buffer and then eluted with 20 mM EDTA. Eluants were incubated with 5 μg/mL mAbs against either the α_2, α_3, α_6, or β_1 integrin subunit or normal mouse IgG. Precipitated proteins were then analyzed by 7.5% SDS−PAGE with detection by autoradiography. Under nonreducing conditions, two proteins of 150 and 120 kDa were immunoprecipitated from the EDTA eluant by the anti-β_1 (lane 2) and anti-α_3 (lane 4) integrin subunit mAbs (Figure 8). The apparent molecular masses correspond to the α_3 and β_1 integrin subunits, respectively (*10*, *26*). No corresponding proteins were observed using normal mouse IgG (lane 1), anti-α_2 integrin subunit mAb (lane 3), or anti-α_6 integrin subunit mAb (lane 5) immunoprecipitations (Figure 8). Prior studies had shown that the $\alpha_3\beta_1$ integrin from melanoma and ovarian carcinoma cells binds directly to L-Hep-III (*10*).

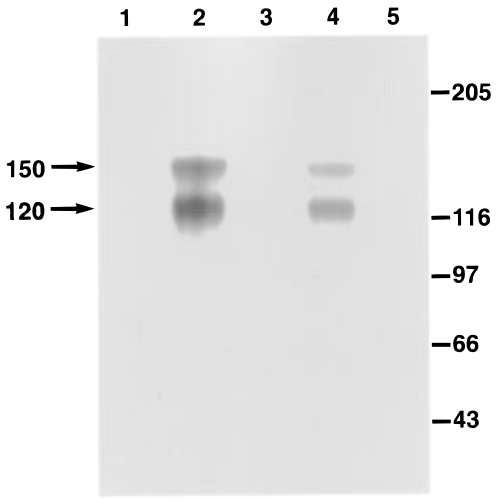

FIGURE 8: Anti-integrin immunoprecipitation analysis of human melanoma cell surface proteins eluted from the D-Hep-III peptide affinity column. The proteins eluted by EDTA from the peptide column were immunoprecipitated with normal mouse (n.m.) IgG (lane 1), anti-β_1 (lane 2), anti-α_2 (lane 3), anti-α_3 (lane 4), and anti-α_6 (lane 5) integrin mAbs. Immunocomplexes were separated on a 7.5% SDS−PAGE under nonreducing conditions. The gel was dried and autoradiographed.

The enzymatic stability of D-Hep-III was studied by comparing D-Hep-III and L-Hep-III susceptibility to trypsin. The Hep-III sequence contains two Lys residues and thus should be degraded by trypsin. Untreated D-Hep-III eluted at 8.46 min by CZE (data not shown). No change in the elution profile of D-Hep-III was seen following overnight treatment with trypsin (data not shown). In contrast, a 10−15 min treatment of L-Hep-III with trypsin showed 50% conversion of the intact peptide (elution time, 8.48 min) into two products (elution times, 7.35 and 11.29 min) (data not shown). D-Hep-III was thus far more stable with respect to trypsin degradation than L-Hep-III.

DISCUSSION

Functional assays using well-defined proteolytic fragments of type IV collagen have demonstrated that endothelial cells, melanoma cells, and other cell types interact with multiple domains of this protein. Cells adhere, spread, and migrate on the pepsin-generated, triple-helical rich domains of type IV collagen, and cells also adhere and spread but do not migrate in response to the noncollagenous domain (*1*, *2*, *18*). As a result of molecular complexities of type IV collagen, applying a reductionist approach for mapping the multiple active sites of the protein has been successful. There are three type IV collagen regions within the triple-helical domain that have been characterized as cell adhesion and/or migration sites: $[\alpha1(IV)414-452]_2\alpha2(IV)432-469$, $\alpha1(IV)$-531−543, and $\alpha1(IV)1263-1277$. The $\alpha1(IV)531-543$

sequence (L-Hep-III) supports the adhesion of keratinocytes, corneal epithelial, melanoma, and ovarian carcinoma cells (*5*, *8*, *21*, *22*). The α1(IV)531−543 sequence retains cell adhesion-promoting activity when conformationally constrained in a triple-helix, suggesting that this region is biologically active in type IV collagen. However, there is no significant increase in activity when L-Hep-III is in a triple-helical form (*5*), suggesting that primary structure alone is responsible for the activity of L-Hep-III. In addition, an all-D amino acid version of Hep-III (D-Hep-III) can inhibit cell adhesion to L-Hep-III as efficiently as L-Hep-III itself (*5*), indicating that ligand chirality does not effect the binding specificity of the cell surface receptor for the α1(IV)531−543 region.

It is of great general interest to better understand how receptors interact with protein/peptide backbones and side chains. Specifically, if a receptor binds both the side chains and backbone of the ligand, then enantiomeric models of the ligand would have altered binding affinity for the receptor. The molecular interactions of integrins with proteins/peptides are not well-understood, particularly the role of the ligand backbone (see further discussion below). A refined view of the interaction between complementary molecular surfaces, i.e., the "molecular topology", is essential for rational drug design (*27*). Due to the similar behaviors of its peptide enantiomers, the Hep-III sequence provides a unique opportunity to examine the relationship between integrin binding and subsequent cellular responses and ligand configuration.

The cellular activities of L- and D-Hep-III have been compared in this study. The peptide enantiomers promote human melanoma and breast carcinoma cell adhesion, spreading, and migration, inhibit cell adhesion to type IV collagen, and inhibit melanoma cell invasion through Matrigel. The concentration dependencies of the peptides for all activities are very similar. We have thus shown that D-Hep-III mimics the biological activities of L-Hep-III for a variety of cell types, including A375M melanoma, SKOV3 ovarian carcinoma, and Jurkat cells in our prior study (*5*) and M14 melanoma and Hs578T breast carcinoma cells in this study. Interestingly, both L- and D-Hep-III inhibit cell adhesion to fibronectin as well as adhesion to type IV collagen. It is possible that some of the inhibitory properties of these peptides are based on their ability to induce certain signal transduction pathways[2] and effect cell surface receptor expression and/or activation. Hep-III binding to the $\alpha_3\beta_1$ integrin may result in trans-dominant suppression of other integrin functions, as has been demonstrated previously by ligand binding to the $\alpha_{IIb}\beta_3$ integrin and subsequent inhibition of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrin functions (*28*).

D-Hep-III promotion of human melanoma and breast carcinoma cell adhesion can be inhibited by mAbs against both the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins. But, in a fashion similar to that of L-Hep-III (*10*), only the $\alpha_3\beta_1$ integrin binds directly to D-Hep-III. We speculate that the $\alpha_2\beta_1$ integrin probably has lower affinity for D-Hep-III than the $\alpha_3\beta_1$ integrin. An $\alpha_2\beta_1$ integrin binding site is reported within the sequence α1(IV)453−551 (*6*, *29*, *30*). It is possible that the α1(IV)-531−543 sequence may be a partial binding site for the $\alpha_2\beta_1$ integrin, and the receptor−ligand binding affinity is very weak between either L-Hep-III or D-Hep-III and the $\alpha_2\beta_1$ integrin.

The all-D enantiomeric sequence could directly bind the $\alpha_3\beta_1$ integrin, presenting the first evidence that $\alpha_3\beta_1$ does not discriminate on the basis of chirality for the specific sequence. Indeed, both L-Hep-III and D-Hep-III could bind to the $\alpha_3\beta_1$ integrin (*10*; Figure 8). These results indicate that the $\alpha_3\beta_1$ integrin does not strongly utilize the Hep-III peptide backbone for binding. So far, a key feature of integrin ligands is the fact that they contain acidic residues, usually Asp, within their integrin-binding motifs (*31*). It had been shown previously that acidic residues in L-Hep-III play an important role in cell adhesion, as the substitution of either Asp^{536} or Asp^{542} with Leu in L-Hep-III greatly reduced cell adhesion for melanoma, ovarian carcinoma, and Jurkat cells (*5*). Thus, the Asp residue side chains in either Hep-III peptide enantiomer may be critical for $\alpha_3\beta_1$ integrin binding, and are being evaluated presently.

Several recent studies, including the work presented here, have examined the chiral specificity of cell surface receptors. Chiral discrimination has been shown during epithelial cell binding to calcium tartrate crystals (*32*). Several epithelial-like cell lines had different adhesive responses to calcium (*R*,*R*)-tartrate versus calcium (*S*,*S*)-tartrate crystals. The cell surface receptor for calcium tartrate crystals was not identified. D-Amino acid-substituted analogs of a Gly-Arg-Gly-Asp-Ser-Pro peptide were studied for inhibition of rat kidney cell adhesion to either fibronectin (via the $\alpha_5\beta_1$ integrin) or vitronectin (via the $\alpha_v\beta_3$ integrin) (*33*). Substitution of Arg with D-Arg had no effect on the inhibitory activities of the peptide, while substitution of Asp with D-Asp resulted in an inactive peptide. Thus, inhibition of integrin binding to either fibronectin or vitronectin by Arg-Gly-Asp sequences is sensitive to the peptide inhibitor stereochemistry (*33*). Additional studies which correlated the NMR-derived structures of cyclic Arg-Gly-Asp analogs with inhibition of $\alpha_v\beta_3$ integrin binding to vitronectin indicated that the $\alpha_v\beta_3$ integrin interacts with both the Arg-Gly-Asp peptide side chains and backbone (*34*).

In contrast to the above examples, the laminin-derived synthetic peptide LAM-L (A chain residues 2097−2108) and its all-D enantiomer had nearly identical concentration-dependent activities for promotion of rat pheochromocytoma cell (PC12) attachment, inhibition of PC12 adhesion to laminin, and promotion of murine melanoma cell growth in mice (*13*). The cell surface receptor for LAM-L or LAM-D was not identified. A synthetic combinatorial library has been used to select an all-D peptide (acetyl-Arg-Phe-Trp-Ile-Asn-Lys-NH_2) as a potent ligand for the μ opioid receptor (*35*). The peptide was shown to be a full agonist, binding to the μ receptor and inducing a conformational change which allowed for signal transduction (*35*). In this case, the all-L peptide was not active.

There are certainly precedents to warrant the further investigation of all-D peptides as inhibitors of receptor function. We have presented the first evidence that an integrin binds directly to an all-D peptide. Further development of all-D peptide ligands may be acheived by using "mirror-image" phage display libraries (*36*). This approach has been used to identify the all-D Pep-D1 (Arg-cyclo[Cys-Leu-Ser-Gly-Leu-Arg-Leu-Gly-Leu-Val-Pro-Cys]-Ala) which interacts with the Src homology 3 domain of c-Src (*36*).

[2] Lauer, J. L., and Fields, G. B., manuscript submitted for publication.

Using all-D peptides to enhance *in vivo* activities is based upon the premise that proteases have reduced activity toward these potential substrates. Schnolzer and Kent (*12*) synthesized all-L and all-D HIV-1 proteases and then examined the chiral specificity of the two enzymes using the substrate 2-(aminobenzoyl)-Thr-Ile-Nle-Nph-Gln-Arg-NH_2 (where Nph is nitrophenylalanine). The synthetic all-L enzyme cleaved only the all-L, not the all-D, version of 2-(aminobenzoyl)-Thr-Ile-Nle-Nph-Gln-Arg-NH_2, while the synthetic all-D enzyme cleaved only the all-D substrate. The chiral specificity of enzymes was established by these results.

The results of other enzyme studies are consistent with those from the HIV-1 study, in that native (all-L) enzymes cleave only all-L substrates, not all-D substrates. For example, trypsin cleaves all-L cecropin A but does not cleave all-D cecropin A (*11*). We have found that trypsin cleaved L-Hep-III rapidly but did not hydrolyze D-Hep-III. Due to resistance to proteolysis, all-D receptor-binding peptides such as D-Hep-III have great potential for *in vivo* studies and as therapeutic agents.

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