Development of the Fibronectin–Mimetic Peptide KSSPHSRN(SG)₅RGDSP as a Novel Radioprobe for Molecular Imaging of the Cancer Biomarker α₅β₁ Integrin

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Regular Article

Integrins are a family of transmembrane glycoproteins consisting of 2 noncovalently associated α and β subunits. α₅β₁ Integrin (α₅β₁) mediates cell-extracellular matrix protein interactions through its major ligand, fibronectin. α₅β₁ is overexpressed in various cancers and in tumor vascular endothelium and plays important roles in tumor progression, angiogenesis, and metastasis. Suppression of α₅β₁ activities has been shown to potentiate the efficacy of chemo- or radiotherapy and induce apoptosis. Overexpression of α₅β₁ revealed that it had prognostic value for patients with non-small cell lung cancer, colorectal cancer, cervical cancer, and gastric cancer.

A number of α₅β₁ antagonists including antibodies, peptides, and small nonpeptidic molecules have been developed as therapeutic agents, and some are in clinical trials. However, there is still a lack of efficient molecular probes for noninvasive in vivo imaging of α₅β₁ expression, for use in evaluating malignancy, predicting prognoses, and assessing and monitoring of anti-tumor efficacy of α₅β₁-targeted drugs, assessing and monitoring of anti-tumor efficacy of α₅β₁-targeted drugs in clinical trials, and for contributing to the development of new drug therapies.

The efficient binding of α₅β₁ to its natural ligand, fibronectin, requires the involvement of 2 peptide sequences, Pro-His-Ser-Arg-Asn (PHSRN, the synergy binding site) and Arg-Gly-Asp (RGD, the primary binding site) on the 9th and 10th type-III repeats of fibronectin, respectively. PR_b, KSSPHSRN(SG)₅RGDSP, is an α₅β₁-specific fibronectin–mimetic peptide developed by Kokkoli and colleagues. The presence of (SG)₅ was designed to closely mimic both the distance and the hydrophobicity/hydrophilicity between PHSRN and RGD in native fibronectin, while the motif KSS served as a spacer for functional conjugation. The binding affinity of the PR_b ligand to the isolated α₅β₁ receptor was determined to have a dissociation constant, Kᵩ, of 0.0763 ± 0.0063 µM, which is strikingly stronger than that of the GRGDSP peptide (Kᵩ, 270 ± 353 µM), and only moderately weaker than that of the fibronectin (Kᵩ, 0.03 µM). In terms of integrin specificity, studies performed by Kokkoli and colleagues demonstrate that as compared to simple RGD peptides, PR_b is more specific to α₅β₁ integrin. So far PR_b has been applied for the development of targeted drug-delivery systems using various nanoparticles.

Positron emission tomography (PET), with high sensitivity and spatial resolution, has become the predominant strategy for noninvasive visualization and quantification of molecular events in vivo, and fluorine-18 (¹⁸F; 109.8 min half-life) is the most commonly used radionuclide in clinical PET. Peptides are usually radiolabeled with ¹⁸F in a conventional multi-step and time-consuming procedure requiring synthesis and purification of an ¹⁸F-labeled synthon or prosthetic group. McBride et al. developed a novel and facile ¹⁸F-labeling method based on the chelation of ¹⁸F-aluminum fluoride (Al¹⁸F) complex using a peptide-conjugated 1,4,7-triazacyclonane-triacetic acid (NOTA) ligand. The present study explored the development of a novel and facile ¹⁸F-labeling method based on the chelation of ¹⁸F-aluminum fluoride (Al¹⁸F) complex using a peptide-conjugated 1,4,7-triazacyclonane-triacetic acid (NOTA) ligand.
velopment of PR_b as a potential 18F-labeled PET probe for noninvasively visualizing and quantifying $\alpha_\beta$ expression in vivo. PR_b was modified for conjugation with a bifunctional NOTA-based chelator and labeled using the Al18F method. The biological characteristics of 18F-labeled PR_b were evaluated in vitro, ex vivo, and in vivo.

MATERIALS AND METHODS

Peptide Synthesis The PR_b peptide, KSSPHSRN(SG)5RGDSP was synthesized by the standard 9-fluorenylmethoxy carbonyl (Fmoc)-based solid phase method using an Applied Biosystems 433 A peptide synthesizer. Deblocking of the protected peptide was carried out in a solution containing trifluoroacetic acid (TFA), thioanisole, ethanedithiol, phenol, and water at room temperature (RT) for 2 h. For 18F-labeling, one $\beta$-alanine was added to the N-terminus of PR_b to produce the sequence $\beta$-Ala-KSSPHSRN(SG)5RGDSP, which was then conjugated at the N-terminus with the chelating agent 2-S-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid ($p$-SCN-Bn-NOTA, Macroyclics Inc., Dallas, TX, U.S.A.). In brief, this peptide resin (0.1 mmol), irradiation for the production of 18F by the 18O(p, n)18F nuclear reaction using a CYPRIS HM-18 cyclotron (Sumitomo Heavy Industries, Ltd., Osaka, Japan), was then conjugated with the chelating agent and the pH value of the 18F solution was adjusted to 4 with 12 µL of water–acetonitrile (9 : 1, v/v) for 15 min for chelating of Al18F by NOTA. The reaction mixture was diluted with 200 µL of water–acetonitrile (9 : 1, v/v) containing 0.1% TFA and was purified with a semi-preparative RP-HPLC (solvent A=0.1% TFA in water, solvent B=0.1% TFA in acetonitrile, flow rate=5.0 mL/min, linear gradient=10 to 60% solvent B in 16 min, UV absorbance detection at 210 nm, and a Atlantis T3 column [10×150 mm; Nihon Waters K.K.]). The fraction containing the 18F-labeled peptide was collected and dried using a rotary evaporator. After reconstitution in normal saline (NS), purified 18F-PR_b was analyzed by RP-HPLC under the same conditions as described above except for the use of a 1 mL/min flow rate and a 4.6×150 mm Atlantis T3 column, and was analyzed again after 3 h of storage at RT to evaluate its stability.

In vitro plasma stability was examined by incubation of 10 µL 18F-PR_b mixed with 90 µL mouse plasma and 100 µL phosphate-buffered saline (PBS) at 37°C for 1 min and for 30 min. An equivalent quantity of 18F-PR_b was incubated in 190 µL PBS at 37°C for 1 h as a standard control. For in vivo blood stability, 18F-PR_b (ca. 11.1 MBq) was injected into normal mice via the tail vein, and at 10 and 30 min post-injection the mice were euthanized, and blood was collected and centrifuged at 2300 $\times g$ for 5 min at 4°C. The plasma supernatants were collected with an extraction efficiency of >85%. All samples were directly analyzed by RP-HPLC without precipitating plasma proteins because we observed that, after protein precipitation with the addition of either ethanol or acetonitrile, the peptide tended to be retained on the column. The relative radioactivity of the radiolabeled peptide was expressed in milivolts (mV).

Cells and Tumor Models B16-F10 murine melanoma cells (high expression of $\alpha_\beta$, integrin subunit) and SW48 human colorectal carcinoma cells ($\alpha_\beta$-negative) were obtained from the American Type Culture Collection (B16-F10: ATCC CRL-6475; SW48: ATCC CCL-231) and cultured according to their instructions. Animal procedures were approved by the Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS, Chiba, Japan). Four to six-week old female BALB/c-nu/nu athymic mice and C57BL/6 mice (the original source of the B16-F10 melanoma tumor cells) were obtained fromCLEA Japan, Inc. (Tokyo, Japan). Tumor models were established by subcutaneous injection of 1×10⁶ B16-F10 cells or 10×10⁶ SW48 cells into the right shoulder of the mice, and the tumors were used for experiments after reaching 7–10 mm in diameter.

In Vitro $\alpha_\beta$ Expression and Binding Assay Expression status of $\alpha_\beta$ and its 2 subunits in B16-F10 and SW48 cells was validated by flow cytometric analysis. B16-F10 cells (1×10⁶ cells) were incubated on ice for 30 min with primary rat anti-mouse monoclonal antibody to the integrin $\alpha_\beta$ subunit (rat IgG2a, clone 5H10-27, 1:100 dilution; BD Pharamingen, BD Biosciences), $\beta_\alpha$ subunit (rat IgG2b, clone KM16, 1:100 dilution; eBioscience, San Diego, CA, U.S.A.), or $\alpha_\beta$ (rat IgG2b, clone BM85, 1:100 dilution; Millipore, Temecula, CA, U.S.A.), monoclonal rat IgG2a (clone eBR2a, 1:100 dilution; eBioscience) or IgG2b isotype control (clone eB149/10H5, 1:100 dilution; eBioscience), or antibody diluent only (PBS containing 1% bovine serum albumin (BSA) and 1 mm CaCl₂). After washing with cold PBS, the cells were incubated on ice for 30 min with fluorescein-conjugated goat anti-rat immunoglobulin G (IgG) secondary antibody (1:100 dilution; Thermo Scientific, Rockford, IL, U.S.A.).
The cells were washed, resuspended in PBS, and analyzed by flow cytometry using a Guava EasyCyte Plus System (Guava Technologies-Millipore, Hayward, CA, U.S.A.).

Similar procedures were performed to test the expression status of α5β1 in SW48 cells. The cells were directly stained with fluorescein-conjugated mouse anti-human α5 subunit antibody (clone SAM-I, 1:10 dilution; Millipore) or β1 subunit antibody (clone P4G11, 1:100 dilution; Millipore), or indirectly stained by incubation with different clones of mouse monoclonal antibodies against the human α5 subunit (clone SAM-I, 1:50 dilution; LifeSpan BioSciences, Seattle, CA, U.S.A.; clone P1D6, 1:100 dilution; Abcam, Cambridge, U.K.), or mouse anti-human α5β1 monoclonal antibody (clone HA5, 1:100 dilution; Millipore) and the secondary antibody fluorescein-conjugated goat anti-mouse IgG (1:100 dilution; Millipore). Cells treated in the absence of primary antibody or with mouse IgGl isotype control (clone DAK-G01, 1:100 dilution; Millipore) were used as negative controls.

In vitro cell-binding activities of 18F-PR_b were assessed according to our published method. In brief, trypsinized and resuspended cells were incubated with 18F-PR_b or 18F-PR_b control using various buffers, divalent cations, incubation time, temperature, or cell density conditions as indicated in Fig. 4. Competitive inhibition assays were performed by co-incubation of 18F-PR_b with increasing molar concentrations of unlabeled PR_b or NOTA-PR_b. The cell-bound radioactivity was measured using a gamma counter (1480 Wizard 3, PerkinElmer, Inc., Waltham, MA, U.S.A.) with decay correction, and the relative cell-binding ratio was defined as the percentage of the total added radioactivity that was cell-bound. IC50 values of PR_b and NOTA-PR_b were determined by nonlinear regression analysis.

Cell Adhesion Assay The Innocyte Cell Adhesion Microplate Assay Kit (EMD Biosciences, La Jolla, CA, U.S.A.) was used to determine the effect of NOTA-PR_b on B16-F10 cells binding to fibronectin and performed according to the manufacturer’s instructions. In brief, B16-F10 cells suspended in the binding buffer (Hank’s Balanced Salt Solution [HBSS] with 25 mM HEPES and 0.1% BSA for 20 min, and incubated for 1 h in optimized binding buffer (determined by in vitro binding assays) containing 18F-PR_b (ca. 370 kBq). The blocking study was performed in serial sections by incubation of ca. 25 kBq 18F-PR_b or 18F-PR_b control with or without co-incubation of unlabeled PR_b at a concentration of 200 μM, a dose estimated from the results of an in vitro blocking assay. After incubation, the sections were autoradiographed on BAS-MS 2040 imaging plates (FUJIFILM, Tokyo, Japan) for 1 or 12 h and analyzed using a bioimaging analyzer system (FLA-7000; FUJIFILM). Photo-stimulated luminescence per unit area was measured for quantitative comparison of tissue-bound radioactivity.

PET Imaging and Biodistribution Study An Inveon small-animal PET system (Siemens, Knoxville, TN, U.S.A.) was used for dynamic scanning (30 scans of 1 min each) after injection of the imaging agents via tail vein. Three groups of mice were studied, including 2 groups of B16-F10 tumor-bearing mice administered with 18F-PR_b (8.3±2.2 MBq, n=12) and 18F-PR_b control (10±1.9 MBq, n=9), and another group of SW48 tumor-bearing mice administered with 18F-PR_b (8.6±2.6 MBq, n=5). All scanned mice were displayed as a three-dimensional (3D) decay-corrected maximum-intensity-projection (MIP) image using the Inveon Research Workplace software (version 4.0; Siemens). The 3D volume of interest-derived percentage injected dose per gram (%ID/g) values were calculated as described previously. Images were reconstructed using a 3D maximum a posteriori (MAP) method (18 iterations with 16 subsets, β=0.2) without attenuation correction. Image analysis was performed using the ASIPro VM Micro PET Analysis software (Siemens). The total injected dose was calculated using a decay correction of the total activity present at the time of injection (t=0). For radioactivity quantification in the tumor, both kidneys, and urinary bladder, regions of interest (ROIs) encompassing the whole tissue area on each coronal slice were drawn manually, and all ROIs were linked to form a 3D volume of interest (VOI) using the 3D (VOI) dimensionality tool. For each VOI, the percentage of the total injected dose (%ID) was calculated to represent the total activity accumulated in the urinary bladder and both kidneys and the mean %ID/g was used to represent tumor uptake, assuming a tissue density of 1 g/mL. To estimate the radioactivity in the blood pool, a ROI with a fixed size of 0.1 cm² was
placed over the heart, and the radioactivity was quantified as the mean %ID/g. The mean %ID/g of the muscles contralateral to the tumors was also calculated.

Immediately after PET scan, the mice were euthanized, and their blood was drawn. Tumors and major organs were sampled and measured for radioactivity using a gamma counter with decay correction. Radioactivity was expressed as %ID/g normalized to a body weight of 20 g. It should be mentioned that the mice participated in this biodistribution study were randomly selected due to the convenience of experiment schedules.

Statistical Analysis Quantitative data were expressed as mean±standard deviation (S.D.) and compared using a one-way ANOVA with the Dunnett’s multiple comparisons test. A p value <0.05 was considered significant.

RESULTS

Peptide, Radiolabeling, and Stability Peptide synthesis and radiolabeling procedures are schematically summarized in Fig. 1. RP-HPLC analysis and purification of the labeling mixture (Fig. 2A) demonstrated the presence of unbound $^{18}$F or AlCl$_3$-$^{18}$F (retention time $t_R=1.8$ min) and $^{18}$F-labeled PR$_b$ ($t_R=9.2$ min). Two UV peaks with $t_R$ 9.0–9.4 min revealed the mixture of labeled and unlabeled PR$_b$ peptides as well as a possible complex of AlCl$_3$-NOTA-PR$_b$. The process of radio-labeling, including the purification procedure, was completed within 1 h, with a decay-corrected labeling yield of 22.3±1.9% (n=7) based on $^{18}$F-F$^-$. $^{18}$F-PR$_b$ was obtained with >99% radiochemical purity and a specific activity of 30–70 GBq/µmol and was highly stable in NS, with >99% of $^{18}$F-PR$_b$ remained intact for at least 3 h at RT (Fig. 2B). $^{18}$F-PR$_b$ was found to be relatively stable in plasma in vitro (Fig. 2C, left panel). In vivo, the intact probe and a major fraction of the radioactive metabolites were detected in the plasma within 30 min post-injection (Fig. 2C, right panel), demonstrating that $^{18}$F-PR$_b$ was metabolized rapidly, but detectable levels of intact probe remained.

Cellular $\alpha_5\beta_1$ Expression and $^{18}$F-PR$_b$ Binding Flow cytometric analysis confirmed the positive expression of $\alpha_5\beta_1$ in B16-F10 cells (Fig. 3A) and the negligible expression of $\alpha_5\beta_1$ in SW48 cells (Fig. 3B). The binding of $^{18}$F-PR$_b$ to B16-F10 cells was influenced by the choice of incubation buffer, divalent cation composition, incubation time, and temperature (Figs. 4A–D). Based on these results and considering the relatively short half-life of $^{18}$F, the optimized binding conditions were: HBSS containing 25 mM HEPES, 0.1% BSA, and 1 mM CaCl$_2$ as the binding buffer, and 1 h-incubation on ice, and were used for the subsequent in vitro assays unless otherwise stated.

The relative cell binding ratios of $^{18}$F-PR$_b$ to B16-F10 cells increased with increasing cell numbers, in contrast to the negligible binding of $^{18}$F-PR$_b$ to SW48 cells and $^{18}$F-PR$_b$ control to B16-F10 cells (Fig. 4E). Unlabeled PR$_b$ and NOTA-PR$_b$ inhibited the binding of $^{18}$F-PR$_b$ to B16-F10 cells in a similarly concentration-dependent manner, with IC$_{50}$ values of 1.9±1.1 µM and 4.8±2.3 µM, respectively (Fig. 4F).

Effect of NOTA-PR$_b$ versus PR$_b$ on Cell Adhesion to Fibronectin Effects of NOTA-PR$_b$ and PR$_b$ on $\alpha_5\beta_1$-positive B16-F10 cell adhesion to fibronectin-coated surface were examined. NOTA-PR$_b$ was found to be nearly as effective as PR$_b$ for functionally blocking B16-F10 cell attachment to fibronectin (Fig. 5).

Tumoral $\alpha_5\beta_1$ Expression and $^{18}$F-PR$_b$ Binding $\alpha_5\beta_1$
Fig. 2. (A) Representative RP-HPLC Chromatograms (UV at 210nm and Radioactivity [RI]) of \( ^{18}\text{F}-\text{PR}_b \) Labeling Mixture; (B) RP-HPLC Analysis of the Purified \( ^{18}\text{F}-\text{PR}_b \) after Reconstitution in NS (\( T_0 \)) and Again after 3 h of Storage at RT (\( T=3 \) h at RT); A 20\( \mu \text{L} \)-Sample Loop Was Used for Sample Injection; (C) RP-HPLC Analysis of Biological Stability of \( ^{18}\text{F}-\text{PR}_b \) Incubated in Mouse Plasma at 37°C for 1 and 30 min (Left Panel) or in Mouse Plasma Sampled at 10 and 30 min Post-Injection (p.i.)

Different sizes of sample loops, 321-\( \mu \text{L} \) (left panel) and 1-mL (right panel), were used for sample injection.

Fig. 3. Flow Cytometric Analysis of \( \alpha_\beta \) Expression

(A) Representative cytograms of B16-F10 cells incubated with rat anti-mouse \( \alpha_\beta \) or \( \alpha_\beta \) monoclonal antibodies, antibody diluent (BKG), or rat IgG isotype control (IgG2a for \( \alpha \) and \( \beta \); IgG2b for \( \alpha_\beta \)). (B) Representative cytograms of SW48 cells. Upper panel: Cells directly stained with fluorescein-conjugated mouse anti-human \( \alpha_\beta \) or \( \beta \) monoclonal antibodies. Middle and lower panels: Cells incubated with mouse anti-human \( \alpha_\beta \) (clone P1D6 or SAM-1) or \( \alpha_\beta \) monoclonal antibodies, or mouse IgG isotype control.
Immunohistofluorescence showed positive staining in B16-F10 tumors developed in C57BL/6 and nude mice (Fig. 6A, upper panel) and negative staining in murine kidneys (Fig. 6A, upper panel) and SW48 tumors (Fig. 6A, lower panel).

 Autoradiographic evaluation of frozen tissue sections after incubation with 18F-PR_b showed a higher degree of radioactive staining in B16-F10 tumors in C57BL/6 mice than that in nude mice (Fig. 6B, upper panel). A B16-F10 tumor model in C57BL/6 mice was used for the subsequent studies unless otherwise stated.

18F-PR_b binding properties were further investigated in serial sections of B16-F10 tumors, SW48 tumors, and murine kidney (Fig. 6B, middle panel). In contrast to the negligible radioactivity signals in SW48 tumors and kidney, strong radioactivity was observed in B16-F10 tumors, >90% of which could be blocked by unlabeled PR_b. As compared to the 18F-PR_b, 18F-PR_b control showed weaker binding to B16-F10 tumors, with ca. 80% reduction observed after blocking with unlabeled PR_b (Fig. 6B, lower panel).

PET and Biodistribution Studies B16-F10 tumors were clearly visualized using 18F-PR_b with high contrast relative to the contralateral background at 25–30 min post-injection (Fig. 7A), in comparison to the weak radioactivity exhibited by the 18F-PR_b control in the peripheral region of B16-F10 tumors.
between 18F-PR_b and its control probe during the 30-min lateral muscle uptake did not show significant difference ± vs. uptake in B16-F10 tumors than in SW48 tumors (1.35 ± 0.16%ID/g, respectively, at 30 min post-injection, p = 0.0002) and significantly higher uptake in B16-F10 tumors compared to its control probe (1.35 ± 0.16 vs. 1.12 ± 0.25%ID/g at 30 min post-injection, p = 0.023). In contrast, the contralateral muscle uptake did not show significant difference between 18F-PR_b and its control probe during the 30-min scanning period and between C57BL/6 and nude mice from 14 to 30 min post-injection (Fig. 7E). Rapid blood clearance of radioactivity was observed for all the mice evaluated, with no significant difference between 18F-PR_b and its control probe and between C57BL/6 and nude mice (Fig. 7F). Time-activity curves for the total activities of both kidneys plus bladder (Fig. 7G) demonstrated rapid and steady excretion of the radiolabeled peptides by the renal system within 30 min after injection, and showed some difference between the mouse strains and between the different probes.

As shown in Table 1, the ex vivo biodistribution study performed following PET imaging demonstrates tissue distribution profiles of 18F-PR_b and its control probe and confirms the results of PET quantification.

DISCUSSION

There are a few reports on the development of αβ1-specific radiotracers. Neubauer et al. recently reported on a 68Ga-labeled radiotracer based on an aza-glycine derivative of an αβ1 antagonist that was initially characterized by Heckmann et al. based on a nonpeptidic tyrosine scaffold. PET imaging of mice bearing an αβ1-positive tumor and an αβ1-positive tumor on each side of the shoulder demonstrated promising results of this peptidomimetic radiotracer for selective imaging of αβ1. Koivunen et al. isolated a lead peptide GACRRETAWACGA from a phage display library with a high specificity and affinity for αβ1. This peptide was recently radiolabeled with 18F, and it was found to be stable in human serum. However, biodistribution studies demonstrated this radiotracer was not suitable for in vivo imaging due to its considerably high and constant radioactivity accumulation in the blood and other major organs. In contrast to the above-mentioned approaches, PR_b was developed to mimic the natural states of 2 αβ1-binding regions PHSRN and RGD in fibronectin. To the best of our knowledge, the present study is the first to explore the possible functionalization of αβ1-specific fibronectin–mimetic peptide for in vivo imaging of αβ1 expression, and encompasses the design, synthesis, radiolabeling, stability examination, and in vitro, ex vivo, and in vivo bioactivity studies.

Several peptide ligands, including an octreotide analog, a bombesin peptide, and cyclic RGD-containing peptides, have been radiolabeled with 18F, and it was found to be stable in vivo and between C57BL/6 and nude mice (Fig. 7B) and the negligible radioactivity of 18F-PR_b at 30 min post-injection, which was not influenced by the addition of either Mg2+ or Mn2+. These differences may be due to the different states of αβ1 (the isolated αβ1 versus that existing in cells) as well as the different bioevaluation systems. Besides the divalent cations, the binding activities of 18F-PR_b to B16-F10 cells was also influenced by the choice of incubation buffer, incubation time, and temperature, and the involved mechanisms should be clarified in future study.

The αβ1 integrin specificity of PR_b has been extensively studied and validated by Kokkoli and colleagues using blocking studies with specific anti-integrin antibodies and peptides and using scrambled peptide sequences, and further confirmed in αβ1-targeted drug delivery studies. As mentioned, the αβ1-binding affinity of PR_b is only moderately weaker than the native ligand fibronectin. PR_b is hence distinguished from other simple linear RGD peptides that may also bind to several αv integrins, such as αβ1 and αβ5, with very low binding affinity. For example, our previous study demonstrated that a linear fibronectin-derived monomeric AVTGRGDPS peptide, labeled with either 125I or a fluorescent dye Cy5.5, bound in a negligible level to αβ1-overexpressing HEK293(β1) cells under the similar cell-binding assay conditions used for evaluation of 18F-PR_b. In the present work, under in vitro conditions, the αβ1 specificity of 18F-labeled PR_b was supported by comparison of (1) the binding of 18F-PR_b to αβ1-positive B16-F10 and αβ1-negative SW48 cells and (2) the cell-binding activities between 18F-PR_b and its control probe. It should be mentioned that B16-F10 cells had minimal expression of αv inte-

Fig. 5. Inhibitory Effects of NOTA-PR_b or PR_b on B16-F10 Cell Adhesion to Fibronectin-Coated Wells

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grin subunit, and SW48 cells did not express both αVβ3 and αVβ5. Such two cell lines were used in the present study for excluding the influence of other RGD-recognizing integrins, especially αVβ3 and αVβ5. In addition, binding assays using αVβ3-overexpressing HEK293(β3) demonstrate that 18F-PR_b had almost negligible binding activity to αVβ3 integrin (Supplementary Fig. 1). The α5β1-binding specificity of 18F-PR_b was further confirmed by the dose-dependent inhibition of

Table 1. Biodistribution of 18F-PR_b or 18F-PR_b Control in Mice with B16-F10 or SW48 Tumor

| Organ          | 18F-PR_b (B16-F10) | 18F-PR_b control (B16-F10) | 18F-PR_b (SW48) |
|----------------|--------------------|-----------------------------|-----------------|
| Blood          | 1.82±0.26          | 1.61±0.43                   | 1.49±0.42       |
| Tumor          | 1.07±0.14          | 0.88±0.23*                  | 0.79±0.08*      |
| Muscle         | 0.36±0.03          | 0.37±0.13                   | 0.43±0.21       |
| Bone           | 0.72±0.12          | 0.67±0.24                   | 0.68±0.36       |
| Brain          | 0.05±0.003         | 0.04±0.005*                 | 0.04±0.01       |
| Heart          | 0.61±0.02          | 0.47±0.11*                  | 0.49±0.04       |
| Lung           | 1.18±0.08          | 0.78±0.08*                  | 1.04±0.20       |
| Liver          | 0.48±0.04          | 0.30±0.05*                  | 0.39±0.07       |
| Pancreas       | 0.40±0.05          | 0.33±0.02*                  | 0.26±0.01*      |
| Spleen         | 0.41±0.02          | 0.34±0.04*                  | 0.36±0.04       |
| Stomach        | 0.82±0.34          | 0.25±0.04*                  | 0.47±0.11       |
| Small intestine| 0.63±0.04          | 0.45±0.05                   | 0.65±0.36       |
| Large intestine| 0.50±0.06          | 0.52±0.03                   | 0.31±0.08       |
| Kidney         | 73.4±0.62          | 63.8±0.65                   | 62.3±15.8       |
| Skin           | 1.43±0.30          | 1.04±0.27                   | 0.90±0.27       |

Tissue radioactivity was assessed immediately after the PET scan, i.e., at 30 min post-injection of 18F-PR_b or 18F-PR_b control and is expressed as %ID/g (mean±S.D.). n=6–10/group for blood and tumor and n=3–4/group for other organs; *p<0.05 vs. group of B16-F10-bearing mice that received 18F-PR_b.

Fig. 6. Immunohistofluorescence Staining of α5β1 and ex Vivo 18F-PR_b Autoradiography

(A) Upper panel: Frozen sections of B16-F10 tumors in nude mice and in C57BL/6 mice, and murine kidney were incubated with rat anti-mouse α5β1 monoclonal antibody or rat IgG isotype control; Lower panel: Frozen sections of SW48 tumor were incubated with rabbit polyclonal antibody against human and mouse α5 or rabbit IgG isotype control. B16-F10 tumors were used as a positive control. Scale bar=50µm (tumors) or 100µm (kidneys). (B) Upper panel: Frozen sections of B16-F10 tumors in nude mice and in C57BL/6 mice were incubated with 18F-PR_b; Middle panel: Frozen sections of B16-F10 tumor (C57BL/6 mice), SW48 tumor, and murine kidney were incubated with 18F-PR_b in the absence or presence (blocking) of an excess of unlabeled PR_b; Lower panel: B16-F10 tumor (C57BL/6 mice) sections were incubated with 18F-PR_b control in the absence or presence (blocking) of an excess of unlabeled PR_b.

Fig. 7. (A) Representative MIP PET Images of B16-F10 Tumor-Bearing C57BL/6 Mice at 25–30min after Injection with 18F-PR_b; (B) with 18F-PR_b Control; (C) SW48 Tumor-Bearing Mice Administered with 18F-PR_b; (D–E) Time Course of the Radioactivity Concentration in Tumors (D) and Contralateral Muscles (E); (F) Clearance of Radioactivity from the Blood Pool over Time; (G) Time Course of Total Accumulation of Radioactivity in Both Kidneys and the Urinary Bladder

Dotted and solid circles indicate tumor and contralateral muscle locations, respectively. K=kidneys; B=urinary bladder. (a) vs. (b): *p<0.05; (a) vs. (c): *p<0.05.
the binding of $^{18}$F-PR$_b$ to $\alpha_5\beta_1$-positive cells in the presence of unlabeled PR$_b$ or NOTA-PR$_b$. The introduction of the p-SCN-Bn-NOTA-$\beta$-Ala moiety to PR$_b$ was considered to not markedly compromise the receptor binding, because PR$_b$ and NOTA-PR$_b$ showed similar inhibitory effects on the binding of $^{18}$F-PR$_b$ to $\alpha_5\beta_1$-positive cells and on the adhesion of $\alpha_5\beta_1$-positive cells to fibronectin-coated wells.

Ex vivo autoradiographic studies in frozen tissue sections demonstrated similar results as those obtained under in vitro conditions. $^{18}$F-PR$_b$ showed a much higher degree of radioactive staining in $\alpha_5\beta_1$-positive B16-F10 tumor than in $\alpha_5\beta_1$-negative SW48 tumor. The radioactive staining produced by $^{18}$F-PR$_b$ was also found weak in our previously reported $\alpha_4\beta_1$-overexpressing HEK293($\beta_1$) and $\alpha_5\beta_1$-overexpressing HEK293($\beta_3$) tumor models. The low binding affinity of $^{18}$F-PR$_b$ for the $\alpha_5\beta_1$ and $\alpha_5\beta_3$ integrins. The selective binding of $^{18}$F-PR$_b$ to $\alpha_5\beta_1$-positive tumors was further supported by the blocking effect of an excess of unlabeled PR$_b$. Under ex vivo conditions, the $^{18}$F-PR$_b$ control probe showed weak, but specific binding to $\alpha_5\beta_1$-positive tumors. This could be attributed to the receptor-binding activity of the PHSRN motif alone because, in the control probe, the primary binding motif RGD was replaced by RAD, which abolished integrin-binding activity. Feng et al. reported that PHSRN specifically bound to $\alpha_5\beta_1$ but with lower affinity than did RGD.

In vivo, $\alpha_5\beta_1$-positive tumors were visualized using PET in mice injected with $^{18}$F-PR$_b$, and the binding specificity was demonstrated by paralleled comparative studies using $\alpha_5\beta_1$-negative tumors and the RAD-containing control probe. It should be emphasized that RAD probes have been conventionally used as an in vivo control for the corresponding RGD probes because they have similar structural properties (differing only by a single methyl group), but possess discretely different binding abilities for integrin. $^{18}$F-PR$_b$ PET and biodistribution studies did not show obvious radioactivity accumulation in bone and other major normal organs, except the kidneys and urinary bladder, with no obvious sign of bone radioactivity accretion. Since $^{18}$F alone or non-chelated Al$^{18}$F showed considerably high uptake in bone, the above results suggest that, under in vivo conditions, Al$^{18}$F remained predominantly chelated to NOTA, which could be conjugated with the intact peptide or intermediate metabolites, with no significant detachment of $^{18}$F from NOTA. Stability studies (Fig. 2C) demonstrated that $^{18}$F-PR$_b$ was relatively stable in mouse blood plasma, but rapidly metabolized in vivo within 30 min post-injection. The rapid blood clearance and relatively low metabolic stability of $^{18}$F-PR$_b$, which could be attributed to the linear structure of the peptide as observed for other linear peptide-based radiotracers, possibly compromised the efficient accumulation of the probe in the tumor. That is maybe why the differences in tumor uptake between $^{18}$F-PR$_b$ and its control probe were not as large as the differences in cell- and tissue-bound activities between the two probes obtained from in vitro and ex vivo studies. The high kidney uptake is considered to be primarily due to the predominant renal excretion of $^{18}$F-PR$_b$ and its radioactive metabolites because neither the binding of $^{18}$F-PR$_b$ nor the expression of $\alpha_5\beta_1$ by renal tissues was detected by ex vivo autoradiography and immunohistofluorescence staining. Finally, it is necessary to mention that in addition to our extensive discussion of the specificity of $^{18}$F-PR$_b$ for the $\alpha_5\beta_1$ in the present study, a series of blocking studies after further optimization of this probe in terms of metabolic stability, pharmacokinetics, and $\alpha_5\beta_1$-binding affinity would further demonstrate the specificity of the probe.

In summary, the $\alpha_5\beta_1$-specific fibronectin–mimetic peptide PR$_b$ was successfully radiolabeled with $^{18}$F based on the facile Al$^{18}$F method, and investigated in vitro, ex vivo, and in vivo. $^{18}$F-PR$_b$ exhibited its potential for use as an imaging probe for $\alpha_5\beta_1$ positive tumor detection, and is worth further investigation to optimize radiolabeling and to improve its binding activity and pharmacokinetic properties by peptide modification strategies such as multimerization and PEGylation.

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Conflict of Interest The authors declare no conflict of interest.

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