Multivalent interactions are frequently used to enhance ligand-receptor binding affinity. In this study, mono-, di- and trimeric Ala-Val-Thr-Gly-Arg-Gly-Ser-Tyr (AVTGRGDSY) peptides, labeled with $^{125}$I or Cy5.5, were compared in vitro and in vivo. Using human embryonic kidney HEK293 (naturally $\alpha_\beta$-positive and $\beta_\beta$-negative), HEK293($\beta_\beta$)-transfected and $\alpha_\beta$-$\beta_\beta$-negative), HEK293($\beta_\beta$) ($\beta_\beta$-transfected and strongly $\alpha_\beta$-$\beta_\beta$-positive), and human glioblastoma U87MG (naturally $\alpha_\beta$-$\beta_\beta$-positive) cell lines we evaluated their binding affinity and specificity. In vitro, the monomeric AVTGRGDSY showed specific binding to both HEK293($\beta_\beta$) and HEK293($\beta_\beta$) cells. Multimerization resulted in no change toward HEK293 cells, diminished binding with HEK293($\beta_\beta$) cells, but substantially enhanced binding with $\alpha_\beta$-$\beta_\beta$-positive HEK293($\beta_\beta$) and U87MG cells. Moreover, multimeric AVTGRGDSY peptides were found to be nearly comparable to the same molar concentration of a well-known $\alpha_\beta$-$\beta_\beta$-specific cyclo(RGDfV) (c(RGDfV)) peptide in specificity and affinity for targeting $\alpha_\beta$ integrin. Non-invasive in vivo optical imaging demonstrated that as compared to its monomeric analogue, the Cy5.5-labeled dimeric AVTGRGDSY peptide produced markedly enhanced tumor-to-background contrast in HEK293($\beta_\beta$) tumor-bearing mice than in HEK293($\beta_\beta$) tumor-bearing mice. In conclusion, the present study showed the difference of monomer and multimeric linear Arg-Gly-Asp (RGD)-containing compound in integrin selectivity and affinity. Our data provide useful information for the design of novel RGD peptides.

Key words linear Arg-Gly-Asp peptide; multimerization; integrin $\alpha_\beta$-$\beta_\beta$; fibronectin; divalent cation

Integrins are a family of transmembrane glycoproteins playing important roles in tumor invasion, metastasis, and neovascularization by mediating cell-extracellular matrix (ECM) interaction and initiating intracellular signaling. Integrin consists of 2 noncovalently associated subunits ($\alpha$ and $\beta$), and currently 18 $\alpha$ and 8 $\beta$ subunits have been identified in humans, which are able to form 24 distinct integrin heterodimers. Many integrins are known to recognize the tripeptide sequence Arg-Gly-Asp (RGD), which has been found in a wide variety of ECM proteins such as fibronectin (FN), vitronectin (VN), osteopontin, thrombospondin, fibrinogen, von Willebrand factor, collagens and laminin. Recognition and binding between integrin receptor and RGD ligand may depend on intrinsic integrin affinity (via conformational changes) and spatial distribution of the ligands, which can be regulated by microenvironmental divalent cations and RGD-neighbouring sequence, respectively. Various RGD-containing peptides have been increasingly developed for adapting to versatile applications including tumor imaging and therapy, drug delivery vector, targeted gene transfer, and biomaterial or tissue engineering. FN is a very important RGD-containing ECM protein, involved in malignant progression and metastasis, and used as a target for tumor therapy. FN has been found to serve as a ligand for many integrins including $\alpha_\beta$-$\beta_\beta$, $\alpha_\beta$-$\beta_\beta$, $\alpha_\beta$-$\beta_\beta$, $\alpha_\beta$-$\beta_\beta$, $\alpha_\beta$-$\beta_\beta$, $\alpha_\beta$-$\beta_\beta$, and $\alpha_\beta$-$\beta_\beta$ via its RGD motif. Multivalent interactions are frequently used in nature to increase the affinity of weak ligand-receptor interactions, and multimerization has become a principal strategy for the development of cyclic RGD peptides (cRGD) for targeting integrin $\alpha_\beta$-$\beta_\beta$. This protein is highly expressed on activated endothelial cells during angiogenesis, a requirement for tumor growth and metastasis, and also frequently overexpressed on many types of tumor cells. Its overexpression has been known to correlate well with tumor progression, invasion and metastasis. In this study, we evaluated the effect of multimerization of a linear RGD peptide, Ala-Val-Thr-Gly-Arg-Gly-Ser-Tyr (AVTGRGDSY), on the binding affinity and specificity of integrin $\alpha_\beta$-$\beta_\beta$ or $\beta_\beta$, a subunit that forms complexes with various $\alpha$ integrin subunits, using $\alpha_\beta$-$\beta_\beta$-positive cells and $\alpha_\beta$-$\beta_\beta$-negative/$\beta_\beta$-overexpressing cells. AVTGRGDSY was derived from the amino acid sequence AVTGRGDSYP in FN, with “P” being replaced by the tyrosine residue “Y” for $^{125}$I-labeling. Here we describe the synthesis of mono-, di- and trimeric AVTGRGDSY peptides, the radio- or fluorescence dye-labeling of these peptides, and evaluation of their biological characteristics both in vitro and in vivo.

MATERIALS AND METHODS

Synthesis of AVTGRGDSY Peptides Monomeric AVTGRGDSY peptide (RGD-monomer) was synthesized using peptide synthesizer by standard 9-fluorenylmethoxycarbonyl (Fmoc) method. The monomer was dissolved in dimethyl sulfoxide (DMSO) and pH was adjusted to 9 with triethylamine. Cross-linking reactions were performed using disuccinimidyl glutarate (DSG) for obtaining a dimeric-AVTGRGDSY peptide.
GRGDSY (RGD-dimer) and tris-succinimidyl aminotriacetate (TSAT) for a trimeric AVTGGRGDSY (RGD-trimer). DSG and TSAT were purchased from Pierce (Rockford, IL, U.S.A.), and the monomer was reacted with 1/2 molar ratio of DSG or with 1/3 molar ratio of TSAT for 16 h at room temperature (RT). The products were purified with HPLC and freeze-dried. Mass analysis showed the results: RGD-monomer (Fig. 1A) [calculated: 925.4379; observed: 925.40]; RGD-dimer (Fig. 1A) [calculated: 1946.1; observed: 1946.19]; RGD-trimer (Fig. 1A) [calculated: 2912.2; observed: 2912.23].

Synthesis of Cy5.5-AVTGRGDSY Peptides

RGD-monomer-resin was synthesized using peptide synthesizer by standard Fmoc method. Boc-Glu-COOH (0.025 mmol) (Boc: t-Butoxycarbonyl) in 0.1 mmol HBTU/HOBT solution (HBTU: 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate; HOBT: 1-Hydroxybenzotriazole) was incubated with four equivalents of RGD-monomer in 0.1 mmol HBTU/HOBT solution for 16 h to obtain an amino-containing RGD-dimer (Glu(AVTGRGDSY)-AVTGGRGDSY). This was followed by cleavage of peptide from resin with TFA (trifluoroacetic acid) cocktail solution (125 ml TFA, 0.25 ml H2O, 0.375 g phenol, 0.125 ml ethanedithiol and 0.25 ml thioanisole). The crude product was purified by RP (reversed phase)-HPLC. RGD-monomer and amino-containing RGD-dimer were conjugated with 1 mg Cy5.5 mono NHS (N-hydroxysuccinimide) ester (GE Healthcare UK Ltd., Buckinghamshire, U.K.), respectively, per manufacturer’s instructions. Briefly, 1 mg peptide in 400 μl DMSO was added to two equivalents of Cy5.5 in 100 μl DMSO and incubated for 16 h to yield Cy5.5-labeled peptide that was purified with RP-HPLC and freeze-dried. Mass analysis showed the results: Cy5.5-RGD-monomer (Fig. 1A) [calculated: 1824.0; observed: 1825.56]; Cy5.5-RGD-dimer (Fig. 1A) [calculated: 2860.1; observed: 2860.33].

All RGD peptides and Cy5.5-RGD peptide conjugates, as mentioned above, were purchased from Scrum Inc. (Tokyo, Japan) by consignment synthesis. HPLC purities of all compounds were >95%.

Cells and Culture

Human embryonic kidney HEK293 (naturally αv-positive and β3-negative) and human glioblastoma U87MG (naturally αvβ3-positive) cell lines were purchased from American Type Culture Collection (Manassas, VA, U.S.A.) and cultured in Dulbecco’s modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin. HEK293(β3) and HEK293(β1) cells (kindly provided by J.-F. Gourvest, Aventis, France) are HEK293 stable transfectants of human integrin β1 and β3 subunits, respectively, and cultured in DMEM+GlutaMAX™ (Gibco/Invitrogen, Grand Island, NY, U.S.A.) with the same supplements as mentioned above and 700 μg/ml geneticin (G418 sulfate, Roche Diagnostics GmbH, Mannheim, Germany). HEK293(β3) cells are strongly αvβ3-positive, while HEK293(β1) cells are αvβ3-negative but overexpress β1. All cell lines were cultured at 37 °C in a humidified 95% air/5% CO2 atmosphere.

125I-Radiolabeling

RGD-monomer, -dimer and -trimer were labeled with iodine-125 (125I) using the chloramine-T method. The peptide (4.55 μl, 4.55×10-11 mole) was added to a 1.5 ml tube containing 23.45 μl of phosphate buffer (0.3 M PB, pH 7.5). One μl of Na125I (3.7 GBq/ml, 4.55×10-11 mole, PerkinElmer Japan Co., Ltd., Yokohama, Japan) (peptide :125I 1 : 1 molar ratio) was added to the tube, followed by 1 μl of the oxidant chloramine-T (2 mg/ml in H2O). In addition, 1 : 2 and 1 : 3 peptide :125I molar ratios were also tested for labeling of dimer and trimer, respectively. The reaction mixture was gently mixed and incubated at RT for 5 min, and radioiodination was then stopped by addition of

Fig. 1. (A) Structural Formula of RGD-Monomer, RGD-Dimer, RGD-Trimer, Cy5.5-RGD-Monomer and Cy5.5-RGD-Dimer and (B) a Representative of Gel Filtration of Radioiodinated Linear RGD Peptides

The RGD-monomer was labeled with 125I by the chloramine-T method. It was then purified on a column (0.7×15 cm) of Sephadex G-15, with fractions of 160 μl each being collected. Aliquots of 1 μl of each fraction were measured for radioactivity.
5 μl of reductant, Na₂S₂O₃ (1 mM in H₂O). An aliquot (1 μl) of the mixture was retained for counting the radioactivity employed, and the rest was used for purification by size exclusion chromatography using Sephadex G-15 media (GE Healthcare Bio-Sciences, Uppsala, Sweden) packed into a glass Econo-Column column (0.7×15 cm, 6 ml, Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.) with phosphate-buffered saline (PBS, pH 7.4) as elution buffer. Fractions (160 μl/fraction) were automatically collected, and 1-μl aliquots from each fraction were counted for radioactivity. An elution profile of radioactivity against the fraction number was plotted (Fig. 1B), and the fractions containing radiiodinated peptide were identified, pooled and stored at 4 °C for experiments. Throughout the present study, radioactivity (cpm) was measured using an auto-well γ-counter (Aloka ARC370M, Tokyo, Japan).

**Cell-Binding Assay** Binding assay was performed in a similar manner as described in the literature. In brief, cells were harvested with trypsin/ethylenediaminetetraacetic acid (EDTA), rinsed with Hank’s Balanced Salt Solution (HBSS), and resuspended in binding buffer (HBSS, 25 mM N-(2-hydroxyethyl)pipеразинуN’-2-ethanesulfonic acid (HEPES), 0.1% bovine serum albumin (BSA)) containing divalent cations as indicated. Each 1 ml of cell suspension (2.5×10⁶ cells) was then transferred into 1.5 ml tubes, followed by addition of 125I-labeled RGD-monomer, -dimer or -trimer (60000 cpm/10 μl), and incubated for 1 h on ice. After rinsing with ice-cold PBS, the supernatant was aspirated and the radioactivity bound to cells was counted. The purpose of using low temperature is to keep cells in a low metabolic state for minimizing endocytosis and exocytosis. The relative cell-binding ratio was defined as the % of cell-bound radioactivity to the total radioactivity added.

For competitive inhibition assays, the radioactivity bound to cells was measured after co-incubation of radiolabeled RGD peptides with increasing concentrations of unlabeled corresponding peptides or cyclo(RGDfV) (c(RGDfV), a highly effective α₅β₃ antagonist (Peptide Institute, Inc., Osaka, Japan)).

**Cell Adhesion Assay** The InnoCytetm Cell Adhesion Microplate Assay Kit (EMD Biosciences Inc., La Jolla, CA, U.S.A.) was used for determining the inhibitory effects of RGD-monomer, -dimer and -trimer as well as c(RGDfV) on HEK293(β3) cell attachment to FN or VN. The assay was performed according to the manufacturer’s instructions. In brief, HEK293(β3) cells were harvested and resuspended in binding buffer containing 1 mM Mn²⁺ at a density of 500000 cells/ml. The cell suspension was incubated with these peptides (1.6 μM), respectively, for 20 min at 37 °C, and then each 100 μl of the reaction mixture was added to the FN- or VN-coated 96-well plates and incubated for 1 h at 37 °C. After removal of the binding buffer and gentle washing with PBS, fluorescent dye calcein-AM solution was added and incubated for 1 h at 37 °C. The relative cell attachment was determined by measuring the fluorescence of the samples by microplate reader at excitation wavelength of 485 nm and emission wavelength of 520 nm.

**Confocal Microscopy** HEK293(β3) and HEK293(β3) cells were grown on Lab-Tek™ chambered coverglass (Nunc Thermo Fisher Scientific, Roskilde, Denmark). The culture medium was replaced with ice-cold binding buffer, and the cells were incubated for 1 h at 4 °C with 1 μM Cy5.5-RGD-monomer or -dimer in the absence of divalent cation (HEK293(β3)) or presence of Mn²⁺ or Ca²⁺/Mg²⁺ (1 mM for each cation) (HEK293(β3)). Afterwards, the cells were rinsed with ice-cold PBS and observed by Olympus Fluoview™ FV1000 confocal laser scanning microscopy. Cy5.5 fluorescence was detected using an UPlanSApo 20× objective lens and HeNe (R) (633 nm) excitation and a variable barrier filter set for Cy5.5 acquisition. Brightfield images were simultaneously acquired. In Vivo Fluorescence Imaging Animal procedures were approved by the pertinent committee of our institution and performed in compliance with national and institutional rules for the conduct of animal experimentation. Tumor xenografts were established by subcutaneous (s.c.) injection of 10×10⁶ HEK293(β3) or HEK293(β3) cells into the right flank of the female athymic nude mice (BALB/c Jcl-nu, CLEA Japan, Inc.). When approximate 10 mm-diameter tumors developed, the mice were used for fluorescence imaging study by receiving intravenous (i.v.) injection of 10 nmol Cy5.5-RGD-monomer, -dimer or Cy5.5 alone. Imaging was performed using the Maestro™ In-Vivo Imaging System (CRI Inc., Woburn, MA, U.S.A.) with a red excitation filter (615 to 665 nm) and a 700 nm long-pass emission filter. A tunable filter was automatically stepped in 10-nm increments from 680 to 950 nm with the same exposure time of 100 ms for images captured at each wavelength.

**Statistical Analysis** Results are shown as mean±standard deviation (S.D.) for each index, respectively. Significant levels were calculated by ANOVA and multiple comparisons (Dunnnett’s test). Values of p<0.05 were considered significant.

**RESULTS**

**Radiolabeling of Peptides with 125I** When 1:1 of peptide:125I molar ratio was used, the yield of radioiodination reaction for RGD-monomer, -dimer and -trimer was 80—90%, and the specific radioactivity was around 80, 37 and 23 MBq/μg, respectively. The ratios 1:2 and 1:3 resulted in decreased labeling yield for dimer (67%) and trimer (56%), respectively. Hence, all the labeling was performed at the ratio of 1:1 for the following studies. The purified 125I-peptides were kept at 4 °C, with the addition of BSA at final 0.1% for preventing the labeled peptides from adhering to the container surface. Gel filtration analysis confirmed that no significant radioisolation occurred within one week of storage.

**In Vivo Cell-Binding Studies** The cell-binding activities of 60000 cpm of 125I-labeled RGD-monomer, -dimer and -trimer, at final concentrations of about 2.4×, 2.6×, and 2.9×10⁻¹¹M respectively, were assessed and compared using HEK293, HEK293(β3), HEK293(β3) and U87MG cells (Fig. 2). All of the peptides showed negligible binding to HEK293 cells in the presence of Ca²⁺/Mg²⁺ or Mn²⁺, while the multimeric analogs did not. In α₅β₃-positive HEK293(β3) and U87MG cells (Figs. 2C, D, respectively), none of the RGD-monomer, -dimer and -trimer showed notable binding in the presence of Ca²⁺/Mg²⁺.

In contrast, the binding of the RGD-monomer onto
HEK293(β₃) was significantly improved in the presence of Mn²⁺, and further substantial increase was observed with the RGD-dimer and -trimer (Fig. 2D). No marked difference in cell binding by multimerization was also observed in U87MG cells (Fig. 2D). We also examined the cellular binding of ¹²⁵I-labeled RGD-monomer, -dimer and -trimer in the absence and presence of divalent cations (Ca²⁺, Mg²⁺ or Mn²⁺ alone, or their various combinations). The presence of divalent cations did not affect the binding of these peptides to HEK293 and HEK293(β₃) cells (data not shown). However, as represented in Fig. 2E, the presence of Mn²⁺ was the only cation markedly enhancing the binding of the RGD-monomer, -dimer and -trimer to HEK293(β₃) and U87MG cells. The following study was thus performed in the presence of Mn²⁺ when HEK293(β₃) cells were used or without any cation with HEK293(β₃).

Different radioactivity doses of ¹²⁵I-labeled RGD-monomer, -dimer or -trimer (ranging from 10000 to 60000 cpm) were also compared and not found to lead to a significant difference in the cell-binding ratios (data not shown). For example, the binding ratios of ¹²⁵I-labeled RGD-monomer, -dimer and -trimer to HEK293(β₃) cells were 80%±2.9%, 86%±5.4% and 83%±5.1% at 10000, 30000, and 60000 cpm, respectively.

The cell-binding specificity of ¹²⁵I-labeled RGD-monomer, -dimer and -trimer was evaluated by competitive inhibition assay using the unlabeled counterparts as well as α₅β₃-specific c(RGDfV) (Fig. 3). The binding of ¹²⁵I-RGD-monomer to HEK293(β₃) cells (Fig. 3A) was inhibited by the unlabeled RGD-monomer in a dose-dependent manner, with >90% inhibition achieved at 10 μM and nearly 99% inhibition at 100 μM. No inhibition was seen with c(RGDfV). Similar results with the RGD-monomer were observed in HEK293(β₃) cells except that c(RGDfV) showed a blocking effect with a maximal 40% inhibition at 0.1 μM (Fig. 3B), with no stronger blocking at higher concentrations. The binding of ¹²⁵I-RGD-dimer to HEK293(β₃) cells was dose-dependent and almost identically inhibited by both the unlabeled RGD-dimer and c(RGDfV), with around 99% inhibition at 1 μM (Fig. 3C). Similar results were obtained for the ¹²⁵I-RGD-trimer (Fig. 3D).

**Effects of AVTGRGDSY Peptides on Cell Adhesion to FN or VN**

The effect of RGD-monomer, -dimer and -trimer on α₅β₃-positive HEK293(β₃) cell adhesion to FN or VN was examined and compared with the reference c(RGDfV) (Fig. 4). Both FN and VN are known as natural ligands for α₅β₃ integrin, but their α₅β₃-positive cell adhesion ability could be differently influenced by cRGD-containing peptides.²⁷ For FN (Fig. 4A), none of the peptides showed a significant effect on cell attachment. For VN (Fig. 4B), the RGD-monomer showed a low but significant inhibitory effect, whereas the RGD-dimer and RGD-trimer were nearly as effective as c(RGDfV) in significantly inhibiting cell attachment when same molar concentration was used.

**Confocal Microscopic Observation of Cy5.5-RGD Peptide Conjugate Distribution**

We examined the binding and subcellular localization of Cy5.5-RGD-monomer and Cy5.5-RGD-dimer by observing the fluorescence of cells after incubation with them (Fig. 5). It might be necessary to mention that the present microscopy study used cells cultured in wells without specific coating for visualizing the binding of RGD peptide to ligand-unoccupied integrins to avoid confusion with the result shown in Fig. 4B that RGD-dimer and -trimer strongly blocked cell adhesion to VN-Mnr.

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**Fig. 2. (A—D) Comparison of ¹²⁵I-Labeled RGD-Monomer, -Dimer and -Trimer for in Vitro Binding to Various Cell Lines**

A: HEK293; B: HEK293(β₃); C: HEK293(β₃); D: U87MG cells. The binding assay was performed in the presence of divalent cations: Ca²⁺/Mg²⁺ or Mn²⁺ (1 mM for each cation) as indicated on the abscissa. The binding ratio is presented as % of cell-bound radioactivity to the total radioactivity added (n=3).

(E) Effects of Divalent Cations on the Binding of ¹²⁵I-Labeled RGD-Dimer to HEK293(β₃) Cells

The binding assay was performed in the absence of divalent cations (control) or presence of 1 mM Ca²⁺, 1 mM Mg²⁺, or 1 mM Mn²⁺, added individually or in combinations, as indicated on the abscissa. The data are presented as the fold of control (n=3). *p<0.05; ns: p>0.05. Symbols without error bars indicate that S.D. is too small to be displayed.
coated wells by competing with VN in binding to \( \alpha v \beta_3 \). Very weak or negligible signal was detected for HEK293(b1) cells treated with either Cy5.5-RGD-monomer (Fig. 5A) or Cy5.5-RGD-dimer (data not shown) in the absence of Ca\(^{2+}\)/Mg\(^{2+}\)/H\(^{11001}\) and Mn\(^{2+}\)/H\(^{11001}\). In the presence of Mn\(^{2+}\) (Figs. 5B, D) instead of Ca\(^{2+}\)/Mg\(^{2+}\)/H\(^{11001}\) (Figs. 5C, E), HEK293(b3) cells could be stained with either Cy5.5-RGD-monomer (Fig. 5B) or Cy5.5-RGD-dimer (Fig. 5D), with the latter producing stronger fluorescence than the former. In addition, while most of the Cy5.5 signal was found on the cell membrane, intracellular signal was also easily visualized in Cy5.5-RGD-dimer-treated cells (Fig. 5D, a—c).

**In Vivo Fluorescence Imaging**  
Nude mice bearing s.c. tumor xenografts of HEK293(b1) or HEK293(b3) cell line received an i.v. injection of 10 nmol Cy5.5-RGD-monomer, Cy5.5-RGD-dimer, or Cy5.5 alone and were imaged at different time points during 24 h. As expected, Cy5.5 alone was not retained in the tumor at any time point observed, whereas Cy5.5-RGD conjugates generally could produce better tumor contrast as soon as 2 h postinjection. Figure 6 shows representative monochrome images collected at 720 nm (the peak emission wavelength for the 2 Cy5.5-RGD conjugates in mice) at 3 h. It was shown that Cy5.5-RGD-monomer produced signals in both \( \alpha v \beta_3 \)-negative/\( \beta_3 \)-overexpressed HEK293(b1) and \( \alpha v \beta_3 \)-positive HEK293(b3) tumors, but also accompanied with non-specific binding in the skin.

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**Fig. 3.** Competitive Inhibition of the Binding of \(^{125}\)I-Labeled RGD-Monomer, -Dimer or -Trimer to HEK293(b1) (A) or HEK293(b3) (B, C, D) Cells by Unlabeled Corresponding Peptides or c(RGDfV)

The binding assay was performed in the absence of divalent cations (A) or presence of 1 mM Mn\(^{2+}\) (B, C, D). In A—D, horizontal and vertical axes are presented as logarithmic and linear scales, respectively. Data are presented as % of control (without blocking) (n=2—3). Symbols without error bars indicate that S.D. is too small to be displayed.

**Fig. 4.** Effects of RGD-Monomer, -Dimer, -Trimer or c(RGDfV) on the Adhesion of HEK293(b3) Cells to FN (A) or VN (B)

The adhesion assay was performed in the presence of 1 mM Mn\(^{2+}\). Data are presented as % of control (without peptide) (n=4). *, **p<0.05 vs. control, monomer, respectively.
contrast, Cy5.5-RGD-dimer accumulated in αvβ3-positive HEK293(β3) tumor, but not in the αvβ3-negative HEK293(β1) tumor, demonstrating its specificity for αvβ3. In addition, the obvious kidney uptake of Cy5.5-RGD-dimer may reflect the prominent renal excretion of this probe.

**DISCUSSION**

The effect of the multivalency for RGD-based peptides has been reported in literature by a number of groups as reviewed by Vives et al. 18) Cyclic RGD structures are proved highly specific for αvβ3 integrin receptor, 28) and multimerization can lead to increased binding affinity for the receptor. 23,24,27,29—33) On the other hand, linear RGD peptides may interact with several integrins, including αvβ3 and β1 integrins. 34—37) Although the multivalent effects were also reported for the linear RGD, 38—40) less information was put forward concerning an effect on the integrin specificity. In this
study, we compared the in vitro cell-binding activities of 125I-or Cy5.5-labeled RGD-monomer, -dimer and -trimer, and found that multimerization could bring 3 different effects: no change in αⅢβ3-negative HEK293 cells, diminished binding in αⅢβ3-negative but β3-transfected HEK293(β3) cells, and substantially enhanced binding in αⅢβ3-positive HEK293(β3) and U87MG cells. Moreover, using paired αⅢβ3-positive and αⅢβ3-negative (but β3-overexpressed) tumor models, the multimerization-induced increased specificity for αⅢβ3 integrin was also demonstrated in vivo.

125I-RGD-monomer exhibited significant binding to HEK293(β3) and HEK293(β3) cells as compared to HEK293 cells. Since the former 2 cell lines are stable transfectants of HEK293 cells with the integrin β3 and β3 subunits, respectively, binding of the monomer might be mediated by β3 and β3 integrins. This was further investigated by competitive inhibition assay using the "cold" counterpart and αⅢβ3-specific c(RGDfV). The result that the known specific binder c(RGDfV) did not compete 125I-RGD-monomer binding to HEK293(β3) cells demonstrates that the binding site is different. This is not the case with HEK293(β3) cells since competition with the monomer occurred. This clearly indicates that the RGD-monomer can adapt to bind either β3 or β3 integrin. Unlike the monomeric analog, both of the RGD-dimer (or -trimer) and c(RGDfV) showed almost complete inhibition of 125I-RGD dimer (or -trimer) binding to HEK293(β3) cells in a similar concentration-dependent manner, and competitions of dimer and trimer with c(RGDfV) gave the similar displacement magnitude effect. These demonstrate that the binding site of the RGD-dimer and -trimer is shared, at least a part, with c(RGDfV), and that adding a third monomer does not promote much binding of the trimer compared to the dimer. The binding specificity was further evidenced by the strong inhibitory effect of RGD-multimers on the adhesion of αⅢβ3-positive HEK293(β3) cells onto αⅢβ3-specific VN-coated surface. This result also suggests the functional integrity of our linear RGD peptides with αⅢβ3 integrin. FN is a ligand for αⅢβ3, but also a ligand for several other integrin receptors. That is possibly why blocking αⅢβ3 by pre-incubation with these peptides was not sufficient to influence cell adhesion to FN. Li et al. reported similar results using c(RGD-containing peptides against VN versus FN.

It is thus demonstrated that the RGD-dimer and -trimer exhibit, compared to the monomer, more specificity to αⅢβ3 integrin. This is the most important point since it points out that adding one more AVTGRGDYS sequence to the monomer greatly influences the binding mode. The binding site of dimer and trimer shares with the high specific binder c(RGDfV) common contact points to αⅢβ3 integrin. This difference with the monomer originates from the multivalent presentation of AVTGRGDYS sequence which could bias the binding mode of AVTGRGDYS sequence possibly due to conformational constrains. The specificity of the αⅢβ3-RGD recognition is attributed to the sequences flanking the RGD-triad, the auxiliary binding motifs in the ligand, and to a large extent the conformational presentation of the triad.

The RGD-triad in our multimers might have a conformational change compared to that in the monomer, and such change possibly improve the αⅢβ3 specificity. Multivalent effect should be the most possible mechanism for the enhanced affinity, and based on this, a number of studies have used the multimeric structures to improve the in vitro binding affinity of cRGD-containing peptides for αⅢβ3 integrin.

In the present study, we compared the αⅢβ3-binding activity of our linear RGD peptides using their radiolabeled forms, which was based on the same dose of radioactivity (60000 cpm/10 μl). Because the RGD-monomer, -dimer and -trimer constructs contain 1, 2 and 3 tyrosines (125I-labeling site) or 1, 2 and 3 RGD sequences, respectively, these factors should be considered when comparing the binding ratios. According to the specific radioactivity, the calculated molar concentrations of 125I-labeled RGD-monomer, -dimer and -trimer were 2.4×10–11 M, 2.9×10–11 M, and 2.9×10–11 M respectively. The cooperativity of the binding related to the multivalency of the molecule is visible when comparing the binding ratios of 125I-labeled RGDs. Indeed, the binding ratios of the multimers, as compared to the monomer, increased more importantly than can be accounted for by the sole additive effect of 2 or 3 RGD units. On the other hand, there was no significant difference in the cell-binding ratios by using different radioactive doses (ranging from 10000 to 60000 cpm) of 125I-labeled RGD-monomer, -dimer or -trimer. This may indicate that a certain variation in molar concentrations at the picomolar level would not influence the final cell-binding ratio calculated from the percentage of cell-bound radioactivity to the total radioactivity added. It should also be noticed that although there is no doubt that multimerization enhances the affinity, the explanation why is not always obvious to prove. In the present study, there is not much difference between the dimer and trimer. The chemical structures seem compatible only for the 2 AVTGRGDYS sequences, and grafting the third may impose different distance between each binding motif.

Divalent cations including Ca2+, Mg2+ and Mn2+ are important for in vitro interactions between RGD ligands and integrin receptors. All of Ca2+, Mg2+ and Mn2+, or the former two, are usually presented together in assay buffer when evaluating the binding affinity of cRGD peptides for αⅢβ3 integrin. There are also reports that Mn2+ may have a different regulatory effect from Ca2+ and Mg2+ in certain cases. In this study, none of the 3 cations influenced the interaction between RGD-monomer (also dimer and -trimer) and β3 integrins. In contrast, we observed Mn2+ dependent binding of RGD-monomer, -dimer and -trimer to αⅢβ3 integrin. Arnaout's group reported the crystal structure of integrin αⅢβ3 in complex with cRGD ligand, showing that 2 Mn2+ cations are present in the structure and are required for the adhesion of RGD. Some researchers suggested a combined use of all Ca2+, Mg2+ and Mn2+ because they found that experiments in buffers lacking one or more of these cations showed lower absolute binding of a 125I-RDDep-protein conjugate. However, in the present study, the binding buffer containing either Mn2+/Ca2+/Mg2+ or Mn2+/Ca2+ resulted in significantly lowered binding of 125I-labeled RGD-monomer, -dimer or -trimer to αⅢβ3 integrin, as compared to the buffer containing Mn2+ alone (data not shown). Hence, we suggest that when evaluating a newly developed RGD-containing product for integrin binding activity, the presence of divalent cations should be documented.
labeled RGD-monomer, the Cy5.5-RGD-monomer showed a weak or negligible binding to HEK293(β1) cells in culture. This may be due to the fact that spread cells or cells in suspension may not react identically to the RGD ligand. With cultured HEK293(β1) cells, on the other hand, fluorescence imaging showed similar results to those observed with the radiolabeled peptides, in terms of the multimeric effect and divalent cation requirement. Cell membrane was the main binding site for the Cy5.5-RGD-monomer and -dimer, and also intracellular Cy5.5 signals were clearly observed with divalent cation requirement. Cell membrane was the main binding site for radiolabeled peptides, in terms of the multimeric effect and imaging showed similar results to those observed with the Cy5.5-RGD-dimer, indicating an active internalization. 

In vivo fluorescence imaging of tumor-bearing mice with the fluorescent dye conjugates also showed the difference in the integrin-binding specificity between RGD-monomer and RGD-multimer. We previously presented an interesting duo fluorescent dye conjugates also showed the difference in the impact of chemical modifications possibly affecting the multimerization-induced in vivo tumor uptake. Such a phenomenon observed in vivo was in good agreement with in vitro results.

In conclusion, comparing the in vitro cell-binding properties of FN-derived mono-, di- and trimeric linear AVT-RGDFSY peptides, we found that the RGD-monomer recognized αβ1β1 integrin but also β1 integrins, and multimerization resulted in substantially enhanced affinity and specificity for the αβ1β1 integrin but loss of affinity for β1 integrins. Using paired αβ1β1-positive and αβ1β1-negative (but β1-overexpressed) tumor models, the multimerization-induced increased specificity for the αβ1β1 integrin was also demonstrated in vivo. Our present study provides useful information for the design of novel RGD peptides.

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