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Near-Infrared Fluorescent RGD Peptides for Optical Imaging of Integrin $\alpha_v\beta_3$ Expression in Living Mice

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Abstract

Near-infrared fluorescence optical imaging is a powerful technique for studying diseases at the molecular level in preclinical models. We recently reported that monomeric RGD peptide c(RGDyK) conjugated to the NIR fluorescent dye specifically targets integrin receptor both in cell culture and in living subjects. In this report, Cy5.5-conjugated mono-, di-, and tetrameric RGD peptides were evaluated in a subcutaneous U87MG glioblastoma xenograft model in order to investigate the effect of multimerization of RGD peptide on integrin avidity and tumor targeting efficacy. The binding affinities of Cy5.5-conjugated RGD monomer, dimer, and tetramer for $\alpha_v\beta_3$ integrin expressed on U87MG cell surface were determined to be 42.9 ± 1.2 , 27.5 ± 1.2 , and 12.1 ± 1.3 nmol/L, respectively. All three peptide–dye conjugates had integrin specific uptake both in vitro and in vivo. The subcutaneous U87MG tumor can be clearly visualized with each of these three fluorescent probes. Among them, tetramer displayed highest tumor uptake and tumor-to-normal tissue ratio from 0.5 to 4 h postinjection. Tumor-to-normal tissue ratio for Cy5.5-conjugated RGD monomer, dimer, and tetramer were found to be 3.18 ± 0.16 , 2.98 ± 0.05 , and 3.63 ± 0.09 , respectively, at 4 h postinjection. These results suggest that Cy5.5-conjugated monomeric, dimeric, and tetrameric RGD peptides are all suitable for integrin expression imaging. The multimerization of RGD peptide results in moderate improvement of imaging characteristics of the tetramer, compared to that of the monomer and dimeric counterparts.

INTRODUCTION

Near-infrared fluorescence (NIRF)¹ optical imaging is emerging as a powerful technology for studying diseases at molecular level in small animal models (1–5). Water and biological tissues have minimal absorbance and autofluorescence in the NIR window (650–900 nm), thus allowing efficient photon penetration into, and out of tissue with low intra-tissue scattering. Recent advances in the field of molecular imaging have demonstrated that this technology can be used for monitoring the biological activity of a wide variety of molecular

targets such as intracellular enzymes (6–10), cell surface receptors (11–16), and antigens (17–18), as well as other targets (19–20), providing a unique opportunity to achieve insight into the biological events involved with these molecules of interest. Moreover, NIR optical imaging technology is a highly sensitive imaging modality, which does not require use of ionizing radiation or radioactive materials (1–5). It is expected that NIR optical imaging will make a significant impact in disease detection and staging, drug development, and treatment assessment.

The cell adhesion molecule integrin $\alpha_v\beta_3$ is a very important factor involved in angiogenesis and metastasis in many tumor types (20–22). It is found that $\alpha_v\beta_3$ is significantly up-regulated in activated endothelial cells and fast-growing solid tumor cells, compared to its minimum expression in quiescent blood vessels and most normal tissues (22, 23). The ability to noninvasively image the integrin $\alpha_v\beta_3$ expression in living subjects would render a novel approach to diagnose tumor and its metastasis and to help to better understand tumor biology of angiogenesis, as well as to greatly facilitate the development of integrin targeted therapy. For this purpose, numerous molecular probes have been developed for imaging integrin expression using different modalities such as magnetic resonance imaging (24), ultrasound (25–27), optical imaging (28–30), positron emission tomography (PET) (31–40), and single-photon emission computed tomography (SPECT) (41–44).

We reported recently that NIRF dye Cy5.5-labeled monomeric arginine-glycine-aspartic acid (RGD) peptide c(RGDyK) specifically targets integrin $\alpha_v\beta_3$ both in cell culture and in living subjects (28). Cy5.5-c(RGDyK), however, had only moderate integrin binding affinity and thus might provide suboptimal tumor targeting efficacy. We and others found that dimeric and multimeric RGD peptides, consisting of multiple cyclic RGD monomer appropriately linked together, have significantly higher integrin avidity than the monomeric counterpart, presumably due to the polyvalency effect (35–37, 43, 44). It was hypothesized that the receptor binding of one RGD cyclic peptide moiety will significantly enhance the “local concentration” of the other RGD peptide moieties in the vicinity of the receptor, which may lead to a faster rate of receptor binding or a slower rate of dissociation of the oligomer from the integrin $\alpha_v\beta_3$ receptor. In this report, we evaluate the effect that multimerization of RGD peptide has on integrin binding affinity and tumor targeting efficacy in three novel NIRF probes: Cy5.5-labeled monomer c(RGDyK), dimer E[c(RGDyK)]₂, and tetramer E{E[c(RGDyK)]₂}₂ (structures are shown in Figure 1).

MATERIALS AND METHODS

General

All commercially available chemical reagents were used without further purification. 9-Fluorenyl-methoxycarbonyl (Fmoc) amino acids and 2-chlorotrityl chloride resin were purchased from Novabiochem (San Diego, CA). Arginine and lysine were protected by the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) and *tert*-butoxycarbonyl (Boc) groups, respectively, while D-tyrosine and aspartic acid were protected as a *tert*-butyl ester. Cy5.5 monofunctional *N*-hydroxysuccinimide (NHS) ester (Cy5.5-NHS) and ¹²⁵I-echistatin labeled by the lactoperoxidase method to a specific activity of 2000 Ci/ mmol were purchased from Amersham Biosciences (Piscataway, NJ). Echistatin was purchased from

Sigma (St. Louis, MO). The purification of the crude product was carried out on a semipreparative reversed-phase high-performance liquid chromatography (HPLC) system equipped with a 170U 4-Channel UV–Vis absorbance detector (Dionex Summit HPLC system, Dionex Corporation, Sunnyvale, CA) using a Vydac protein and peptide column (218TP510, 5 μ m 250 $\times$ 10 mm). The flow was 5 mL/min, with the mobile phase starting from 95% solvent A (0.1% TFA in water) and 5% solvent B (0.1% TFA in acetonitrile) (0–3 min) to 35% solvent A and 65% solvent B at 33 min. The analytical HPLC method was performed with the same gradient system, but with a Vydac 218TP54 column (5 μ m, 250 $\times$ 4.6 mm) and flow rate of 1 mL/min.

Peptide Synthesis

Monomeric cyclic RGD peptide c(RGDyK) (MW = 617.6) was synthesized via solution cyclization of the fully protected linear pentapeptide H-Gly-Asp(OtBu)-D-Tyr(OtBu)-Lys(Boc)-Arg(Pbf)-OH, followed by trifluoroacetic acid (TFA) deprotection (45). Dimeric RGD peptide, E[c(RGDyK)]₂, was prepared according to the literature method (37). The tetrameric RGD peptide E{E[c(RGDyK)]₂}₂ was obtained by coupling Boc-protected glutamic acid activated ester Boc-E(OSu)₂ with RGD dimer E[c(RGDyK)]₂ in the presence of diiso-propylethylamine (DIPEA) (pH = 8.5–9) followed by semipreparative HPLC purification (46).

Cy5.5-RGD Conjugates Synthesis

Appropriate RGD peptide (monomer, dimer, or tetramer) (5 μ mol) was dissolved in 1 mL of 0.1 M sodium borate (Na₂B₄O₇) buffer (pH = 8.3) and then mixed with 1.2 equiv of Cy5.5-NHS (6.7 mg, 6 μ mol) in H₂O (1 mL). The reaction mixture (pH = 8.1) was incubated in the dark at 4 °C overnight and then quenched by adding 200 μ L of 5% acetic acid (HOAc). The crude product was purified by a semipreparative reversed-phase HPLC and characterized by an analytical HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). α -Cyano-4-hydroxycinnamic acid (α -CHCA, prepared as 10 g/L in 33.3% CH₃CN: 33.3% EtOH: 33.3% H₂O: 0.1% TFA) was used as solid matrix. Fractions containing RGD-Cy5.5 conjugates were collected, lyophilized, redissolved in saline at a concentration of 1 mg/ mL, and stored in the dark at –20 °C until use.

U87MG Glioblastoma Xenograft Model

U87MG human glioblastoma cell line was obtained from American Type Culture Collection (Manassas, VA) and was maintained at 37 °C in a humidified atmosphere containing 5% CO₂ in Iscove's modified Dulbecco's medium and 5% fetal bovine serum (Life Technologies, Inc., Grand island, NY). Animal procedures were performed according to a protocol approved by the Stanford University Administrative Panels on Laboratory Animal Care (APLAC). Female athymic nude mice (*nu/nu*), obtained from Charles River Laboratories, Inc. (Cambridge, MA) at 4–6 weeks of age, were injected subcutaneously in the right foreleg with 5 $\times$ 10⁶ U87MG glioblastoma cells suspended in 100 μ L of phosphate-buffered saline (PBS). The tumor bearing mice were subjected to in vivo imaging studies when the tumors reached 0.4–0.6 cm in diameter (14–21 d after implant).

Receptor Binding Assay

The $\alpha_v\beta_3$ receptor binding assay was performed to determine binding affinities of NIR fluorescent RGD conjugates. Briefly, 2×10^5 U87MG cells/well in a 96-well plate were incubated with ^{125}I -echistatin (0.06 nM) in the binding buffer [25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride (Tris-HCl), pH 7.4, 150 mM NaCl, 1 mM CaCl_2 , 0.5 mM MgCl_2 and 1 mM MnCl_2 , 0.1% bovine serum albumin (BSA)] in the presence of different concentrations of Cy5.5-RGD conjugates at room temperature for 3 h. After incubation, the plate was washed three times with binding buffer, and the radioactivity was solubilized with 2 N NaOH followed by γ -counting. Nonspecific binding of ^{125}I -echistatin to $\alpha_v\beta_3$ was determined in the presence of 100 nM echistatin. The IC_{50} values were calculated by nonlinear regression analysis using the GraphPad Prism computer-fitting program (GraphPad Software, Inc., San Diego, CA). Each data point is a result of the average of triplicate wells.

Fluorescence Microscopic Studies

For fluorescence microscopic studies, 1×10^4 cells were cultured on 35 mm MatTek glass bottom culture dishes (cat. no.: P35G-0-14-C, Ashland, MA). After 24 h, the cells were washed with PBS and then incubated at 37 °C with appropriate Cy5.5-RGD conjugate (10 nM) for 30 min. Integrin specificity of the RGD-Cy5.5 conjugates in cell culture was verified by incubating U87MG cells with or without blocking dose of the nonfluorescent monomeric RGD peptide c(RGDyK) (10 $\mu\text{mol/L}$). After the incubation period, cells were washed three times with ice-cold PBS. The fluorescence signal of the cells was recorded using an Axiovert 200M fluorescence microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a Cy5.5 filter set (Exciter, HQ 650/20 nm; Emitter, HQ 675/35 nm). An AttoArc HBO 100W microscopic illuminator was used as a light source for fluorescence excitation. Images were taken using a thermoelectrically cooled charge-coupled device (CCD) (Micromax, model RTE/CCD-576, Princeton Instruments Inc., Trenton, NJ) and analyzed using MetaMorph Software version 6.2r4 (Molecular Devices Corporation, Downingtown, PA).

In Vivo Optical Imaging of Tumors

In vivo fluorescence imaging was performed with an IVIS 200 small animal imaging system (Xenogen, Alameda, CA). A Cy5.5 filter set was used for acquiring Cy5.5-conjugated RGD monomer, dimer, and tetramer fluorescence in vivo. Identical illumination settings (lamp voltage, filters, f/stop, field of views, binning) were used for acquiring all images, and fluorescence emission was normalized to photons per second per centimeter squared per steradian ($\text{p/s/cm}^2/\text{sr}$). Images were acquired and analyzed using Living Image 2.5 software (Xenogen, Alameda, CA). For the control experiment, mice ($n = 3$ for each probe) were injected via tail vein with 500 pmol Cy5.5-RGD (monomer, dimer, or tetramer) and subjected to optical imaging at various time points postinjection (pi). For the blocking experiment, mice ($n = 3$ for each probe also) were injected with the mixture of 10 mg/kg of c(RGDyK) and 500 pmol Cy5.5-conjugated RGD peptides. All NIRF images were acquired using 1 s exposure time (f/stop = 4). The mice in the control experiment were euthanized at 24 h pi. The tumor and major tissues and organs were dissected, wet weighted, and placed

on black papers. The fluorescence images were acquired, and total fluorescence flux (p/s/cm²/sr) for each sample was obtained. Aliquots with known amount of the injectates were also put on the black papers and imaged together with the samples. The results were presented as the percentage injected dose per gram (%ID/g). Values were quoted as mean $\pm$ standard deviation (SD) for a group of three animals.

Data Processing and Statistics

All the data are given as means $\pm$ SD of n independent measurements. Statistical analysis was performed using a Student's t -test. Statistical significance was assigned for P values < 0.05 . For determining tumor contrast, mean fluorescence intensities of the tumor area (T) at the right foreleg of the animal and of the area (N) at the right flank (normal tissue) were calculated by the region-of-interest (ROI) function of Living Image software integrated with Igor (Wavemetrics, Lake Oswego, OR). Dividing T by N yielded the contrast between tumor tissue and normal tissue.

RESULTS

Conjugation and Purification of Cy5.5-RGD Conjugates

The synthesis of Cy5.5-RGD conjugates was achieved through conjugation of Cy5.5-NHS ester with the ϵ -amino group of the lysine residue (in the case of monomer) or glutamate amine (in the case of dimer and tetramer) of the RGD peptides. The desired products were purified by semipreparative HPLC. The purity of Cy5.5-labeled peptides were over 95% from analytical HPLC analysis. The retention times on analytical HPLC for monomer, dimer, and tetramer are found to be: 15.5, 15.8, and 16.2 min, respectively. The yields of Cy5.5-RGD conjugates were typically over 70%. The purified RGD-Cy5.5 conjugates were characterized by MALDI-TOF-MS. Cy5.5-c(RGDyK) (monomer): $m/z = 1518.4$ for $[M + H]^+$ (C₆₈H₈₃N₁₁O₂₁S₄, Calculated MW = 1518.7); Cy5.5-E[c(RG-DyK)]₂ (dimer): $m/z = 2249.0$ for $[M + H]^+$ (C₁₀₀H₁₂₉N₂₁O₃₁S₄, calculated MW = 2249.5); Cy5.5-E{E[c(RGDfK)]₂}₂ (tetramer): $m/z = 3711.8$ for $[M + H]^+$ (C₁₆₅H₂₂₂N₄₀O₅₁S₄, calculated MW = 3710.02); The absorption and fluorescence emission characteristics of Cy5.5-RGD conjugates were identical to those of free Cy5.5, as apparent from the spectrums measured in H₂O (data not shown).

Integrin Binding Characteristics in Vitro

The receptor binding affinity study of Cy5.5-RGD conjugates for integrin $\alpha_v\beta_3$ was carried out using $\alpha_v\beta_3$ positive U87MG cells. We compared the receptor binding affinity of the three probes by performing competitive displacement studies with ¹²⁵I-echistatin as radioligand (Figure 2). All the peptides inhibited the binding of ¹²⁵I-echistatin to U87MG cells in a dose dependent manner. The IC₅₀ values for echistatin, Cy5.5-E{E[c(RGDyK)]₂}₂, Cy5.5-E[c(RGDyK)]₂, and Cy5.5-c(RGDyK) were 1.2 ± 0.1 , 12.1 ± 1.3 , 27.5 ± 1.2 , and 42.9 ± 1.2 nmol/L, respectively.

To demonstrate the $\alpha_v\beta_3$ integrin specificity and subcellular localization of the Cy5.5-RGD conjugates, cyanine dye-labeled RGD peptides were incubated with U87MG tumor cells and images taken with a fluorescent microscope. Receptor-mediated endocytosis of the

fluorescent dye was observed for all three RGD peptide conjugates (Figure 3 A–C) after 30 min of incubation at 37 °C as indicated by the appearance of fluorescence in the perinuclear region. Integrin receptor specificity of the Cy5.5-RGD conjugates was further demonstrated by the absence of the cell associated fluorescence in the presence of a blocking dose of monomeric RGD peptide c(RGDyK) (1 μ mol/L) (Figure 3D – F).

Imaging of U87MG Tumor in Vivo

Whole-body imaging of subcutaneous U87MG tumor xenografted mice was accomplished by using tungsten-halogen lamp light source to excite the probe at 610–665 nm wavelength and monitoring the distribution of the imaging agents with IVIS200 system equipped with a cooled charged-coupled device (CCD) camera. Figure 4A shows typical NIRF images of athymic nude mice bearing subcutaneous U87MG glioblastoma tumor after intravenous (iv) injection of 500 pmol of Cy5.5-RGD monomer, dimer, and tetramer, respectively. For all three probes, the subcutaneous U87MG tumor could be clearly visualized from the surrounding background tissue from 30 min to 24 h pi. Quantification analysis of these images was performed using software Living Image2.5. The fluorescence intensities in the tumor and the normal tissues as a function of time for monomer, dimer, and tetramer are depicted in Figure 4B–D, respectively. Monomer, dimer, and tetramer all exhibited fast tumor targeting character in vivo. The tumor uptake reached a maximum at 30 min pi and slowly washed out over time. Most importantly, at early time points (from 0.5 to 4 h) pi, both tetramer and dimer had significantly higher accumulation in tumor than monomer ($P < 0.05$). Tetramer also showed higher tumor uptake than dimer, although the difference is not significant ($P > 0.05$). The differences in tumor uptakes among the three probes were minimal at 24 h pi. The fluorescence intensity ratio between the tumor and normal tissue (T/N) for Cy5.5-conjugated RGD peptides were calculated and shown in Figure 4E. The tetramer shows significantly higher T/N value than both monomer and dimer at early time points (0.5, 1, 2, and 4 h) ($P < 0.05$). For example, T/N ratio for tetramer, dimer, and monomer are found to be 3.63 ± 0.09 , 2.98 ± 0.05 , and 3.18 ± 0.16 , respectively, at 4 h pi, while the difference diminished at 24 h time point ($P > 0.05$). No significant difference in T/N is observed between dimeric and monomeric peptide probes ($P > 0.05$ for all time points examined in this experiment). Kidneys were clearly visualized for dimeric and tetrameric peptides, indicating that dimer and tetramer have significantly higher renal activity accumulation than the monomer.

Receptor specificities of these probes were validated by a blocking experiment. Unlabeled monomeric RGD peptide, c(RGDyK), successfully reduced tumor contrast for all three tracers (Figure 5). Tumor contrast as quantified by ROI analysis of noninvasive optical imaging indicated that the T/N was reduced from 2.22 ± 0.04 to 1.27 ± 0.05 for monomer, 2.26 ± 0.27 to 1.28 ± 0.02 for dimer, and 2.98 ± 0.09 to 1.09 ± 0.18 for tetramer, respectively.

To further characterize the distribution of RGD peptide–dye conjugates in U87MG in vivo, the tumor-bearing mice were sacrificed right after noninvasive imaging at 24 h pi. Tumors and other normal organs and tissues were removed and subjected to IVIS200 imaging (Figure 6). The total fluorescence of the whole organ was obtained, and %ID/g was

calculated for each organ. The biodistribution of the probes at 24 h pi is shown in the Figure 6B. Tumor-to-blood, tumor-to-muscle, tumor-to-kidney, and tumor-to-liver ratios are illustrated in Figure 6C. The renal uptake for tetramer and dimer are much higher than monomer, consistent with the imaging data obtained in living mice. The tumor accumulation and tumor/muscle and tumor/blood ratios for monomer are similar to that of dimer and tetramer. This corroborated the noninvasive imaging data obtained in living mice.

DISCUSSION

NIRF imaging, which uses neither ionizing radiation nor radioactive materials, is emerging as a complement to nuclear imaging methods. A relatively low-cost, safe, and easy-to-use NIRF imaging system has been proven to be a very powerful tool for noninvasive imaging diseases in preclinical models such as rodents (3). Furthermore, NIRF imaging may provide both surgical anatomy and functional/molecular information when exogenous fluorescent probes are used; thus, it has a potential in the clinical setting by allowing easy visualization and guidance in surgery (47). We are very interested in applying this NIRF imaging technique for studying an important molecular target integrin $\alpha_v\beta_3$, so we can better understand tumor angiogenesis and eventually develop methods for integrin-directed tumor therapy. To achieve this goal, a highly specific and sensitive NIRF agent needs to be developed first.

We have previously demonstrated that Cy5.5-labeled monomeric RGD peptide c(RGDyK) targets integrin $\alpha_v\beta_3$ -expressed tumor in vivo (28). However, this agent only provides moderate tumor contrast in a highly integrin positive U87MG tumor model. Cell receptor binding assay using ^{125}I -echistatin as radioligand indicated that the IC_{50} value for c(RGDyK) was approximately 50 nM. Because the cyclic pentapeptide c(RGDyK) has already been optimized for receptor–ligand interactions (48), it is very unlikely that one can significantly improve receptor avidity of monomeric RGD peptide by fine-tuning of the peptide configuration. An alternative is to apply the concept of multivalency or multimerization and to develop oligomeric RGD peptide probes. In principle, the introduction of a homogeneous multimeric RGD peptide system is expected to improve integrin targeting by cooperative receptor interactions and receptor shielding to endogenous competition. We and others have previously reported that suitably labeled dimeric RGD peptide tracers had incremental improvement on tumor targeting efficiency and pharmacokinetics, compared to the monomeric RGD peptide analogues (35–37, 43, 44). We hypothesize that multimerization to introduce tetrameric RGD peptides might further increase the receptor binding affinity. In this study, we designed a new Cy5.5-labeled tetrameric RGD peptide with repeating c(RGDyK) units connected through glutamate linkers (Figure 1). As expected, integrin affinity followed the order of tetramer > dimer > monomer. Fluorescence microscope imaging studies also confirmed the effectiveness of all three probes for integrin positive U87MG cell staining and receptor mediated endocytosis.

We recently radiolabeled DOTA-conjugated RGD monomer, dimer, and tetramer with ^{64}Cu and compared their tumor targeting efficacy and in vivo kinetics (33, 37, 46). Tetrameric RGD peptide tracer had almost twice as much tumor uptake than the corresponding dimer. The dimer also had significantly higher tumor uptake than the monomeric counterpart. Renal

and hepatic activity accumulation of tetramer was also somewhat higher than that of dimer and monomer. In agreement with these data, we observed significant ($P < 0.05$) increase of tumor fluorescence intensity for Cy5.5-conjugated tetramer and dimer, compared to the monomeric analogue at early time points (from 0.5 to 4 h) pi (Figure 4). However, between tetramer and dimer, no significant difference in tumor accumulation is observed. Another important finding in this research is that tetramer demonstrated the highest T/N contrast from 0.5 to 4 h pi (Figure 4). This attribute is apparent from the high tumor uptake and relatively low normal tissue uptake. The monomer displayed low uptake in the tumor, but it was quickly cleared from normal tissue. This results in a moderate T/N contrast. For the dimer, although it showed a higher tumor uptake than that of the monomer, the relatively high uptake in normal tissue reduced its T/N contrast. We did not observe significant differences in the magnitude of tumor uptake or T/N contrast at 24 h after administration of the NIRF probes by which most of the tumor uptake has been cleared. Moreover, the tumor uptake of NIRF RGD peptides could be specifically inhibited by the unlabeled c(RGDyK) (Figure 5). This demonstrates that Cy5.5-conjugated monomeric, dimeric, and tetrameric RGD peptides are all specific agents for NIR fluorescence imaging of integrin expression. Overall, these data suggest that based on the highest tumor uptake and contrast at early time points pi, the tetramer appears to be the best integrin $\alpha_v\beta_3$ targeting agent among these three compounds. However, only moderate improvement was achieved through multimerization of the peptide strategy. Compared to the monomer, the tetramer only shows 1.20–1.45-fold increase in tumor accumulation and 1.08–1.30-fold increase in T/N ratio at different time points postinjection.

The small animal optical imaging studies above also highlight the importance of evaluation of polyvalency effects of the contrast agents in vivo. Initially we expected that higher tumor uptake, retention, and T/N ratio would be achieved for Cy5.5-labeled RGD multimers due to their increased binding affinities. However, although it was found that the absolute tumor uptake of Cy5.5-labeled RGD probes was generally enhanced with increasing number of RGDs in each probe, multimerization of RGD also changes the molecular size, overall molecular charge, and hydrophilicity of the resulting peptides, thus altering their in vivo pharmacokinetics and clearance from non-target tissues. These results are consistent with the data reported in the literature (43, 46). In both reports, the multimeric RGD peptides showed significantly higher IC_{50} values in vitro and the magnitude of tumor uptake in vivo; however, the improvement of tumor-to-background ratio is marginal. Apparently, for applying the strategy of peptide multimerization extensively and efficiently, more studies are needed to investigate what the essential effects are required to improve the biodistribution and achieve greater targeting selectivity.

Fluorescence intensities of the dissected tumors and normal organs and tissues agreed well with the aforementioned in vivo observations, except that the ex vivo tumor-to-muscle ratio was substantially higher than that observed noninvasively, which might be attributed to the minimal loss of excitation and emission of light density without the need of penetration in and out of skin in addition to the scatter caused by the skin. Quantification analysis verified there were no significant differences in tumor/blood and tumor/muscle ratios for these NIRF probes at 24 h pi. Furthermore, both dimer and tetramer had much lower tumor-to-kidney

ratios than the monomer, likely reflecting the more net positive charge character of the dimer and tetramer than the monomer. The membranes of the renal tubular cells contain negatively charged sites to which the positively charged groups (such as guanidines in the RGD sequence) are expected to bind. There is no significant difference in liver uptake of the three probes.

To further optimize the NIRF RGD probes, it also should carefully choose fluorescent labels itself. The emission maximum of Cy5.5 at 694 nm is at the lower limit of NIRF window. It is expected that fluorescent dyes with more red-absorbing character may provide deeper tissue penetration and thus a closer reflection of an actual distribution of the probe in vivo. Another major problem with Cy5.5 dye is the very small Stokes shift (20 nm). Development of fluorescent dyes with a large Stokes shift would provide better fluorescence detection sensitivity by eliminating the interference with the excitation source, minimizing the rejection of excitation light by the emission filter, and reducing the error by excitation light and scattered light. A good alternative to the fluorescent dyes would be quantum dots (QDs) as fluorescent biolabeling reagents. In comparison with organic fluorophores, QDs have unique optical and electronic properties, such as size- and composition-tunable fluorescence emission from visible to NIR wavelengths, a large absorption coefficient across a wide spectral range, and very high level of brightness and photostability (49). Because of their broad excitation profiles and narrow, symmetric emission spectra, high-quality QDs are suited for optical multiplexing. Whether conjugation of RGD peptides with novel reporter components such as fluorescent dyes with a large Stokes shift or QDs could generate better NIRF imaging agents for integrin $\alpha_v\beta_3$ requires further investigation.

Initially we also expected that NIRF imaging using Cy5.5-labeled RGD peptides might provide the opportunity for rapid and cost-effective structure–activity studies to screen newly developed ligands, rather than the more costly radionuclide-based imaging studies such as SPECT and PET. However, this hypothesis oversimplifies the complex effect that a fairly bulky Cy 5.5 label has on the pharmacokinetics of a probe. The in vivo behavior is the combination of many factors, including, but not limited, to the receptor affinity and specificity, molecular size, overall molecular charge, hydrophilicity, and metabolic stability. Cy5.5 is a rather large chromophore with a molecular weight of ca. 1000. Tumor contrast obtained with a Cy5.5-RGD conjugate may not truly reflect the same RGD peptide labeled with a radionuclide. Additional studies to evaluate the impact of cyanine dye structure on the receptor targeting properties of RGD peptide in vivo is needed to elucidate the discrepancy between NIRF imaging and radionuclide imaging.

CONCLUSION

Cy5.5-RGD monomer, dimer, and tetramer all exhibit specific uptake in the U87MG glioblastoma both in vitro and in vivo. Among these three probes, Cy5.5-RGD tetramer displays the highest tumor uptake and tumor-to-background ratio up to 4 h postinjection and as such appears to be the most promising probe for integrin receptor NIRF imaging. However, the improvement of imaging character of tetramer is only moderate, compared to that of monomer. More research is needed to further optimize such probes.

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Figure 1.

Schematic structures of Cy5.5-conjugated RGD monomer Cy5.5-c(RGDyK), dimer Cy5.5-E[c(RGDyK)]₂, and tetramer Cy5.5-E{E[c(RGDyK)]₂}₂.

Figure 2.

Competitive integrin receptor assay of unlabeled echistatin (■), monomer Cy5.5-c(RGDyK) (▲), dimer Cy5.5-E[c(RGDyK)]₂ (●), and tetramer Cy5.5-E[E[c(RGDyK)]₂]₂ (◆) to U87MG human glioma cells. All experiments were done in triplicate. The IC₅₀ values for echistatin, Cy5.5-monomer, dimer, and tetramer were determined to be 1.2 ± 0.1 , 12.1 ± 1.3 , 27.5 ± 1.2 , and 42.9 ± 1.2 nmol/L, respectively.

Figure 3.

Specific binding and endocytosis of the Cy5.5-labeled cyclic RGD peptides. NIRF images of U87MG were obtained after the cells were incubated for 30 min at 37 °C in the presence of 10 nmol/L monomer Cy5.5-c(RGDyK), dimer Cy5.5-E[c(RGDyK)]₂, tetramer Cy5.5-E{E[c(RGDyK)]₂}₂ with (D, E, F) or without (A, B, C) blocking dose of nonfluorescent RGD peptide c(RGDyK) (10 μ mol/L). Complete blocking of NIR fluorescence of U87MG cells demonstrating high $\alpha_v\beta_3$ integrin specificity of these NIR dye-labeled RGD peptides.

Figure 4.

(A) In vivo fluorescence imaging of athymic nude mice bearing subcutaneous U87MG glioblastoma xenografts after intravenous injection of 500 pmol Cy5.5-RGD monomer, dimer, or tetramer. The location of the tumor was indicated by an arrow. Fluorescence signal from Cy5.5 was pseudo-colored red. The tetramer and dimer display higher tumor uptake and tumor/normal contrast than that of monomer from 0.5 to 4 h pi. (B–D) In vivo targeting characters of Cy5.5-RGD monomer, dimer, and tetramer, respectively. Both tetramer and dimer had significantly higher accumulation in tumor than monomer ($P < 0.05$) at early time

points (from 0.5 to 4 h) pi. But the differences in tumor uptakes among the three probes were minimal at 24 h pi. (E) Tumor contrast (tumor-to-normal tissue ratio) as a function of time post-administration of Cy5.5-RGD monomer (■), dimer (●), and tetramer (▲). The fluorescence intensity for the region of interest was recorded as photons per second per centimeter squared per steradian ($\text{p/s/cm}^2/\text{sr}$). The tumor contrast of Cy5.5-RGD-tetramer from 0.5 to 4 h postinjection is significantly higher than that of Cy5.5-RGD-dimer and monomer ($P < 0.05$).

Figure 5.

Representative NIRF images of mice bearing subcutaneous U87MG tumor on the right foreleg demonstrating blocking of Cy5.5-RGD conjugates (500 pmol) uptake in the tumors by coinjection with 10 mg/kg of c(RGDyK). Pseudo-color fluorescence images of tumor-bearing mice were acquired 1 h after intravenous injection of Cy5.5-RGD conjugates (experiment) or Cy5.5-RGD conjugates + c(RGDyK) (block). (B) Tumor contrast (tumor-to-normal tissue ratio) calculated from region of interest measurement at 1 h postadministration of Cy5.5-RGD conjugates.

1: Blood; 2: Heart; 3: Lung; 4: Liver; 5: Spleen; 6: Pancreas; 7: Muscle; 8: Tumor 9: Stomach; 10: Kidney; 11: Intestines; 12: Skin; 13: Brain; 14: Bone

Figure 6.

Representative images of dissected organs of mice bearing U87MG tumor sacrificed 24 h after intravenous injection of Cy5.5-RGD monomer (A), dimer (B), and tetramer (C) at a dose of 500 pmol per mouse. (D) Biodistribution of the probes at 24 h postinjection. (E) Tumor-to-normal organ ratios.