Optical Imaging of Integrin αv β3 Expression with Near-Infrared Fluorescent RGD Dimer with Tetra(ethylene glycol) Linkers

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Abstract
Integrin αv β3 plays great roles in tumor angiogenesis, invasion, and metastasis. We report here the noninvasive visualization of tumor integrin αv β3 expression by using near-infrared fluorescence (NIRF) imaging of an IRDye800-labeled new cyclic RGD (arginine-glycine-aspartic acid) dimer with tetra(ethylene glycol) (PEG4) linkers (ie, E[PEG4-c(RGDfK)]2. PEG4 = 15-amino-4,7,10,13-tetraoxapentadecanoic acid) in a U87MG tumor model. Fluorescent dye-labeled E[PEG4-c(RGDfK)]2 were subjected to in vitro cell staining, in vivo NIRF imaging, ex vivo NIRF imaging, and histologic studies. The in vitro and in vivo characterization of dye-labeled E[PEG4-c(RGDfK)]2 were compared with dye-labeled RGD dimer without PEG4 linkers (namely, E[c(RGDfK)]2). Both Cy5.5-E[PEG4-c(RGDfK)]2 and Cy5.5-E[c(RGDfK)]2 exhibited integrin αv β3 binding specificity in a cell-staining experiment. In vivo NIRF imaging showed higher tumor accumulation and tumor to background contrast of IRDye800-E[PEG4-c(RGDfK)]2 over IRDye800-E[c(RGDfK)]2. The tumor integrin αv β3 specificity of IRDye800-E[PEG4-c(RGDfK)]2 was confirmed by successful inhibition of tumor uptake in the presence of an excess dose of c(RGDfK). Histologic examination revealed both tumor vasculature and tumor cell integrin αv β3 binding of IRDye800-E[PEG4-c(RGDfK)]2 in vivo. In summary, NIRF imaging with IRDye800-E[PEG4-c(RGDfK)]2 offers an easy, fast, and low-cost way to detect and semiquantify tumor integrin αv β3 expression in living subjects.

Integrin family members that consist of two non-covalently bound heterodimeric subunits (α and β) have been well documented as important cell adhesion molecules for cell survival, proliferation, and migration. Among all of the integrins, integrin αv β3 has been identified as the most important member with overexpression pattern among vascular cells during tumor angiogenesis and vascular remodeling. Integrin αv β3 is highly expressed on activated and proliferating endothelial cells and some tumor cells, but not on quiescent vessels and normal cells. It is suggested that noninvasive imaging of integrin αv β3 expression is a promising strategy to characterize the biologic aggressiveness and prognosis of a malignant tumor.

In the last decade, Arg-Gly-Asp (RGD)-based probes that specifically bind integrin αv β3 have been extensively investigated for imaging of integrin αv β3 expression using different
modalities, such as magnetic resonance imaging, optical imaging, positron emission tomography (PET), and single-photon emission computed tomography (SPECT). To develop the RGD tracers with enhanced integrin binding affinity and increased tumor uptake, it was proposed by others and us that the binding affinity of dimeric and multimeric RGD peptides would be better than that of monomeric RGD peptides based on the polyvalency effect. We were particularly interested in the development of RGD dimer-based radiotracers for PET and SPECT imaging of integrin expression because the RGD dimer had higher tumor uptake than the monomer and lower background signal than the tetramer, which make it a promising lead compound for tumor imaging in a short time (e.g., < 2 hours) after injection. To further improve the receptor binding affinity of RGD dimer, we recently developed a series of new RGD dimers with PEG4 (PEG4 = 15-amino-4,7,10,13-tetraoxapentadecanoic acid) or Gly3 (Gly3 = Gly-Gly-Gly) linkers between the two RGD motifs in the dimeric molecule. 68Ga, 18F, and 99mTc labeled the new RGD dimers exhibited improved receptor binding affinity and increased tumor uptake compared with the corresponding RGD dimers with no linkers. Optical imaging techniques such as near-infrared fluorescence (NIRF) imaging are emerging as a powerful technology for noninvasive imaging of the molecular basis of disease states in animal models. NIRF imaging uses wavelengths in the 700 to 900 nm range, where the absorbance and autofluorescence spectra for water and biomolecules are minimal, thus allowing for efficient photon penetration of tissue with minimal intratissue light scattering. Compared with radioisotope-based PET or SPECT imaging, NIRF optical imaging technology does not require use of ionizing radiation or radioactive materials, and the NIRF imaging system is relatively low cost, safe, and easy to use. Moreover, optical imaging allows multicolor imaging to be performed based on the use of fluorophores with differing emission wavelengths and allows the ex vivo analysis of the exact location of the tracers in the targeted tissues.

In this study, we labeled the RGD dimer with two tetra(ethylene glycol) (PEG4) spacers, E[PEG4-c(RGDfK)]2 (PEG4-RGD2), with the fluorescent IRDye800 (Figure 1), and investigated whether E[PEG4-c(RGDfK)]2 will be superior to E[c(RGDfK)]2 (RGD2) when labeled with dye for optical imaging of integrin expression in a nude mice model.

Materials and Methods

Materials

All chemicals obtained commercially were of analytical grade and used without further purification. c(RGDfK), E[PEG4-c(RGDfK)]2 (PEG4-RGD2), and E[c(RGDfK)]2 (RGD2) were custom-made by Peptides International, Inc. (Louisville, KY). Cy5.5 monofunctional N-hydroxysuccinimide (NHS) ester (Cy5.5-NHS) and IRDye800-NHS were purchased from Amersham Biosciences (Piscataway, NJ). 125I-echistatin was obtained from Perkin-Elmer (Norwalk, CT). The syringe filter and polyethersulfone membranes (pore size, 0.22 µm; diameter, 13 mm) were obtained from Nalge Nuc International (Rochester, NY).

Synthesis of Dye-RGD Dimers

The dye-RGD dimer conjugates IRDye800-E[PEG4-c(RGDfK)]2 (IRDye800-PEG4-RGD2) IRDye800-E[c(RGDfK)]2 (IRDye800-RGD2), Cy5.5-E[PEG4-c(RGDfK)]2 (Cy5.5-PEG4-RGD2), and Cy5.5-E[c(RGDfK)]2 (Cy5.5-RGD2) were synthesized by coupling RGD dimers with IRDye800-NHS and Cy5.5-NHS in 0.1 M sodium borate (Na2B4O7) buffer (pH 8.5) followed by high-performance liquid chromatography purification, as we previously described.
Cell Culture and Animal Model

The U87MG human glioblastoma cells purchased from American Type Culture Collection were cultured in Dulbecco’s Modified Eagle’s Medium (Gibco, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C with 5% CO₂. Animal procedures were performed according to a protocol approved by the Stanford University Institutional Animal Care and Use Committee. The U87MG tumor model was generated by subcutaneous injection of 5 × 10⁶ cells into the right front flank of female athymic nude mice (Harlan, Indianapolis, IN). The mice were subjected to in vivo imaging studies when the tumor volume reached 100 to 300 mm³ (3 to 4 weeks after inoculation).

Cell Competition Binding Assay

The in vitro integrin αvβ3 binding affinity and specificity of cyclic RGD dimers (PEG₄-RGD2 and RGD2) and their IRDye800-conjugates (IRDye800-PEG₄-RGD2 and IRDye800-RGD2) were assessed via cell binding assay using ¹²⁵I-echistatin as the integrin-specific radioligand. Experiments were performed on U87MG glioblastoma cells as we previously described. The best-fit 50% inhibitory concentration (IC₅₀) values were calculated by fitting the data by nonlinear regression using GraphPad Prism (GraphPad Software, San Diego, CA) and reported as an average of triplicate samples plus the standard deviation. Experiments were repeated twice with similar results.

Cell Fluorescence Staining

U87MG cell staining study was performed as previously described with some modifications. Briefly, cells grown on 35 mm MatTek glass-bottomed culture dishes were incubated at room temperature with Cy5.5, Cy5.5-PEG₄-RGD2, or Cy5.5-RGD2 (100 nmol/L in phosphate-buffered saline [PBS]) for 60 minutes. Receptor binding specificity of Cy5.5-PEG₄-RGD2 in cell culture was verified by incubating U87MG cells with a blocking dose of the nonfluorescent c(RGDfK) (10 μmol/L). After the incubation period, cells were washed with PBS and then visualized 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).

In Vivo and Ex Vivo Optical Imaging

U87MG tumor–bearing nude mice were injected via the tail vein with 0.5 nmol of IRDye800, IRDye800-PEG₄-RGD2, or IRDye800-RGD2. At 1, 2, and 4 hours postinjection, the mice were subjected to optical imaging using an Maestro In-Vivo Imaging System (CRI, Woburn, MA; excitation = 735 nm, emission = 780 nm long pass) as we previously described. Images were acquired and analyzed using Maestro 2.4 software (CRI, Woburn, MA) (n = 3/group). For the blocking experiment, mice (n = 3) were injected with the mixture of 10 mg/kg of c(RGDfK) and 0.5 nmol IRDye800-PEG₄-RGD2 and then subjected to optical imaging using the same condition. After the last images were collected at 4 hours postinjection, the tumor-bearing nude mice were sacrificed; tumors and major tissues and organs were harvested, wet weighted, and placed on the blank papers. Aliquots with a known amount of injectates were also put on the black papers and were subjected to optical imaging together with the samples. The results were presented as the percentage injected dose per gram (%ID/g). Values were expressed as mean ± SD for a group of three animals.

Immunofluorescence Staining

One U87MG tumor–bearing nude mouse was injected via the tail vein with 1 nmol of Cy5.5-PEG₄-RGD2. At 30 minutes postinjection, the mouse was sacrificed and perfused with 20 mL PBS. The U87MG tumor was then collected and frozen cut into slices. Tumor
sections were incubated with antihuman integrin αvβ3 (Abegrin, [MedImmune, Inc., Gaithersburg, MD] 50 μg/mL) and hamster antimouse integrin β3 (1:100; BD Biosciences, San Jose, CA) antibodies for 1 hour at room temperature and then visualized with FITC-conjugated secondary antibodies (1:200, Jackson ImmunoResearch Laboratories, Inc., West Grove, CA) using the fluorescence microscope.

Statistical Analysis
Quantitative data were expressed as mean ± SD. Means were compared using one-way analysis of variance and Student t-test; p values < .05 were considered statistically significant.

Results

In Vitro Receptor Binding Studies
The integrin αvβ3 binding affinity of PEG$_4$-RGD2, RGD2, and their IRDye800 conjugates was determined by competition displacement of $^{125}$I-echistatin (integrin αvβ3 specific) bound to U87MG cells. As shown in Figure 2A, the IC$_{50}$ values for PEG$_4$-RGD2, RGD2, IRDye800-PEG$_4$-RGD2, and IRDye800-RGD2 were determined to be 41.83 ± 5.81, 88.83 ± 5.42, 56.76 ± 3.41, and 94.31 ± 7.14 nM, respectively (mean ± SD, n = 3). The binding affinity of PEG$_4$-RGD2 was higher than that of RGD2, whereas the IRDye800-RGD dimer conjugates possessed slightly lower affinity than the corresponding unmodified RGD dimers.

Direct staining of U87MG tumor cells using dye-labeled RGD dimers was performed. We used Cy5.5 instead of IRDye800 conjugates for cell staining because the emission wavelength of IRDye800 (806 nm) is beyond the optimal detection capacity of our inverted epifluorescence microscope. Both Cy5.5-PEG$_4$-RGD2 and Cy5.5-RGD2 showed strong staining of U87MG cells in vitro, whereas Cy5.5 cannot bind the U87MG cells. An excess dose of nonfluorescent c(RGDfK) can significantly block the staining of Cy5.5-PEG$_4$-RGD2, which also confirmed the receptor binding specificity of Cy5.5-PEG$_4$-RGD2 on U87MG tumor cells (Figure 2B).

In Vivo and Ex Vivo Optical Imaging
The in vivo behaviors of IRDye800-PEG$_4$-RGD2 and IRDye800-RGD2 were tested in U87MG tumor–bearing nude mice using the Maestro In-Vivo Imaging System. After image acquisition, spectral unmixing yielded the pseudocolor images of the pure spectrum of IRDye800. Figure 3A showed the representative optical images of nude mice after injection of 0.5 nmol of IRDye800-RGD dimers. For the two probes, the subcutaneous U87MG tumors can be clearly visualized from 1 to 4 hours postinjection. The region of interest (ROI) was drawn for each tumor or muscle over time, and the total signals were normalized by the exposure time and tumor size (total signal/ms·mm$^2$). As shown in Figure 3B, both probes showed the highest tumor uptake at 1 hour postinjection and then decreased with time. The tumor uptake of IRDye800-PEG$_4$-RGD2 was higher than that of IRDye800-RGD2 at any time points examined with the values of 1.38 ± 0.12 versus 1.24 ± 0.15, 1.21 ± 0.21 versus 1.02 ± 0.13, and 1.15 ± 0.10 versus 0.71 ± 0.06 (total signal/ms·mm$^2$) at 1, 2, and 4 hours, respectively (mean ± SD, n = 3). At 4 hours postinjection, the tumor uptake of IRDye800-PEG$_4$-RGD2 was significantly higher than that of IRDye800-RGD2 (p < .05). The light intensity ratios between the tumor and muscle (tumor to muscle ratio) for IRDye800-PEG$_4$-RGD2 and IRDye800-RGD2 were calculated at each time point and are shown in Figure 3C. Both IRDye800-PEG$_4$-RGD2 and IRDye800-RGD2 had increased tumor to muscle ratios from 1 to 4 hours. The ratio of IRDye800-PEG$_4$-RGD2 was higher than that of IRDye800-RGD2 after 2 hours postinjection. At 4 hours postinjection, the tumor

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to muscle ratio of IRDye800-PEG$_4$-RGD2 (4.63 ± 0.28) was significantly higher than that of IRDye800-RGD2 (3.31 ± 0.22) ($p < .05, n = 3$).

The in vivo receptor targeting specificity of IRDye800-PEG$_4$-RGD2 was tested by blocking study at 4 hours postinjection. As shown in Figure 4, the coinjection of a blocking dose of c(RGDfK) with IRDye800-PEG$_4$-RGD2 can significantly reduce the tumor signals (from 1.15 ± 0.10 to 0.22 ± 0.07 total signal/ms·mm$^2$), demonstrating the specific targeting of IRDye800-PEG$_4$-RGD2 in U87MG tumors. IRDye800 only also showed low tumor uptake at 4 hours postinjection (0.42 ± 0.17 total signal/ms·mm$^2$), which was significantly lower that of IRDye800-PEG$_4$-RGD2 in the U87MG tumors ($p < .01$).

To investigate the biodistribution of IRDye800-PEG$_4$-RGD2 or IRDye800-RGD2 in the U87MG tumors and normal tissues, we performed ex vivo optical imaging after the last in vivo scanning at 4 hours postinjection (Figure 5, A and B). The total signal of the whole organ was obtained and %ID/g was calculated. The biodistribution data of the two probes at 4 hours postinjection are shown in Figure 5C and the tumor to blood, tumor to liver, tumor to kidney, and tumor to muscle ratios are illustrated in Figure 5D. Both probes showed high kidney uptake, indicating that they were mainly excreted by the renal route. IRDye800-PEG$_4$-RGD2 and IRDye800-RGD2 had similar normal organ uptake, but the tumor uptake of IRDye800-PEG$_4$-RGD2 was significantly higher than that of IRDye800-RGD2 (5.87 ± 1.21 vs 2.71 ± 0.77 %ID/g, $p < .01$), which is consistent with the in vivo optical imaging data in the living mice. With the higher tumor uptake and similar normal organ uptake compared with IRDye800-RGD2, IRDye800-PEG$_4$-RGD2 showed much higher tumor contrast and tumor to nontumor ratios. Taken together, IRDye800-PEG$_4$-RGD2 provided better tumor image quality than IRDye800-RGD2 at 4 hours postinjection.

**Immunofluorescence Staining**

To characterize the microdistribution of dye-labeled PEG$_4$-RGD2 in U87MG tumors, we injected Cy5.5-PEG$_4$-RGD2 into the U87MG tumor–bearing nude mouse, and the tumor tissue was collected and frozen cut at 30 minutes postinjection. The tumor sections were stained with antihuman integrin $\alpha_v$ $\beta_3$ and antimurine integrin $\beta_3$ antibodies to localize the integrin $\alpha_v$ $\beta_3$ expressed on the tumor cells and the tumor vasculature, respectively. As shown in Figure 6, Cy5.5-PEG$_4$-RGD2 showed good colocalization with both murine integrin $\beta_3$ and human $\alpha_v$ $\beta_3$, indicating that Cy5.5-PEG$_4$-RGD2 specifically targeted both the tumor newborn blood vessels and tumor cells in the U87MG tumor tissues.

**Discussion**

We recently introduced a new series of RGD dimers with PEG$_4$ or Gly$_3$ linkers between the two RGD motifs in the dimeric molecule. The radiolabeled new RGD dimers possessed increased receptor binding affinity and enhanced tumor uptake as determined by both PET and SPECT imaging.\textsuperscript{21, 25, 27} In this report, we investigated whether fluorescent dye labeled RGD dimer (E[PEG$_4$-c(RGDfK)]$_2$) can be used for in vivo visualization and semiquantification of integrin $\alpha_v$ $\beta_3$ expression in a glioblastoma model.

PEGylation is a useful strategy to improve the in vivo kinetics of various pharmaceuticals.\textsuperscript{34} We previously demonstrated that PEGylation of RGD peptides does improve the pharmacokinetics of the resulting tracers.\textsuperscript{35} To avoid the reduction of receptor binding affinity by inserting a long PEG, in a recent study,\textsuperscript{20} we incorporated a “mini-PEG” spacer, with three ethylene glycol units, onto the glutamate $\alpha$-amino group of the dimeric RGD peptide E[c(RGDyK)]$_2$. The mini-PEG spacer increased the overall hydrophilicity and alleviated the steric hindrance, thereby increasing the $^{18}$F labeling yield. In this study, we inserted two tetra(ethylene glycol) (PEG$_4$) between the two RGD motifs in the
E[c(RGDfK)]_2 molecule and found that the integrin αvβ3 binding affinity of E[PEG_4-c(RGDfK)]_2 (PEG_4-RGD2) is slightly higher than that of E[c(RGDfK)]_2 (RGD2) (41.83 ± 5.81 vs 88.83 ± 5.42 nM; see Figure 2A), which is probably due to the insertion of two PEG_4 that make the two RGD motifs more flexible for receptor binding. In the in vitro cell staining study, Cy5.5-PEG_4-RGD2 and Cy5.5-RGD2, but not unconjugated Cy5.5, can specifically bind the U87 tumor cells (high integrin αvβ3 expression) (see Figure 2B). Coincubation of Cy5.5-PEG_4-RGD2 with unfluorescent c(RGDfK) almost totally blocked the binding of Cy5.5-PEG_4-RGD2 to the tumor cells (see Figure 2B), demonstrating that the binding of dye-labeled PEG_4-RGD2 on tumor cells was integrin αvβ3 mediated.

Optical imaging is wildly used during preclinical probe development for monitoring biomarkers and molecular events in living subjects. We previously reported Cy5.5- and Cy7-labeled RGD monomeric, dimeric, and tetrameric probes for integrin αvβ3 expression.16, 17 For potential clinical applications of integrin αvβ3 optical imaging, it is preferable to use a NIRF dye with longer wavelength absorption and emission than Cy5.5 to obtain NIRF images with reduced background signals. Both unconjugated Cy5.5 and Cy7 showed relatively high tumor-nonspecific targeting (data not shown), which may be due to the high lipophilicity of the Cy dyes. In contrast, IRDye800 showed only a lower nonspecific tumor accumulation (see Figure 4A). So in this study, we used IRDye800 as the NIRF dye for PEG_4-RGD2 labeling and carried out the in vivo and ex vivo optical imaging in a U87MG tumor model.

Figure 3 illustrated the in vivo optical images and the quantified results by ROI analysis after injection of IRDye800-PEG_4-RGD2 in U87MG tumor–bearing nude mice at 1, 2, and 4 hours. Both RGD tracers showed good tumor-targeting properties, and the high tumor contrast was found at 4 hours with the clearance of tracers in normal organs. The tumor uptake of IRDye800-PEG_4-RGD2 was higher than that of IRDye800-RGD2 at 1 and 2 hours postinjection, and the difference is significant at 4 hours postinjection (p < .01). Although the molecular weight of IRDye800-PEG_4-RGD2 was slightly higher than that of IRDye800-RGD2, it is impossible that the increased molecular weight contributes to the enhanced tumor uptake because the tumor to muscle ratios of IRDye800-PEG_4-RGD2 were also higher than those of IRDye800-RGD2 after 2 hours postinjection. We speculate that the longer linkers between two RGD motifs make the new RGD dimer more flexible for receptor binding and even possible bivalent binding with the vicinal receptors, thereby increasing the receptor binding affinity. The increased affinity would lead to a faster rate of receptor binding or a slower rate of dissociation of the tracers in the nude mice model. The in vivo tumor targeting specificity of IRDye800-PEG_4-RGD2 was confirmed by a blocking study. An excess dose of c(RGDfK) significantly reduced the tumor uptake of IRDye800-PEG_4-RGD2 and the tumor uptake of IRDye800-PEG_4-RGD2 was significantly higher than that of the uncoupled IRDye800, all of which demonstrated that the tumor targeting of the probe was specific (see Figure 4). To investigate the exact location of the probe in the tumor tissues, we dissected the U87MG tumors after injection of IRDye800-PEG_4-RGD2 and then stained them with anti-human integrin αvβ3 and antimurine integrin αvβ3 antibodies. In the tumor tissues, the probe accumulated in both the U87MG tumor cells (human integrin αvβ3 positive) and the newborn blood vessels (murine integrin αvβ3 positive), indicating that the RGD probe can bind the receptor-positive tumor cells and the activated endothelial cells during tumor angiogenesis.

Nuclear imaging is a wildly used modality that offers excellent sensitivity and accurate qualification of the tracer distribution. In contrast, optical imaging is a relatively new molecular imaging modality that allows real-time and high-resolution imaging of fluorophores embedded in diseases.36 Optical imaging is limited for deep tissue imaging. Although in this study we also performed ex vivo optical imaging to characterize the in vivo
distribution of IRDye800-PEG₄-RGD2, the quantification of ex vivo imaging of excised organs and tissues may still not be a true reflection of tissue distribution of the probe because the images were planar and the fluorescence signal was distributed heterogeneously throughout the tumor (see Figure 5B). This may partially explain the different in vivo distribution properties of IRDye800-labeled PEG₄-RGD2 (determined by optical imaging) compared with the positron emitter ¹⁸F-labeled PEG₄-RGD2 (determined by PET imaging). With the recent development of state-of-the-art fluorescence imaging techniques, such as three-dimensional fluorescence molecular tomography techniques, the reconstruction of three-dimensional maps of fluorochromes can be quantified on the basis of sophisticated algorithms, leading to accurate quantification of fluorophore distribution. Additionally, the synthesis of a dual-labeled probe composed of both a PET isotope and an NIRF dye and analysis of the biodistribution of this probe under the same conditions would be beneficial to correlate the fluorescence quantification and organ distribution.

In summary, the new RGD dimeric probe IRDye800-PEG₄-RGD2 showed better tumor-targeting properties than the IRDye800-labeled RGD dimer without PEG₄ spacers, which is at least partially due to the increased receptor binding affinity. Despite the limited penetration of optical imaging, IRDye800-PEG₄-RGD2 is a useful probe to conduct fast fluorescence measurements at a low fluorophore dose for the detection and characterization of integrin αᵥ β₃ expression with low cost and without the harmful effects of radiation exposure.

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Figure 1.
Schematic structure of IRDye800-conjugated cyclic RGD dimer with tetra(ethylene glycol) PEG₄ linkers (IRDye800-E[PEG₄-c(RGDfK)]₂).
Figure 2.
A, Inhibition of $^{125}$I-echistatin binding to integrin αv β3 on U87MG tumor cells by E[c(RGDfK)]$_2$ (RGD2, IC$_{50}$ = 88.83 ± 5.42 nM), PEG$_4$-E[c(RGDfK)]$_2$ (PEG$_4$-RGD2, IC$_{50}$ = 41.83 ± 5.81 nM), IRDye800-E[c(RGDfK)]$_2$ (IRDye800-RGD2, IC$_{50}$ = 94.31 ± 7.14 nM), and IRDye800-PEG$_4$-c(RGDfK)]$_2$ (IRDye800-PEG$_4$-RGD2, IC$_{50}$ = 56.76 ± 3.41 nM) (mean ± SD, n = 3). B, Fluorescence staining of U87MG tumor cells with unconjugated Cy5.5, Cy5.5-RGD2, and Cy5.5-PEG$_4$-RGD2 (without or with c(RGDfK) as a blocking agent), respectively.
Figure 3.
A, In vivo fluorescence imaging of U87MG tumor–bearing nude mice at 1, 2, and 4 hours after intravenous injection of 0.5 nmol IRDye800-RGD2 or IRDye800-PEG₄-RGD₂. Fluorescence signal from IRDye800 was pseudocolored red. B, Quantification and kinetics of in vivo tumor targeting characteristics of IRDye800-RGD2 and IRDye800-PEG₄-RGD₂. Light intensity was recorded as total fluorescence signal per millisecond per millimeter squared (total signal/ms·mm²) (mean ± SD, n = 3). C, The tumor contrast (tumor to muscle ratio) as a function of time postadministration of IRDye800-RGD2 or IRDye800-PEG₄-RGD₂ in a U87MG tumor model (mean ± SD, n = 3).
Figure 4. A, In vivo fluorescence imaging of U87MG tumor–bearing nude mice at 4 hours after intravenous injection of 0.5 nmol IRDye800-PEG₄-RGD2 (without or with c(RGDfK) as a blocking agent) or unconjugated IRDye800. Fluorescence signal from IRDye800 was pseudocolored red. B, Quantified U87MG tumor uptake at 4 hours after intravenous injection of 0.5 nmol IRDye800-PEG₄-RGD2 (without or with c(RGDfK) as a blocking agent) or unconjugated IRDye800. Light intensity was recorded as total fluorescent signal per millisecond per millimeter squared (total signal/ms-mm²) (mean ± SD, n = 3).
Figure 5.
A–B, Representative images of dissected organs of U87MG tumor–bearing nude mice sacrificed at 4 hours after intravenous injection of IRDye800-PEG₄-RGD2 (A) or IRDye800-RGD2 (B) at a dose of 0.5 nmol per mouse. C, Biodistribution of IRDye800-PEG₄-RGD2 and IRDye800-RGD2 in U87MG tumor–bearing nude mice at 4 hours postinjection (mean ± SD, n = 3). D, Tumor to nontumor (T/NT) ratios of IRDye800-PEG₄-RGD2 and IRDye800-RGD2 in U87MG tumor–bearing nude mice at 4 hours postinjection (mean ± SD, n = 3).
Figure 6.
U87MG tumor histology of Cy5.5-PEG$_4$-RGD2. The U87MG tumor–bearing nude mouse was intravenously injected with 1 nmol of Cy5.5-PEG$_4$-RGD2. At 30 minutes postinjection, the U87MG tumor was dissected, frozen cut into slices, and then stained for murine integrin β$_3$ and human integrin α$_v$β$_3$ to determine Cy5.5-PEG$_4$-RGD2 localization. A, Left, Cy5.5-PEG$_4$-RGD2; middle, murine integrin β$_3$; right, overlay. B, Left, Cy5.5-PEG$_4$-RGD2; middle, human integrin β$_3$; right, overlay.