

## Evaluation of Radiolabeled Type IV Collagen Fragments as Potential Tumor Imaging Agents

W. Barry Edwards, Carolyn J. Anderson,\* Gregg B. Fields,<sup>†</sup> and Michael J. Welch

Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri 63110 and Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, Florida 33431. Received June 26, 2001; Revised Manuscript Received September 18, 2001

The objective of this study was to examine radiopharmaceuticals that target the  $\alpha3\beta1$  integrin to determine if these agents target tumors for diagnostic imaging and/or targeted radiotherapy of cancer. Prior studies had shown that residues 531–542 from the  $\alpha1$  chain of type IV collagen bind a variety of tumor cell  $\alpha3\beta1$  integrins. A peptide mimic of this sequence containing all D-amino acids (designated D-Hep-III) was synthesized by solid-phase methods. The tetraazamacrocyclic chelator, TETA, was conjugated to the peptide while it was resin-bound. TETA-D-Hep-III and D-Hep-III were radiolabeled with  $^{64}\text{Cu}$  and  $^{125}\text{I}$ , respectively, in high specific activity and radiochemical purity. Heterologous competitive binding assays between D-Hep-III and either  $^{125}\text{I}$ -D-Hep-III or  $^{64}\text{Cu}$ -TETA-D-Hep-III indicated low micromolar affinity of D-Hep-III. The biodistribution of each radiolabeled analogue of D-Hep-III was carried out in rats and tumor-bearing mice. Both analogues were rapidly cleared from the blood in normal rats, with the kidneys receiving the highest accumulation of each. SKOV3 human ovarian tumor cells, known to strongly express  $\alpha3\beta1$ , were xenografted in SCID mice. Localization of  $^{125}\text{I}$ -D-Hep III and  $^{64}\text{Cu}$ -TETA-D-Hep III in the xenografts were low (<2% ID/g), and in the case of  $^{125}\text{I}$ -D-Hep III, not inhibited by a competitive dose of D-Hep III. The low tumor accumulation is likely not due to receptor down-regulation, but rather due to the weak affinity of the radioligands for the  $\alpha3\beta1$  integrin.

### INTRODUCTION

Radiolabeled analogues of peptide hormones are under current investigation as diagnostic imaging agents and radiotherapeutics for the diagnosis or treatment of cancer (for reviews see refs 1–3). These radiolabeled peptide receptor ligands are targeted to tumors that have up-regulated receptors on the tumor cell surface. For example,  $^{111}\text{In}$ -DTPA–octreotide is currently in routine clinical use for imaging neuroendocrine tumors overexpressing the somatostatin receptor (4).

Integrins are a family of receptors that bind various components of the ECM<sup>1</sup>. They are heterodimeric glycoproteins comprised of two subunits,  $\alpha$  and  $\beta$ , that each span the cell membrane once. A  $\beta$ -subunit can pair with a number of different  $\alpha$ -subunits, and this combination confers the unique binding properties of that integrin. Also, cell type-specific factors appear to play a role in determining specificities of integrins. For example,  $\alpha2\beta1$  is a receptor for collagen but not laminin on platelets (5); however,  $\alpha2\beta1$  is a receptor for both ligands on other cell types (6). In addition to promoting adhesion, integrins

provide a linkage between the ligand and intracellular proteins and can promote signal transduction (7). The cells of most normal human tissues express a variety of integrins including  $\alpha1\beta1$ ,  $\alpha2\beta1$ ,  $\alpha3\beta1$ , and  $\alpha6\beta1$ , which are primarily required for adhesion to collagen and laminin (for reviews, see refs 8, 9, 10). Radiolabeled ECM fragments have been investigated as imaging agents (11–14) since their integrins are up-regulated in certain types of tumors and can therefore be a target for diagnosis or therapy.

Immunohistochemical analysis of human tissue showed expression of  $\alpha3\beta1$  in dermal and lung epithelium, in large vessel and capillary endothelium (15), and in certain cell types within rat and human kidney (16, 17). Immunohistochemical methods on surgically excised specimens from human tumors showed strong expression of  $\alpha3\beta1$  on primary tumors from the ovary, colon–rectum, stomach, and skin as well as on tumors from their metastatic sites (18). Moreover,  $\alpha3\beta1$  is frequently expressed on cultured human tumor cell lines such as ovarian carcinoma (19), breast carcinoma (20), and melanoma (21, 22). A noninvasive means of assessing  $\alpha3\beta1$  expression could provide useful information about the extent of metastasis prior to therapy or surgery by possibly revealing metastatic sites too small for conventional imaging techniques.

Since the natural ligands for several integrins are collagens, radiolabeled fragments of collagen have the potential to serve as imaging agents. Recently, peptides incorporating segments of type IV collagen have demonstrated specific binding to the  $\alpha3\beta1$  integrin. One of these peptides, Hep-III (GEFYFDLRLKGDK), consisting of residues 531–543 from the  $\alpha1$  chain of type IV collagen, promoted 40–50% adhesion of human ovarian, breast

\* Corresponding author: Carolyn J. Anderson, Ph.D., Mallinckrodt Institute of Radiology, Washington University School of Medicine, 510 S. Kingshighway Blvd., Campus Box 8225, St. Louis, MO 63110. Phone: (314) 362-8427; fax: (314) 362-9940; e-mail: andersoncj@mir.wustl.edu.

<sup>†</sup> Florida Atlantic University.

<sup>1</sup> ECM, extracellular matrix; PET, positron emission tomography; TETA, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; HBTU, *O*-(1*H*-benzotriazol-1-yl)-*N,N,N,N*-tetramethyluronium hexafluorophosphate; BSA, bovine serum albumin; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; Mab, monoclonal antibody; ATCC, American Type Culture Collection).

carcinoma, and melanoma cell lines at concentrations of 7–11  $\mu\text{M}$  (23). An all D-isomer of Hep-III (D-Hep-III) was as effective as the L-isomer in promoting cell adhesion of these cell lines, indicating that the receptor does not discriminate between the two enantiomers (23, 24). Furthermore, D-Hep-III was found to bind  $\alpha 3\beta 1$  integrins on cultured human tumor cells in a fashion similar to Hep-III (19, 24). Radiopharmaceuticals derived from all D-peptides are particularly attractive due to their anticipated resistance to proteolysis (24, 25).

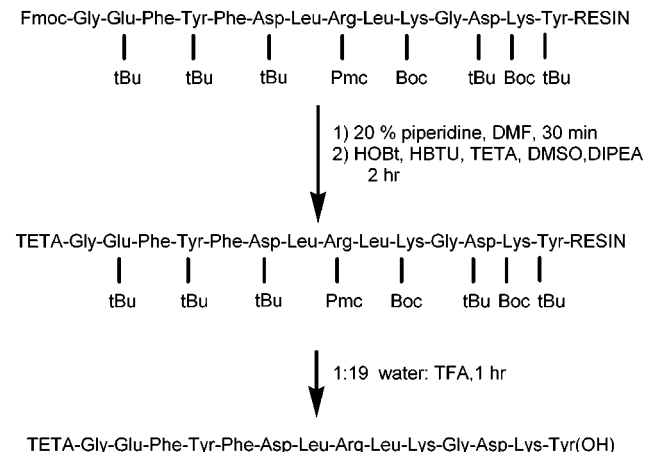
Copper-64 ( $t_{1/2} = 12.7$  h,  $\beta^+ = 0.655$  MeV [19.3%],  $\beta^- = 0.573$  MeV [39.6%]) has emissions suitable for PET imaging ( $\beta^+$ ) as well as radiotherapy ( $\beta^-$ ) (3, 26). A commercially available chelator, TETA, has been conjugated to both peptides and monoclonal antibodies (Mabs) for subsequent labeling with radiometals such as  $^{64}\text{Cu}$ . The resulting complex has demonstrated relatively high in vivo stability (27–29). The N-terminal amine of D-Hep III was chosen as the site for conjugation of one of the carboxylic arms of TETA because the N-terminus did not appear important in binding (23). Iodine radionuclides are also of interest due to the availability of cyclotron-produced  $^{124}\text{I}$  ( $t_{1/2} = 4.2$  d,  $\beta^+ = 2.14$  MeV [25%]) for PET imaging (30–32) and  $^{131}\text{I}$  for therapy. Because  $\alpha 3\beta 1$  is highly expressed in several types of human neoplasms, it was hypothesized that  $^{64}\text{Cu}$ - or  $^{125}\text{I}$ -labeled D-Hep III would localize to tumors in a receptor specific manner. The aim of this investigation was to demonstrate that  $^{64}\text{Cu}$ - and  $^{125}\text{I}$ -labeled D-Hep III peptides were specifically bound to receptor positive cells or tissues, estimate receptor affinities in vitro, and determine whether they would localize within tumor xenografts in a receptor specific manner in vivo.

## EXPERIMENTAL SECTION

**Reagents.** All standard peptide synthesis chemicals were analytical reagent grade or better and obtained from Applied Biosystems (Foster City, CA) or Fisher Scientific (St. Louis, MO). DBU and TETA·4HCl were obtained from Aldrich (Milwaukee, WI). Fmoc-Tyr(*t*-Bu)-HMP resin (substitution level = 0.31 mmol/g) was from Millipore Corporation (Bedford, MA). HBTU was from Richlieu Biotechnologies (St. Hyacinthe, Quebec). Glass test tubes precoated with Iodogen were obtained from Pierce (Rockford, IL). The mouse anti-human integrin,  $\alpha 3$  monoclonal antibody (Mab anti- $\alpha 3$ ) was obtained from Chemicon International (Temecula, CA). BSA and 0.5 M EDTA were from Sigma (St. Louis, MO), and ammonium acetate was from Fluka (Ronkonkoma, NY). C-18 Sep-pak Light cartridges were from Waters (Milford, MA). Sprague–Dawley rats were obtained from either Charles River (Wilmington, MA) or Harlan Bioproducts (Indianapolis, IN), and SCID mice were from Taconic (Germantown, NY). Copper-64 was produced as previously described (33).  $^{125}\text{I}$  was obtained from Amersham (Piscataway, NJ). Radio-TLC detection was accomplished using a BIOSCAN System 200 Imaging Scanner (Washington, DC). Radioactive samples were counted using a Beckman 8000 automated well-type counter (Fullerton, CA).

**High Performance Liquid Chromatography.** HPLC analysis and purification was performed on one of two systems: Spectra Physics 8700 ternary pump and controller with a Waters 486 UV detector, or a Waters 600E quaternary pump and controller with a Waters 991 photodiode array UV–Vis detector and Waters 771 autosampler. Both systems were fitted with Rheodyne 7125 injectors and radioactivity was detected by NaI scintillation.

## Scheme 1. Solid-Phase Synthesis of TETA-D-Hep-III. All Residues Are in the D-Configuration Except for Glycine



## Peptide Synthesis, Purification, and Analysis.

The peptides were assembled on the Fmoc-Tyr(*t*-Bu)-HMP resin by Fmoc chemistry as previously described (34) on an Applied Biosystems 431A Peptide Synthesizer. Purity was assessed on an analytical Vydac C-18 RP-HPLC column (300 Å, 5  $\mu\text{m}$ , 4.6  $\times$  250 mm) using a solvent system of A, consisting of H<sub>2</sub>O (0.1%TFA) and B, consisting of acetonitrile (0.1%TFA):H<sub>2</sub>O (0.1%TFA), 9:1, v:v. The peptides were purified on a Vydac C-18 semipreparative column (300 Å, 15–20  $\mu\text{m}$ , 22  $\times$  250 mm) unless noted. The flow rates were 1.0 and 12.0 mL/min for the analytical and semipreparative column, respectively. Edman degradation analysis was performed on an Applied Biosystems 477A Protein Sequencer/120A Analyzer. Peptide molecular weights were determined on a Finnigan (San Jose, CA) LCQ ion-trap electrospray mass spectrometer by the Protein and Nucleic Acid Chemistry Laboratories in the Departments of Medicine and Molecular Biology and Pharmacology at the Washington University School of Medicine. Data were collected and analyzed using the Finnigan LCQ Bioworks software (version 1.1). Size-exclusion chromatography was performed on a Superdex HR 10/30, 10  $\times$  100 mm, using a solvent system consisting of 20 mM NH<sub>4</sub>OAc pH 7.5 at a flow rate of 0.8 mL/min. The column was calibrated against insulin, D-Hep-III and 3-iodotyrosine, which eluted at 13.6, 18.5, and 38.2 min, respectively (BSA eluted at 10.4 min in the void volume). A plot of the elution volume vs log molecular weight was linear ( $r = 0.98$ ). Peptide concentrations were determined by UV absorbance at 280 nm utilizing the extinction coefficient for tyrosine in water ( $\epsilon = 1200$  L M<sup>-1</sup> cm<sup>-1</sup>) (35). Because D-Hep-III contains two tyrosine residues, the molar extinction coefficient ( $\epsilon$ ) was for D-Hep-III was assumed to be 2400 L M<sup>-1</sup> cm<sup>-1</sup>.

**Solid-Phase Synthesis of TETA-D-Hep III.** TETA-D-Hep III was synthesized by coupling TETA to the N-terminal amine of the resin-bound peptide (Scheme 1). LiOH·H<sub>2</sub>O (34.6 mg, 0.82 mmol) was added to TETA·4HCl (106 mg, 0.18 mmol) and dissolved in 1.5 mL of DMSO. HBTU (63 mg, 0.16 mmol) was added to 208 mg (0.046 mmol) of resin containing the D-Hep-III sequence. The TETA solution was added, immediately followed by 64  $\mu\text{L}$  (0.37 mmol) of DIEA. After 4 h, the mixture was filtered over a fine, fritted, glass funnel, and the resin was rinsed with 3  $\times$  5 mL DMSO, 3  $\times$  5 mL DMF, 2  $\times$  5 mL methanol:H<sub>2</sub>O, 1:1, 2  $\times$  5 mL H<sub>2</sub>O, and 3  $\times$  5 mL methanol and was then dried in vacuo. The peptide was released from the resin and side-chain protecting groups

were simultaneously removed in 3.5 h with 3.5 mL of TFA: H<sub>2</sub>O, 19:1. The resin was filtered through a fine, fritted glass funnel and rinsed with 2 mL of TFA. Greater than 90% of the peptide was cleaved from the resin. The filtrate was concentrated to 1.5 mL in vacuo and added to 35 mL of *tert*-butyl-methyl ether (4 °C). The white precipitate was collected by centrifugation at 3000 rpm for 10 min at 4 °C. The pellet was rinsed twice with 35 mL of *tert*-butyl-methyl ether (4 °C). RP-HPLC analysis showed about 40% coupling of TETA to the *N*-terminal amine. The crude mixture was dissolved in TFA (30 mg/mL) and 4.9 mg was loaded onto a semipreparative Vydac C-18 RP-HPLC column and purified with a gradient of 24 to 34% B in 45 min. TETA-D-Hep III (1.0 mg) was recovered in 20% yield and D-Hep III (0.8 mg) in 16% yield. Both were greater than 98% pure as determined by RP-HPLC. The peptides were identified by ES-MS (D-Hep III: calcd 1751, obsvd 1751; TETA-D-Hep-III: calcd 2165, obsvd 2165).

**Radiolabeling of TETA-D-Hep III with <sup>64</sup>Cu.** TETA-D-Hep III (1.5 μg) was labeled in ammonium acetate (100 mM, pH 5.5, 0.1% BSA) with 200–1000 μCi of <sup>64</sup>Cu-acetate at room temperature. After 15–30 min, 0.5 M EDTA was added to the reaction mixture (for a final concentration of 1 mM EDTA). By HPLC, <sup>64</sup>Cu-TETA-D-Hep III eluted at 20.26 min (22 to 32% B in 20 min, analytical) while unbound <sup>64</sup>Cu or <sup>64</sup>Cu-EDTA both eluted in the void volume. By radio-TLC (C-18, Whatman, 3:2 methanol: 10% ammonium acetate), <sup>64</sup>Cu-TETA-D-Hep III had a *R<sub>f</sub>* of 0.6, compared to <sup>64</sup>Cu-acetate, which remained at the origin, and <sup>64</sup>Cu-EDTA, which migrated to the solvent front. The mixture was purified on a C-18 Sep-Pak preconditioned with ethanol and water and eluted with labeling buffer to remove <sup>64</sup>Cu-EDTA. Elution with ethanol produced <sup>64</sup>Cu-TETA-D-Hep III in ≥ 95% purity as demonstrated by HPLC and TLC.

**Radiolabeling of D-Hep III with <sup>125</sup>I.** D-Hep-III (10 μg, 1.0 mg/mL, 1:9 DMSO:H<sub>2</sub>O) was added to 20 μL of sodium phosphate (200 mM, pH 7.5) in a 1.5 mL polypropylene vial. Sodium (<sup>125</sup>I)-iodide (1.3 mCi) was added to the vial followed by 100 μg of chloramine-T (10 mg/mL in sodium phosphate, 200 mM, pH 7.5). After 2 min, the iodination was quenched with 500 μg of Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (10 mg/mL in sodium phosphate, 200 mM, pH 7.5). The mixture was diluted with 200 μL of TFA and purified on an analytical Vydac C-18 column eluted with 25 to 35% B in 45 min. D-Hep-III eluted at 15.0 min, and the major radioactive product, <sup>125</sup>I-D-Hep-III, eluted at 21.0 min. Radiochemical yield was 24% and purity was ≥95%. Purity was assessed on an analytical Vydac C-18 RP-HPLC column. As a control to test for degradation of D-Hep-III by chloramine-T, 40 μg of D-Hep-III in 1:9 DMSO:H<sub>2</sub>O was added to 40 μL of chloramine-T (10 μg/μL in sodium phosphate, 200 mM, pH 7.5). After 2 min, the mixture was added to 500 μg of Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (10 mg/mL in sodium phosphate, 200 mM, pH 7.5) to quench the chloramine-T.

**Iodination of Mab anti-α3.** A precoated Iodogen tube was rinsed with 1 mL of Tris-HCl (200 mM, pH = 7.5). Tris-HCl (40 μL, 200 mM, pH = 7.5) was added to the glass tube followed by Na(<sup>125</sup>I) (181 μCi) and then Mab anti α3 (5 μg). The mixture was allowed to react for 20 min. The iodination mixture was transferred to a 1.5 mL polypropylene tube and allowed to stand for 5 min before addition of Tris-HCl (200 mM, 1 mg/mL BSA, pH = 7.5) to a final volume of 200 μL. Incorporation of <sup>125</sup>I into the Mab was determined by precipitation of total protein with 10% trichloroacetic acid. Incorporation was 92% and the

<sup>125</sup>I-Mab-anti-α3 was not further purified. Specific activity of <sup>125</sup>I-Mab-anti-α3 was determined to be 33 μCi/μg.

**Serum Stability of Radiolabeled D-Hep III Analogs.** <sup>125</sup>I-D-Hep-III (50 μL) in ethanol was added to 500 μL of rat serum and incubated at 37 °C for 3 h. The incubation mixture was monitored on an analytical Vydac C-18 RP-HPLC column eluted with a gradient of 20 to 90% B in 30 min (1 mL/min, A = H<sub>2</sub>O, 0.1% TFA; B = acetonitrile, 0.1% TFA). Stability was monitored for 3 h by RP-HPLC. To estimate serum protein binding of <sup>125</sup>I-D-Hep-III, it was concentrated from RP-HPLC eluant in vacuo with 20 μL of PBS containing 1 mg/mL BSA and incubated for 40 min at 37 °C and then analyzed by SEC. To investigate whether higher concentrations of D-Hep-III caused aggregation of <sup>125</sup>I-D-Hep-III, 280 μM D-Hep III was incubated with <sup>125</sup>I-D-Hep-III prepared as described above (serum protein binding experiment) in both rat serum (75 μL) and PBS (75 μL) at 37 °C for 30 min followed by analysis by SEC.

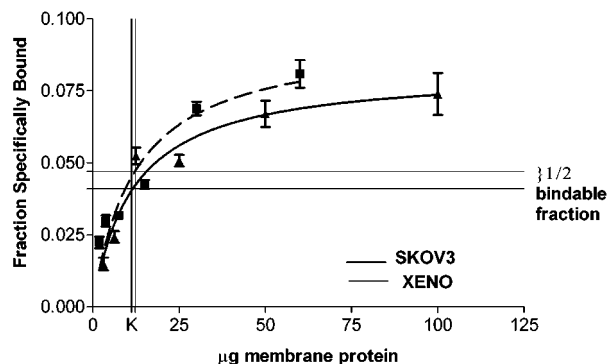
Copper-64-TETA-D-Hep-III in ethanol was concentrated in vacuo with 10 μL of NH<sub>4</sub>OAc (100 mM, pH 5.5, 10 mg/mL BSA). Rat serum (75 μL) was added, and the mixture was incubated at 37 °C for 1 h. Stability was monitored for 1 h by RP-HPLC.

**Cell Maintenance.** The SKOV3 human ovarian tumor cell line was obtained from ATCC (Rockville, MD) and maintained in McCoy's 5A media (Iwakata and Grace modification with glutamine) from Mediatech (Herndon, VA) with 10% fetal bovine serum supplemented with 50 units/mL penicillin and 0.05 mg/mL streptomycin. Cells were passaged for 4–5 weeks and then replaced from frozen stocks of earlier passages to minimize phenotypic drift. SKOV3 cells were detached with either 0.05% trypsin with 0.53 mM EDTA in HBSS or 5 mM EDTA in PBS each without calcium or magnesium salts.

**Preparation of Membranes from Harvested Xenografts and SKOV3 Cultured Cells.** SKOV3 cells were split 1:2 24 h prior to harvesting to ensure they were in the growth phase. Cells were detached at about 80% confluency with 5 mM EDTA in PBS and concentrated by centrifugation at 200g for 10 min at 4 °C. The supernatant was discarded, and the cells were suspended in a homogenizing buffer consisting of 50 mM Tris·HCl, 5 mM MgCl<sub>2</sub>, pH 7.5, containing aprotinin (0.5 μg/mL), leupeptin (10 μg/mL), pepstatin A (10 μg/mL), phenylmethylsulfonyl fluoride (2 μg/mL), and *N*-ethylmaleimide (2 μg/mL). The cells were lysed by sonication and centrifuged at 34000g for 30 min at 4 °C. After Dounce homogenization of the pellet in the homogenizing buffer, the membranes were quantified with the BCA assay (Pierce, Rockford, IL) relative to BSA. The membranes were centrifuged at 34000g for 30 min at 4 °C and then suspended at a concentration of 1.5 mg/mL in PBS, 1 mM MgCl<sub>2</sub>, 1 mg/mL BSA, and stored in aliquots at –80 °C.

Membranes from xenografted tumor were prepared by homogenization of 0.5 g tumor/10 mL homogenizing buffer at 4 °C. After centrifugation at 2000g, the supernatant was stored on ice while the pellet was rehomogenized and recentrifuged twice. The combined supernatants were then recentrifuged at 34000g for 30 min at 4 °C. The pellet was resuspended in 15 mL of homogenizing buffer, and protein content quantified by the BCA method relative to BSA. The membranes were centrifuged at 34000g for 30 min at 4 °C and then suspended at a concentration of 1.5 mg/mL in PBS, 1 mM MgCl<sub>2</sub>, and 1 mg/mL BSA, and stored in aliquots at –80 °C.

**Binding of <sup>125</sup>I-Mab-anti-α3 to Membranes Prepared from Xenografted SKOV3 tumor.** The specific binding of <sup>125</sup>I-Mab-anti-α3 was measured at infinite

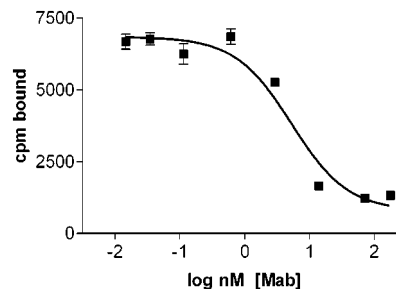


**Figure 1.** Binding of  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  to membranes from SKOV3 cells ( $\blacktriangle$ ) and SKOV xenografts ( $\blacksquare$ ).  $K$  represents the amount of membrane protein required to bind one-half of the bindable fraction of  $^{125}\text{I}$ -Mab-anti- $\alpha 3$ .  $K$  for xenografted SKOV3 membranes =  $12.3 \pm 4.7 \mu\text{g}$  and  $K$  for SKOV 3 membranes from cell culture =  $11.7 \pm 3.0 \mu\text{g}$ .

antigen excess (36) with membranes from SKOV3 cells and SKOV3 xenografted tumor (passage 10). Seven 1:2 serial dilutions of 833  $\mu\text{g}/\text{mL}$  membrane protein were made in PBS containing  $\text{MgCl}_2$  (1 mM) and BSA (1 mg/mL). Iodine-125-labeled Mab-anti- $\alpha 3$  was added to the membranes for a final concentration of 466000 to 497000 cpm/mL. To assess nonspecific binding, Mab-anti- $\alpha 3$  was added to membranes containing the radioiodinated Mab for a final concentration of 33  $\mu\text{g}/\text{mL}$ . Nonspecific binding of  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  to the glass fiber filters was assessed by adding the tracer to the binding buffer alone. The samples were allowed to bind at 24  $^\circ\text{C}$  for 2 h, and then five 125 mL aliquots were filtered in a 96-well plate with 1.0  $\mu\text{m}$  glass fiber filters. The filters were rinsed with  $3 \times 50 \mu\text{L}$  of cold (4  $^\circ\text{C}$ ) binding buffer, dried at  $\sim 40 \text{ }^\circ\text{C}$ , and removed to 1.5 mL polypropylene centrifuge tubes by the Millipore Multiscreen system (Bedford, MA) and counted in a gamma counter. The data (Figure 1) were fit by nonlinear regression according to a one-site binding hyperbola model with the following equation:  $Y = (R_p^{\text{max}}) \cdot (X) / (K + X)$  with Graphpad Prism, where  $X = \mu\text{g}$  membrane protein;  $Y = \text{reactive fraction}$ ;  $R_p^{\text{max}}$  = maximum reactive fraction;  $K$  = amount of membranes ( $\mu\text{g}$ ) required to bind one-half of the  $^{125}\text{I}$ -Mab-anti- $\alpha 3$ .

To establish  $\alpha 3\beta 1$  density, a homologous competitive binding assay was carried out between the  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  and Mab-anti- $\alpha 3$ . Briefly, membranes from SKOV3 xenografted tumor (200  $\mu\text{g}$ , passage 10) were suspended in binding buffer (PBS, 1 mg/mL BSA, 1 mM  $\text{MgCl}_2$ ) along with  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  (0.0144 nM,  $\sim 80000$  cpm) and either 0.02, 0.1, 0.6, 2.9, 14, 72, or 177 nM Mab-anti- $\alpha 3$ . One preparation contained only  $^{125}\text{I}$ -Mab-anti- $\alpha 3$ . The total volume was 370  $\mu\text{L}$ . The samples were allowed to bind at 24  $^\circ\text{C}$  for 2 h, and then four 40  $\mu\text{L}$  aliquots were filtered in a 96-well plate with 1.0  $\mu\text{m}$  glass fiber filters. The filters were rinsed with  $3 \times 50 \mu\text{L}$  cold (4  $^\circ\text{C}$ ) binding buffer, dried at  $\sim 40 \text{ }^\circ\text{C}$ , removed to 1.5 mL polypropylene centrifuge tubes by the Millipore Multiscreen system (Bedford, MA), and counted in a gamma counter. Specifically bound cpm were plotted against  $\log [\text{Mab-anti-}\alpha 3]$  concentration, and the data was fit by nonlinear regression to a one-site competition curve in Prism (Graphpad, San Diego, CA) (Figure 2).

**Heterologous Competitive Binding Assays.** Membranes from SKOV3 xenografts (passage 10) were thawed, Dounce homogenized, and diluted in PBS containing  $\text{MgCl}_2$  (1mM), BSA (1 mg/mL) (binding buffer) to prepare a stock solution of membranes. Either  $^{125}\text{I}$ -D-Hep-III or  $^{64}\text{Cu}$ -TETA-D-Hep-III was diluted to prepare a stock



**Figure 2.** Homologous competitive binding between  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  and Mab anti  $\alpha 3$  to membranes from SKOV3 xenografts, passage 10 ( $\text{IC}_{50} = 5.3 \text{ nM}$ ).

solution of tracer in the binding buffer. D-Hep-III (1 mg/mL in 1:9 DMSO:water) was serially diluted in the same vehicle at a volume of 25  $\mu\text{L}$  in 1.5 mL polypropylene centrifuge tubes. Membranes (500  $\mu\text{L}$ ) and tracer (250  $\mu\text{L}$ ) were added to the D-Hep-III for a final concentration of 400 to 600  $\mu\text{g}/\text{mL}$  membrane protein and 200 to 300 kcpm/mL tracer, respectively. The mixtures were allowed to mix at room temperature for 2 h, and then four to five aliquots of 100–125  $\mu\text{L}$  were layered over 250  $\mu\text{L}$  of dioctylphthalate:dibutylphthalate (55:45) in 1.5 mL polypropylene centrifuge tubes. To separate tracer bound to membrane protein from unbound, the aliquots were centrifuged at 14000 rpm in an Eppendorf 5415 benchtop centrifuge. After removing the supernatant, the pellets were counted in a gamma counter. Specifically bound cpm were plotted against  $\log [\text{D-Hep-III}]$  concentration, and the data was fit by nonlinear regression to a one-site competition curve using Prism.

**Biodistribution Studies: General.** The laboratory rats and mice were maintained in the animal facilities operated by the Division of Comparative Medicine at Washington University School of Medicine in accordance with the American Association for Accreditation of Laboratory Animal Care (AAALAC) requirements. The radiolabeled D-Hep-III analogues and blocking doses were injected into the tail vein of anesthetized rats and mice. The vehicle was either 0.9% NaCl or 10 mM  $\text{NH}_4\text{OAc}$ , pH 6, each containing 0.1% BSA. Animals were sacrificed by cervical dislocation while under Metofane anesthesia at specific time points postinjection. Organs of interest were excised, counted in a gamma counter, and then weighed to generate the percent injected dose/organ (% ID/organ) and percent injected dose/gram tissue (% ID/g).

**Biodistribution of  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep-III in Male Sprague–Dawley Rats.** Copper-64-TETA-D-Hep-III (5.0  $\mu\text{Ci}/100 \mu\text{L}/\text{rat}$  0.1 M ammonium acetate, 1 mg/mL BSA  $\geq 95\%$  radiochemical purity, specific activity 798 Ci/mmol) and  $^{125}\text{I}$ -D-Hep-III (5.0  $\mu\text{Ci}/100 \mu\text{L}/\text{rat}$ ; water, 1 mg/mL BSA,  $\geq 98\%$  radiochemical purity, specific activity 2000 Ci/mmol) were coinjected into male-Sprague–Dawley rats ( $\sim 500 \text{ g}$ ). The gamma counter windows were adjusted so that none of the cpm from the  $^{125}\text{I}$  contributed to the cpm of  $^{64}\text{Cu}$ . After 6 days ( $> 10$  half-lives,  $^{64}\text{Cu}$ ) the tissues were recounted for  $^{125}\text{I}$ .

**SKOV3 Xenografts in SCID Mice.** One hundred microliter aliquots ( $7.5 \times 10^6$  cells) were implanted sc in the right and left flank of  $\geq 5$  week old female SCID mice. Tumor growth was measured every 3–5 days with calipers. The tumors grew to 500 to 1500  $\text{mm}^3$  within 3–4 weeks. This tumor model was maintained by serial passages of solid tumor out to passage 12 by implantation of two tumor pieces ( $\sim 3 \text{ mm}^3$ ) in the right and left flank of female SCID mice.

**Biodistribution of  $^{64}\text{Cu}$ -TETA-D-Hep-III in SCID Mice Bearing SKOV3 Tumors.** Nine mice bearing xenografted SKOV3 tumors (passage 3) implanted bilaterally on the flank were used at 21 days post-implantation. Five mice received  $^{64}\text{Cu}$ -TETA-D-Hep-III (5.8  $\mu\text{Ci}/100 \mu\text{L}/\text{mouse}$ , 0.1 M ammonium acetate, 1 mg/mL BSA, 540 Ci/mmol). The animals were sacrificed at 1 h post-injection, and tissues were removed and counted in a gamma counter for biodistribution as described above.

**Biodistribution of  $^{125}\text{I}$ -D-Hep-III in SCID Mice Bearing SKOV3 Tumors.** Four SCID mice bearing bilateral SKOV3 xenografts (passage 5) received  $^{125}\text{I}$ -D-Hep-III (1  $\mu\text{Ci}/100 \mu\text{L}/\text{mouse}$ , 0.9% NaCl, 1 mg/mL BSA,  $\geq 2000$  Ci/mmol). Four tumor-bearing mice were coinjected with 1  $\mu\text{Ci}$   $^{125}\text{I}$ -D-Hep-III (1  $\mu\text{Ci}/100 \mu\text{L}/\text{mouse}$ , 0.9% NaCl, 1 mg/mL BSA,  $\geq 2000$  Ci/mmol) and 9  $\mu\text{g}$  D-Hep-III. Three mice were coinjected with 1  $\mu\text{Ci}$   $^{125}\text{I}$ -D-Hep-III (1  $\mu\text{Ci}/100 \mu\text{L}/\text{mouse}$ , 0.9% NaCl, 1 mg/mL BSA,  $\geq 2000$  Ci/mmol) and 1.7  $\mu\text{g}$  D-Hep-III. The animals were sacrificed at 1 h postinjection and biodistribution was determined as described above.

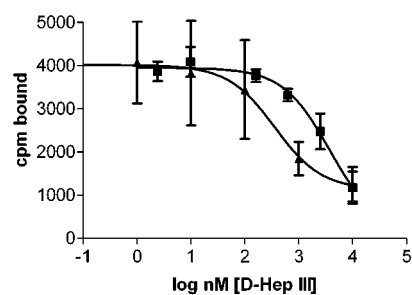
## RESULTS

**Peptide Synthesis.** Following Fmoc solid-phase synthesis of D-Hep-III, the peptide was found to be compositionally correct by Edman degradation sequence analysis. D-Hep-III was isolated by RP-HPLC and further characterized by mass spectrometry. Purities were determined by analytical RP-HPLC, with monitoring both at  $\lambda = 222$  and 280 nm, and were greater than 98%.

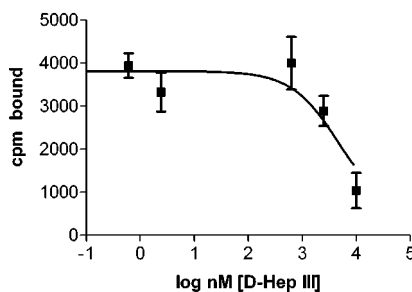
TETA was coupled to D-Hep-III while it was resin-bound. TETA $\cdot$ 4HCl had low solubility in DMF or DMSO; however, neutralization with lithium hydroxide improved its solubility in DMSO. Coupling yields were in the range of 40 to 50% and could not be improved by further coupling reactions. RP-HPLC analysis of the unpurified cleavage mixtures showed that D-Hep-III and TETA-D-Hep-III eluted closely, even with a relatively shallow gradient, which resulted in numerous fractions from the semipreparative purification to be mixtures of both peptides in varying amounts. TETA-D-Hep III was recovered in 20% yield and D-Hep III in 16% yield.

**Radiochemistry.** Radiochemical yields of  $^{64}\text{Cu}$ -TETA-D-Hep-III ranged from 40 to 90% and radiochemical purity  $\geq 95\%$  and specific activities ranged from 500 to 2000 Ci/mmol. D-Hep-III contains two tyrosine residues, each of which has the potential for radioiodination. RP-HPLC of the purified  $^{125}\text{I}$ -D-Hep-III utilizing a shallow gradient showed a small shoulder in the trace for the major component, suggesting a mixture of two mono-radioiodinated species. Therefore, the  $^{125}\text{I}$ -D-Hep-III used in vitro assays and in vivo studies was a probable mixture of two mono-radioiodinated peptides. Whether or not this affected the binding affinity of the peptide is unknown. RP-HPLC investigation of a mixture of D-Hep-III and chloramine-T showed no degradation of the peptide. Separation of the  $^{125}\text{I}$ -D-Hep-III from D-Hep-III yielded specific activities of  $\geq 2000$  Ci/mmol.

**Serum Stability.** The stability of  $^{125}\text{I}$ -D-Hep-III and  $^{64}\text{Cu}$ -TETA-D-Hep-III were monitored by RP-HPLC and no changes in retention time or the appearance of new radiolabeled components were observed within 3 h which is indicative of the stability of the radiolabeled D-Hep III analogues. Although RP-HPLC is sensitive to changes in structure, such as cleavage of the peptide bonds, it may not have revealed whether either of the radiolabeled D-Hep-III analogues bound serum proteins. Furthermore, in cases where there were mixtures of the radiolabeled



**Figure 3.** Heterologous competitive binding of  $^{125}\text{I}$ -D-Hep-III and D-Hep-III to membranes from SKOV3 cultured cells ( $\blacktriangle$ ,  $\text{IC}_{50} = 360$  nM) and SKOV3 xenografts, passage 10 ( $\blacksquare$ ,  $\text{IC}_{50} = 3700$  nM).



**Figure 4.** Heterologous competitive binding between  $^{64}\text{Cu}$ -TETA-D-Hep-III and D-Hep-III on membranes from SKOV3 cultured cells ( $\text{IC}_{50} = 4700$  nM).

**Table 1. Biodistribution of Radiolabeled D-Hep-III Analogues in Normal Male Sprague–Dawley Rats at 4 h Postinjection. The Data Are Reported as %ID/Gram  $\pm$  Standard Deviation**

organ	$^{64}\text{Cu}$ -TETA-D-Hep-III ( $n = 5$ )	$^{125}\text{I}$ -D-Hep-III ( $n = 5$ )
blood	0.03 $\pm$ 0.002	0.06 $\pm$ 0.01
lung	0.06 $\pm$ 0.04	0.13 $\pm$ 0.02
liver	0.26 $\pm$ 0.03	0.61 $\pm$ 0.15
kidney <sup>a</sup>	2.98 $\pm$ 0.48	27.3 $\pm$ 2.61
muscle	0.01 $\pm$ 0.001	0.02 $\pm$ 0.002
fat	0.01 $\pm$ 0.001	0.01 $\pm$ 0.003
heart	0.02 $\pm$ 0.003	0.04 $\pm$ 0.006
bone	0.03 $\pm$ 0.004	0.10 $\pm$ 0.02
thyroid	0.05 $\pm$ 0.02	3.67 $\pm$ 1.33
pancreas	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01
stomach	0.08 $\pm$ 0.05	0.13 $\pm$ 0.01
intestines	0.55 $\pm$ 0.26	0.22 $\pm$ 0.04

<sup>a</sup> Single kidney.

D-Hep-III analogues and D-Hep-III at a concentration equal to or in excess of 10  $\mu\text{M}$ , a SEC HPLC column especially designed for low molecular weight peptides showed there was no aggregation of D-Hep-III and its radiolabeled analogues. This is an important control for biodistributions where  $^{125}\text{I}$ -D-Hep-III was coinjected with D-Hep-III (25  $\mu\text{M}$ ) as well as for the competitive binding assays where  $^{125}\text{I}$ -D-Hep-III analogues were combined with D-Hep-III (10  $\mu\text{M}$ ). D-Hep-III at 280  $\mu\text{M}$  did not cause the aggregation of  $^{125}\text{I}$ -D-Hep-III either in aqueous buffer or rat serum.

**Heterologous Competitive Binding Assays between Radiolabeled D-Hep III Analogues and D-Hep III.** The heterologous competitive binding assay between  $^{125}\text{I}$ -D-Hep-III or  $^{64}\text{Cu}$ -TETA-D-Hep-III and D-Hep-III on membranes from SKOV3 cultured cells and xenografts (Figures 3 and 4, Table 3) show that both the D-Hep-III and the radiotracers are specifically bound to cell surface receptors. The 95% confidence intervals (95% CI) for the  $\text{IC}_{50}$  values are large in cases where the concentration of D-Hep-III was not great enough to completely block tracer

**Table 2. Biodistribution of  $^{125}\text{I}$ -D-Hep-III in SCID Mice Bearing sc Bilateral SKOV3 Tumors at 1 h Postinjection. The Data Are Reported as %ID/Gram  $\pm$  Standard Deviation**

organ	$^{64}\text{Cu}$ -TETA-D-Hep-III ( <i>n</i> = 5)	$^{125}\text{I}$ -D-Hep-III ( <i>n</i> = 4)	$^{125}\text{I}$ -D-Hep-III + 1.7 $\mu\text{g}$ D-Hep-III ( <i>n</i> = 4)	$^{125}\text{I}$ -D-Hep-III + 9 $\mu\text{g}$ D-Hep-III ( <i>n</i> = 3)
blood	0.53 $\pm$ 0.14	1.44 $\pm$ 0.526	1.64 $\pm$ 0.478	1.95 $\pm$ 0.255
lung	1.13 $\pm$ 0.10	2.10 $\pm$ 0.199	2.55 $\pm$ 0.338	3.30 $\pm$ 0.528
liver	3.37 $\pm$ 0.97	2.87 $\pm$ 0.340	5.12 $\pm$ 1.36	9.79 $\pm$ 2.63
spleen	1.82 $\pm$ 1.07	1.59 $\pm$ 0.251	2.59 $\pm$ 0.884	4.86 $\pm$ 1.62
kidney <sup>a</sup>	29.7 $\pm$ 2.82	244 $\pm$ 17	224 $\pm$ 54	237 $\pm$ 45
muscle	0.19 $\pm$ 0.03	0.66 $\pm$ 0.25	0.63 $\pm$ 0.089	0.69 $\pm$ 0.13
fat	0.15 $\pm$ 0.07	0.20 $\pm$ 0.06	0.50 $\pm$ 0.25	0.31 $\pm$ 0.19
bone	0.43 $\pm$ 0.13	0.72 $\pm$ 0.02	0.81 $\pm$ 0.06	0.91 $\pm$ 0.094
ovaries	0.33 $\pm$ 0.18	0.75 $\pm$ 0.19	1.11 $\pm$ 0.41	0.83 $\pm$ 0.25
intestines	0.46 $\pm$ 0.10	1.11 $\pm$ 0.10	1.18 $\pm$ 0.17	2.14 $\pm$ 0.36
tumor <sup>b</sup>	0.55 $\pm$ 0.05	1.25 $\pm$ 0.12	1.93 $\pm$ 0.53	1.72 $\pm$ 0.20

<sup>a</sup> Single kidney. <sup>b</sup> Average of right and left tumors.

**Table 3. IC<sub>50</sub> Values for D-Hep-III in Heterologous Binding Assays**

radioligand	membrane source	IC <sub>50</sub> , nM	95% CI, nM
$^{125}\text{I}$ -D-Hep-III	SKOV3 cultured cells	360	210–614
$^{125}\text{I}$ -D-Hep-III	SKOV3 xenograft passage 10	3700	1682–8061
$^{64}\text{Cu}$ -TETA-D-Hep-III	SKOV3 cultured cells	4700	1402–15620

binding. The concentrations of the radioligands in the binding assays did not exceed 100 pM.

**Biodistributions of  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep III in Normal Rats.** A biodistribution of  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep-III in male Sprague–Dawley rats showed that both radiolabeled analogues cleared from the circulation by 4 h ( $\leq 0.06 \pm 0.01\%$  ID/g) with the highest accumulation for each in the kidney (Table 1).  $^{125}\text{I}$ -D-Hep-III ( $27.3 \pm 2.61\%$  ID/g) accumulated 9-fold more ( $P < 0.01$ ) in a single kidney than did  $^{64}\text{Cu}$ -TETA-D-Hep-III ( $2.98 \pm 0.48\%$  ID/g). Accumulation in the other organs was low except for the thyroid accumulation in the case of  $^{125}\text{I}$ -D-Hep-III ( $3.67 \pm 1.33\%$  ID/g).

**Biodistribution of  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep-III in Tumor Bearing SCID Mice.**  $^{64}\text{Cu}$ -TETA-D-Hep-III was evaluated in SCID mice bearing bilateral SKOV3 human ovarian tumors (Table 2).  $^{64}\text{Cu}$ -TETA-D-Hep-III cleared rapidly from the blood ( $0.53 \pm 0.14\%$  ID/g) but demonstrated low tumor accumulation ( $0.55\% \pm 0.055\%$  ID/g) at 1 h. High accumulation of  $^{64}\text{Cu}$ -TETA-D-Hep III in the kidney was observed ( $29.7 \pm 2.82\%$  ID/g).

$^{125}\text{I}$ -D-Hep-III was evaluated in SCID mice bearing bilateral sc SKOV3 xenografts with and without competitive doses of 1.7 and 9  $\mu\text{g}$  of D-Hep III. Tumor accumulation was low ( $1.25 \pm 0.12\%$ ) and no dose dependent blocking of tumor accumulation was observed ( $\geq 1.72 \pm 0.20\%$  ID/g) at the doses administered ( $P > 0.05$ ). Kidney accumulation after 1 h was very high ( $244 \pm 17\%$  ID/g) but dose-dependent blocking was also not observed ( $\geq 237 \pm 45\%$  ID/g,  $P > 0.05$ ). However, increases in liver (3.4-fold,  $P < 0.05$ ) and spleen (3.1-fold,  $P < 0.01$ ) accumulation of the radioiodinated peptide were observed with coinjection of D-Hep-III (9  $\mu\text{g}$ ), but concomitant decreases in accumulation in other tissues were not apparent.

**Binding of  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  to Membranes from SKOV3 Cells and Xenografted Tumor.** One possible explanation for the low accumulation of either of the radiolabeled D-Hep-III analogues in tumors may be down-regulation of  $\alpha 3\beta 1$  expression. Therefore, binding assays were carried out with a radioiodinated monoclonal antibody specific for the  $\alpha 3$  subunit of the targeted integrin. Equal amounts of  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  at the same specific activity were added to varying amounts of membrane protein. The reactive fraction of  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  was

relatively low,  $9.4 \pm 1.3\%$ , for xenografted membranes and  $8.2 \pm 0.7\%$  for SKOV3 cells grown in culture. Moreover, the amount of membrane protein required to bind one-half the tracer was  $12.3 \pm 4.7 \mu\text{g}$  for xenografted membranes and  $11.7 \pm 3.0 \mu\text{g}$  for SKOV3 membranes (Figure 1). This indicates that  $\alpha 3\beta 1$  is present in membranes from SKOV3 xenografts, as well as from SKOV3 membranes of cells grown in culture. Furthermore, since  $\alpha 3\beta 1$  is proportional to the amount of membrane protein, and since the same amount of membrane protein from each receptor source will bind one-half the  $^{125}\text{I}$ -Mab-anti- $\alpha 3$ , it is concluded that the density of  $\alpha 3\beta 1$  is equivalent between the two preparations.

In the homologous competitive binding assay between the  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  and Mab anti- $\alpha 3$ , all of the radioligand bound at concentrations of competitive Mab of 0.01, 0.02, 0.1, and 0.6 nM because of the low immunoreactivity of the  $^{125}\text{I}$ -Mab (Figure 2). Insufficient data points remained for Scatchard analysis. Therefore, the minimum density of  $\alpha 3\beta 1$  integrin can be estimated from the amount of  $^{125}\text{I}$ -Mab specifically bound in the preparations containing 14 and 72 nM Mab, where 1400 and 970 cpm bound, respectively. Using the following conversion factors, values of 2700 and 9800 pM total bound were obtained: 33  $\mu\text{Ci}/\mu\text{g}$  Mab, 150 KDa, 0.6 dpm/cpm,  $2.22 \times 10(6)$  dpm/ $\mu\text{Ci}$ ,  $1 \times 10(6)$  pmol/ $\mu\text{mol}$ . With 75  $\mu\text{L}$  aliquot containing 40  $\mu\text{g}$  of protein, a log-dose dependent displacement of  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  by Mab anti  $\alpha 3$  was observed (Figure 2; IC<sub>50</sub> = 5.3 nM, 95% confidence interval 3.2 to 8.7 nM).

## DISCUSSION

Radiolabeled derivatives of peptide hormones targeted to cell surface receptors, such as vasoactive intestinal peptide (VIP) (38), somatostatin (39), and melanotropin stimulating hormone ( $\alpha$ MSH) (40), have demonstrated receptor mediated accumulation of the radiotracer at tumor sites in animal models or humans and therefore have potential as tumor imaging agents. Besides peptide hormones, another class of peptides derived from ECM proteins has been investigated as tumor imaging agents (11–14). D-Hep-III, derived from the ECM protein, type IV collagen, was investigated as an imaging agent for the  $\alpha 3\beta 1$  integrin.

The tetraazamacrocyclic TETA was coupled to the N-terminal amine of D-Hep-III while it was resin bound,

as structure–activity studies indicated that the *N*-terminal region of D-Hep-III does not significantly impact receptor binding (23). TETA-D-Hep-III and D-Hep-III were radiolabeled with  $^{64}\text{Cu}$  and  $^{125}\text{I}$ , respectively, for evaluation as a receptor specific imaging agents.

To establish that both  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep-III bound specifically to  $\alpha 3\beta 1$  positive tissues, heterologous competitive binding assays between D-Hep-III and  $^{125}\text{I}$ -D-Hep-III or  $^{64}\text{Cu}$ -TETA-D-Hep-III were carried out on membranes isolated from SKOV3 cultured cells and SKOV3 xenografts. Both  $^{125}\text{I}$ -D-Hep-III and  $^{64}\text{Cu}$ -TETA-D-Hep-III were specifically bound to cell surface receptors on membranes originating from the SKOV3 ovarian tumor cell line. However, the  $\text{IC}_{50}$  value for D-Hep-III was 10-fold higher on membranes isolated from SKOV3 xenografts ( $\text{IC}_{50} = 3700$  nM) than from those isolated from cultured cells ( $\text{IC}_{50} = 360$  nM). The concentration of  $^{125}\text{I}$ -D-Hep-III was 50 and 26 pM in the experiments with SKOV3 membranes and xenografts, respectively. According to the Cheng-Prusoff equation [ $K_D = \text{IC}_{50} / (1 + [\text{radioligand}] / K_D)$ ], where  $K_D$  = affinity of the competitor for the receptor;  $K_D$  = affinity of the radioligand for the receptor;  $\text{IC}_{50}$  = concentration of competitive ligand required to displace one-half the radioligand], a 2-fold decrease in tracer concentration cannot account for a 10-fold increase in  $\text{IC}_{50}$  unless either the  $K_D$  of D-Hep-III or  $^{125}\text{I}$ -D-Hep-III or both were different on membranes isolated from SKOV3 cells or xenografts. This indicates that either or both of the ligands were interacting with different receptors between the two preparations. The higher affinity interaction observed on membranes from SKOV3 cells may have been with an integrin other than  $\alpha 3\beta 1$  since the Hep III sequence is contained within a region of type IV collagen that is known to bind both the  $\alpha 2\beta 1$  and  $\alpha 3\beta 1$  integrins (ref 7 and references therein). Furthermore, L-Hep-III (i.e., all residues except glycine are in the *L*-configuration) binds both the  $\alpha 2\beta 1$  and  $\alpha 3\beta 1$  integrins from human mesangial (kidney) cells (41).

The radioligands were also evaluated in normal and tumor-bearing animal models. The biodistribution of  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep-III in normal rats resulted in rapid blood clearance and low accumulation in most of the tissues examined. The highest accumulation was in the kidney, which is known to highly express  $\alpha 3\beta 1$  (16, 17, 37). While kidney uptake could have been receptor-mediated, it is important to note that renal clearance is also the major excretory pathway of the radiotracers. However, coadministration of up to 9  $\mu\text{g}$  doses of D-Hep-III in SCID mice resulted in no observed decreases in kidney accumulation. The observed accumulation of the tracers in the kidneys could simply be a result of clearance although other radiolabeled peptides, such as  $^{64}\text{Cu}$ - and  $^{125}\text{I}$ -labeled somatostatin analogues, do not accumulate in the kidneys to such a great extent (42, 43). Alternatively, the 9  $\mu\text{g}$  blocking dose may not have been sufficient to block the potentially large number of integrin receptors present in the kidneys.

To determine if  $^{64}\text{Cu}$ -TETA-D-Hep-III would accumulate preferentially in  $\alpha 3\beta 1$  rich tumor tissues, a biodistribution of  $^{64}\text{Cu}$ -TETA-D-Hep-III was obtained in SCID mice bearing bilateral SKOV3 xenografts. SKOV3 cells strongly express  $\alpha 3\beta 1$  in culture (44) but  $\alpha 3\beta 1$  density of SKOV3 or any other xenografts of cultured cells known to express  $\alpha 3\beta 1$  has not been reported. To determine if SKOV3 xenograft accumulation was receptor mediated for  $^{125}\text{I}$ -D-Hep-III, a blocking dose of D-Hep-III (1.7 and 9  $\mu\text{g}$ ) was coinjected with  $^{125}\text{I}$ -D-Hep-III. Accumulation of the radioactivity in the liver and spleen increased with

increasing doses of D-Hep-III. However, the accumulation of the radioactivity in the tumor was low and not significantly different than that of the blood or tumors from animals that received the blocking doses. The accumulation of radiolabeled D-Hep-III analogues in the tumor may not be receptor-mediated; however, if the  $\alpha 3\beta 1$  expression is dense, higher blocking doses may be needed to displace the tracers.

Low tumor accumulation of ECM-derived peptides had been previously observed with peptides derived from other ECM proteins. Peptides incorporating the sequence YIGSR, a fragment proposed to bind to laminin receptors, were radiolabeled with  $^{131}\text{I}$  and investigated in a tumor bearing animal model (12). In mice bearing Lewis lung carcinoma tumors,  $^{131}\text{I}$ -YIGSR uptake in the tumor, while low ( $1.15 \pm 0.28\%$  ID/gram, 24 h), was much higher than blood ( $0.014 \pm 0.007\%$  ID/gram, 24 h). However, this ratio was achieved at 24 h postinjection. In contrast, previous work in mice bearing xenografted melanoma showed that cyclic peptides incorporating the RGD sequence [cyclo-(RGD-D-YV)] and radioiodinated with  $^{125}\text{I}$  displayed low tumor accumulation ( $1.30 \pm 0.13\%$  ID/gram, 1 h) (11). In these studies, clearance from the blood was rapid ( $0.17 \pm 0.02\%$  ID/gram, 1 h), and a tumor-to-blood ratio of  $\sim 8$  was achieved. In our study, the evaluation of the radiolabeled D-Hep-III analogues in tumor-bearing animals extended to only 1 h, and this time interval may not have been long enough to reach optimal tumor-to-blood ratios indicative of receptor-mediated accumulation.

The primary goal of this study was to determine whether  $^{125}\text{I}$ -D-Hep-III or  $^{64}\text{Cu}$ -TETA-D-Hep-III would localize in a receptor specific manner in  $\alpha 3\beta 1$  rich tissues, and the results suggest that the observed accumulation was not receptor-mediated. Receptor down-regulation or degradation of the radiotracers are two factors that could have adversely affected receptor-mediated tumor accumulation. However, the results from the binding assay at infinite  $\alpha 3\beta 1$  excess to  $^{125}\text{I}$ -Mab-anti- $\alpha 3$  indicate that  $\alpha 3\beta 1$  density is equivalent between SKOV3 cultured cells and SKOV3 xenografts. Furthermore, the minimum density of  $\alpha 3\beta 1$  of 5 to 18 pmol/mg protein is a relatively high value since xenografted somatostatin-positive tumors routinely express 300 fmol/mg protein (26). Therefore,  $\alpha 3\beta 1$  down-regulation or sparse density cannot explain the lack of receptor mediated accumulation. Moreover, both  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep-III are stable in serum long enough to reach their targets.

It was previously shown that a monoclonal antibody, GA17 ( $K_D = \sim 2$  nM), directed toward  $\alpha 3\beta 1$  and radiolabeled with either  $^{125}\text{I}$  or  $^{111}\text{In}$ , selectively localized to human tumor xenografts which expressed  $\alpha 3\beta 1$  in vitro, thus demonstrating  $\alpha 3\beta 1$  as a viable target for high affinity radiolabeled ligands (45). Although the epitope of GA17 is unknown, Mab binding is not necessarily dependent on  $\alpha 3\beta 1$  conformation or competition from endogenous ligand, two possibilities that would explain the low tumor accumulation of either  $^{125}\text{I}$ -D-Hep-III or  $^{64}\text{Cu}$ -TETA-D-Hep-III in the xenograft.

Another possible explanation for the low accumulation of the  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep-III is low affinity for the receptor. The  $K_D$  of radiolabeled D-Hep-III analogues would have to approach the concentration of the tracer in the competitive binding experiments, which ranged from 26 to 100 pM, for  $\text{IC}_{50}$  values of D-Hep-III to differ greatly from the  $K_D$  of D-Hep-III. The  $\text{IC}_{50} = 3700$  nM for D-Hep-III on membranes from SKOV3 xenografts suggests a weak affinity for D-Hep-III. Furthermore,  $\alpha 3\beta 1$  is known to be recruited to focal contacts of cells adhering to type IV collagen after the collagen is

bound by integrins ( $\alpha 2\beta 1$  and  $\alpha 1\beta 1$ ) with demonstrated high affinity for type IV collagen, suggesting that  $\alpha 3\beta 1$  has a weak affinity for type IV collagen (7). Successful radiotracers that have accumulated in their target tissue by a receptor-mediated process have demonstrated affinity in the low nanomolar region. Micromolar affinity is likely to be insufficient for receptor-mediated uptake in target tissue (46) and would explain the low accumulation of  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep-III.

#### CONCLUSIONS

Radiolabeled TETA-D-Hep-III and D-Hep-III were evaluated as imaging agents for human neoplasms where the  $\alpha 3\beta 1$  integrin is strongly expressed. On tissues known to express  $\alpha 3\beta 1$  integrin, binding of both of  $^{64}\text{Cu}$ -TETA-D-Hep-III and  $^{125}\text{I}$ -D-Hep-III was inhibited in a log-dose dependent manner on membranes from cultured SKOV3 cells and SKOV3 xenografts, demonstrating that these ligands are bound to cell surface receptors.  $\text{IC}_{50}$  values of D-Hep-III in the low micromolar region suggest a weak affinity of D-Hep-III and radiolabeled analogues of D-Hep-III for  $\alpha 3\beta 1$ . In the in vivo tumor model used in this study low accumulation of both radiotracers was observed within the xenograft at the time points investigated despite the relatively dense expression of  $\alpha 3\beta 1$ . The combination of the results obtained from the in vitro and in vivo data strongly suggest that peptides of this class targeted to the  $\alpha 3\beta 1$  would not be suitable as in vivo imaging agents in humans.

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