Lung Vascular Targeting Using Antibody to Aminopeptidase P: CT-SPECT Imaging, Biodistribution and Pharmacokinetic Analysis

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Key Words

Aminopeptidase P · Biodistribution · Caveolae · CT-SPECT · Lung targeting

Abstract

Background/Aims: Aminopeptidase P (APP) is specifically enriched in caveolae on the luminal surface of pulmonary vascular endothelium. APP antibodies bind lung endothelium in vivo and are rapidly and actively pumped across the endothelium into lung tissue. Here we characterize the immunotargeting properties and pharmacokinetics of the APP-specific recombinant antibody 833c. **Methods:** We used in situ binding, biodistribution analysis and in vivo imaging to assess the lung targeting of 833c. **Results:** More than 80% of 833c bound during the first pass through isolated perfused lungs. Dynamic SPECT acquisition showed that 833c rapidly and specifically targeted the lungs in vivo, reaching maximum levels within 2 min after intravenous injection. CT-SPECT imaging revealed specific targeting of 833c to the thoracic cavity and co-localization with a lung perfusion marker, Tc99m-labeled macroaggregated albumin. Biodistribution analysis confirmed lung-specific uptake of 833c which declined by first-order kinetics ($t_{1/2} = 110$ h) with significant levels of 833c still present 30 days after injection. **Conclusion:** These data show that APP expressed in endothelial caveolae appears to be readily accessible to circulating antibody rather specifically in lung. Targeting lung-specific caveolar APP

provides an extraordinarily rapid and specific means to target pulmonary vasculature and potentially deliver therapeutic agents into the lung tissue. Copyright © 2010 S. Karger AG, Basel

Introduction

Targeting of imaging agents or drugs to a single organ can facilitate in vivo imaging of molecular pathophysiological events and serve as a delivery system for drugs, nanoparticles, and even genes. This could be especially powerful for diseases that are localized to a single organ. For example, cystic fibrosis, tuberculosis, lung cancer, pulmonary fibrosis, pulmonary hypertension or acute respiratory distress syndrome are all lung pathologies whose treatments would benefit from specific targeting if lung-specific markers/probes were available. Most blood vessels are lined by an attenuated monolayer of endothelial cells that control vascular permeability and prevent free movement of molecules out of the blood and into the tissue. Therefore, tissue-specific endothelial markers directly accessible to the blood offer a means to effective targeting.

Recent large-scale proteomic efforts to map endothelial cell surface expression in different vascular beds have revealed distinct protein profiles in each tissue [1, 2]. Several methods, including monoclonal antibodies generat-

ed using isolated endothelial cell plasma membranes and standard hybridoma technology [3] and phage display immunopanning [4] as well as proteomic subtractive screens using mass spectrometry [1, 2], have each independently identified aminopeptidase P (APP) as a reasonably lung-specific vascular biomarker. APP is expressed by endothelial cells rather specifically in the lung vasculature, beginning at the fifth branch point of the pulmonary artery [1, 2]. It is abundantly concentrated in caveolae at the luminal surface of the lung endothelial cells which are readily accessible to antibodies circulating in the blood [1, 3, 5]. APP is an N-terminal proline-specific exopeptidase that cleaves and inactivates circulating polypeptides such as bradykinin [6, 7] to rapidly terminate vasodilation [8, 9]. APP expression has been reported in various cells and tissues, including lung [10], heart [11], liver [12] and possibly breast [13]. However, only the lung appears to express significant targetable levels of APP at the luminal surface of endothelial cells [1, 2].

We have created monoclonal antibodies specific for rat APP and used them as specific probes *in vitro*, *in situ* and *in vivo* [3–5]. Fluorescently labeled APP-specific antibodies co-localized with endothelial cell markers caveolin-1 and podocalyxin [5], and could target the lung vasculature *in vivo* [3–5]. Electron microscopy studies using these antibodies conjugated to gold nanoparticles have shown that they can bind caveolae on lung endothelial cells *in situ*, are taken up by caveolae, and are transcytosed across the endothelial cell barrier to penetrate deeply into the lung tissue [3]. More recent *in vivo* studies using intravital microscopy and engrafted lung tissue have shown that this trafficking occurs remarkably rapidly. Unlike control antibodies, APP-specific monoclonal antibodies bind to the lung endothelium within seconds of intravenous injection and accumulate throughout the rat lung tissue within minutes. This process is clearly dependent on caveolae, which appear to actively pump the antibodies out of the blood into the underlying tissue, even acting against a concentration gradient [5].

Here, we extend earlier analyses of caveolae targeting using a human/mouse chimeric monoclonal antibody (833c) against APP that is functionally identical to the parent antibody used in previous works [3–5, 14]. We extensively quantify 833c binding *in situ* and *in vivo* and describe for the first time the rather novel and complex biodistribution and pharmacokinetics of 833c *in vivo*, which has not been observed previously for any other antibody. Because of the rapid transcytosis, tissue-specificity, and long-term retention of 833c within lung tissue, APP-mediated transendothelial transport offers

considerable potential for the targeting of drugs, imaging agents, or even gene therapy vectors specifically to the lungs.

Materials and Methods

Materials

All chemicals were purchased from Sigma (St. Louis, Mo., USA) unless otherwise stated. Mouse IgG used as a nonspecific control was supplied by Southern Biotech (Birmingham, Ala., USA). All animal experiments were carried out in accordance with protocols approved by the institutional animal care and use committee. Animals were housed in the animal care facility, and those animals which received radiolabeled antibodies were housed and imaged in a separate lead-shielded animal facility.

Cell Line

The MA104 monkey kidney epithelial cell line (ATCC CRL-2378.1) was grown in medium 199 (Gibco, Invitrogen, Carlsbad, Calif., USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Omega Scientific, Tarzana, Calif., USA), 2 mM L-glutamine, penicillin G (100 units/ml) and streptomycin sulfate (100 µg/ml). Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere. MA104 cells were transfected with APP cDNA cloned into a pCDNA3.1 construct using lipofectamine according to the manufacturer's protocol (Invitrogen). Stably transfected clones were selected using G418 and screened for APP expression by Western blotting and immunofluorescent microscopy using the Tx3.833 antibody as described [4].

Recombinant Antibody Production

833c antibody was generated as described [14]. Briefly, light chain and heavy chain sequences of the Tx3.833 antibody were cloned into tSK2-LC and tSK-HC vectors. 293F cells were transfected with both vectors in a 1:2 heavy chain to light chain ratio with 293fectin according to the manufacturer's instructions (Invitrogen). After a 6-day incubation at 37°C in an 8% CO₂, 100% humidity incubator with a constant agitation of 130 rpm on a rotating platform (IKA KS 260, Wilmington, N.C., USA), recombinant chimeric 833c antibody was purified from the culture supernatant by IMAC on an AKTApurifier UPC 10 (GE Healthcare). Recovery yields were reproducibly ~80 µg/ml of the starting culture medium.

Isolating Membrane Fractions from Silica-Perfused Organs

Endothelial cell luminal plasma membranes were prepared as previously described [15–18]. Briefly, a colloidal solution of silica particles was perfused through blood vessels to selectively coat the luminal surface of endothelial cells. After tissue homogenization, coated membranes were sedimented by 2 rounds of ultracentrifugation through high-density media. This subfractionation yielded a membrane pellet highly enriched in endothelial cells surface markers and markedly depleted of markers of other cell types and subcellular organelles [15–19].

Western Blotting

Whole tissue (H) or silica-coated luminal endothelial cell plasma membranes (P) were isolated from brain, heart, kidney, liver

and lung. Proteins (10 µg per lane) were separated by SDS-PAGE under non-reducing conditions and transferred onto nitrocellulose membranes. Recombinant APP (25 ng) was used as a positive control. Membranes were blocked with gelatin and probed with 833c for 2 h at room temperature followed by a goat anti-human IgG/HRP-conjugate (Southern Biotech) for 1 h at room temperature. Proteins were visualized using SuperSignal West Pico chemiluminescent substrate according to the manufacturer's instructions (Pierce, Rockford, Ill., USA).

833c Radiolabeling and Determination of Immunoreactive Fraction

833c was radiolabeled with iodine-125 (PerkinElmer, Waltham, Mass., USA) using iodobeads (Pierce) according to the manufacturer's instructions, to a specific activity of 7 µCi/µg. The immunoreactive fraction after radiolabeling was determined by transformation of saturation binding data [20]. Briefly, 2×10^4 cpm of ^{125}I -833c mAb was incubated with increasing numbers of MA104 cells stably transfected with APP at 37°C for 2 h. Experiments were performed in triplicate on serial dilutions of cells in a range of concentrations from 1.7×10^5 to 5.4×10^6 cells/ml. The cell suspensions were then extensively washed and bound radioactivity was measured by a CobraII Auto-Gamma counter (Packard). Nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled 833c. Reciprocal values of the data were fitted with a least squares linear regression method and immunoreactivity was estimated as the reciprocal value of the fraction bound intercept.

Isolated Perfused Rat Lungs

Sprague-Dawley rats (males, 125 g; Harlan) were anesthetized by intraperitoneal injection of a ketamine (50 mg/kg) and xylazine (10 mg/kg) mixture. Lungs were isolated from anesthetized rats after intracardiac injection of 100 IU heparin. The isolated perfused lung system was set up as described [5] with some modifications. Briefly, isolated lungs were suspended in a water-jacketed, humidified chamber and ventilated at a respiration rate of 80 cycles/min, an inspiratory pressure of 9 cm H₂O, and a positive end-expiratory pressure of 2.5 cm H₂O. The lungs were perfused through the pulmonary artery by a peristaltic pump at a constant flow of 0.04 ml/g body weight/min. The perfusion buffer contained 3% hydroxyethyl-dextran-450, 140 mM NaCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, and 5 mM glucose at pH 7.4. Effluent perfusate drained from a left atrial cannula into a perfusate reservoir. After the blood was flushed out of the lungs with 15 ml of perfusion buffer, 1 µg of ^{125}I -833c or ^{125}I -IgG control was injected through the perfusing line. Lungs were additionally perfused with 50 ml of perfusion buffer to wash unbound radioactivity was measured in perfusate and lungs using a gamma-counter (CobraII Auto-Gamma, Packard).

CT-SPECT Imaging

CT-SPECT imaging was performed using an X-SPECT second generation MicroSPECT imaging system (Gamma Medica, Northridge, Calif., USA). Sprague-Dawley rats (males, 150–175 g; Harlan) were intravenously injected with 30 µCi of ^{125}I -833c mAb. Static planar acquisitions were recorded 10 min, 1, 4, 8 and 24 h after injection using a high-resolution parallel hole collimator over a 10-min counting period with γ-camera heads in anterior and posterior positions relative to the animal body. Then, CT

and SPECT scans were acquired 24 h after injection. SPECT images were collected in a 360° orbit with 30-second sampling every 6° with high-resolution parallel hole collimators and the pulse-height analyzer window set over the 30 keV photopeak of iodine-125 and over the 150 keV photopeak for technetium-99m. Tomographic reconstruction was performed using a standard filtered back projection. Three-dimensional data sets after CT-SPECT fusion were processed on the Amira System (TGS Inc.). Dynamic SPECT analysis of activity accumulated in regions of interest over time was performed at a sequence of 30-second time frames for 10 min using high-sensitivity parallel hole collimators. Data were further analyzed using LumaGEM/Mirage software (Segami, Columbia, Md., USA).

Biodistribution Analysis

Sprague-Dawley rats (males, 110–125 g; Harlan) were used for biodistribution analysis. Rats were intravenously injected with 1 µg of ^{125}I -833c as a bolus dose via the tail vein. At different time points, major tissue types were dissected from 7–10 rats, weighed, and assayed for radioactivity on a CobraII Auto-Gamma counter (Packard) at 60-second counting periods. Uptake values were corrected for radiodecay and expressed as a percentage of the injected dose per gram of tissue (%ID/g).

Pharmacokinetic Analysis

The time profile of the blood activity of ^{125}I -833c after intravenous injection was analyzed according to a 2-compartment open model, selected on the basis of the minimal value of Akaike's information criterion. The bi-exponential pharmacokinetic disposition function, %ID/g (t) = $A\exp(-\alpha t) + B\exp(-\beta t)$, where $t_{1/2}(\alpha) = \ln 2/\alpha$, $t_{1/2}(\beta) = \ln 2/\beta$, was applied to describe decline of ^{125}I -833c uptake in the blood. A non-compartmental method was used to estimate the total blood clearance (CL) as well as the distribution volume at steady state (V_{ss}). Total blood clearance was calculated according to the following equation: CL = injected dose/AUC₀[∞], where the area under the uptake-time curve (AUC₀[∞]) was calculated by numeric integration with extrapolation to infinity. The mean residence time (MRT) was obtained by a non-compartment analysis based on the statistical moment theory: MRT = AUMC₀[∞]/AUC₀[∞], where AUMC₀[∞] is the area under the first moment curve from zero to infinity. The distribution volume at steady state was calculated by the equation V_{ss} = CL × MRT.

Results

833c Monoclonal Antibody Specifically Recognizes APP Expressed on the Luminal Surface of Pulmonary Endothelium

Tx3.833 is a murine monoclonal antibody specific to rat APP that targets rat lung endothelial cells in vivo when injected intravenously [3–5]. We have built a human/mouse chimeric version of the Tx3.833 APP antibody, 833c, by cloning the murine V_H and V_L domains into the tSKC-HC and tSV2-LC vectors, respectively. The variable domains of 833c are identical to the murine antibody Tx3.833 variable domains while the Cκ domain,

Fig. 1. 833c specifically recognizes APP on Western blots. Total tissue homogenate (H) or silica-isolated endothelial plasma membranes (P) were isolated from brain, heart, kidney, liver and lung and were run on non-reducing SDS-PAGE gels. 833c recognized a 90 kDa band that was only visible in lung and highly enriched in the plasma membrane fraction. Recombinant APP (rAPP) was used as a positive control.

the IgG1 hinge region, the IgG1 CH1, CH2, and CH3 domains are of human origin. Expression by large-scale transient transfection in 293 cells yielded 80 mg/l of a chimeric antibody with a human IgG1 backbone [14]. As expected, the reactivity of 833c is identical to that of the parent antibody. 833c specifically recognized recombinant APP on the Western blot (fig. 1). 833c also detected endogenously expressed APP. As shown in the Western blot analysis of silica-coated luminal endothelial cell membranes isolated from multiple organs, only the lung was reactive, showing a single 90-kDa band highly enriched in the isolated endothelial cell membranes relative to the starting tissue homogenate (fig. 1).

833c Retains Immunoreactivity after Radiolabeling

833c was radiolabeled with 125 -iodine to analyze biodistribution and immunotargeting in vivo. Because the radiolabeling process might compromise antibody immunoreactivity, we determined the immunoreactive fraction of 125 I-833c from saturation binding data by extrapolating antibody binding at an infinite excess of antigen. Specific binding was determined by subtracting non-specific binding from total binding (fig. 2a). After

reciprocal transformation, linear regression analysis showed optimal correlation ($r = 0.9989$). The percentage of the immunoreactive fraction was then estimated to be 86.9% by linear extrapolation to the fraction bound intercept (fig. 2b). Small amounts of non-immunoreactive antibody (13.1%) might be a result of the radiolabeling procedure or conditions of production, purification or storage. However, the estimated immunoreactivity, 86.9%, is more than sufficient for in vivo biodistribution and imaging study. Therefore, the radiolabeled APP-specific 833c antibody provided us with a reliable probe to track and quantify binding both in vitro and in vivo.

Uptake of 125 I-833c during the First Pass through Isolated Perfused Lung

We used the isolated perfused rat lung system to analyze the dynamics of binding of 125 I-833c to pulmonary endothelium in situ. Isolated, ventilated lungs were perfused with buffer containing 125 I-833c. Surprisingly, after a single pass through lung vasculature with no recirculation of the perfusate and antibody, we observed that 80.5% of the injected dose (%ID) of 833c remained apparently bound in the lung. In contrast, only 0.7 %ID of control IgG was bound. After correction for the non-reactive fraction, the single-pass lung uptake corresponded to 92.6% of the immunoreactive antibody.

125 I-833c Targeting of Rat Lung Evaluated by CT-SPECT Imaging

To analyze the temporal and spatial distribution pattern of 833c in vivo, rats were intravenously injected via the tail vein with radiolabeled antibody and imaged by γ -scintigraphy and CT coupled with SPECT. The SPECT component provides the 3-dimensional spatial distribution of radiolabeled antibody and CT provides anatomical information. Thus, the CT-correlated SPECT images provide essential data about tissue-specificity of antibody targeting in an anatomical context.

Static/planar imaging at 10 min, 1, 4, 8, and 24 h after injection revealed clear, lung-specific immunotargeting of 125 I-833c (fig. 4a). The maximum lung signal was obtained within 10 min, the first time point acquired. The lung-specific signal persisted 24 h after injection. When compared to the control IgG, it was quite apparent that other tissues/organs beyond lung showed little to no specific accumulation of 125 I-833c. A thyroid signal was also absent, indicating negligible dehalogenation of the antibody to produce free radioiodine, as usually observed for iodinated antibodies circulating for several hours in the blood [5]. When we injected control 125 I-IgG, it was dif-

Fig. 2. Determination of the immunoreactive fraction of ^{125}I -833c from saturation binding curve. **a** Total, non-specific and specific binding of ^{125}I -833c mAb to MA104 cells expressing APP. ^{125}I -833c was incubated with indicated amount of MA104/APP cells at 37°C for 2 h, than washed, lysed and bound radioactivity was measured ($n = 3$). Nonspecific binding was determined by addition of 100-molar excess of unlabeled 833c. **b** The immunoreactive fraction was determined by extrapolation of antibody binding at infinite antigen excess. Reciprocally transformed data were fitted with a least squares linear regression method and immunoreactivity was calculated as the reciprocal value of the fraction bound intercept.

fusely distributed throughout the body with the highest signals seen in heart, liver and other blood-rich and well-vascularized tissues, consistent with signal reflecting radiolabeled antibody circulating in the blood (fig. 4b, and see also biodistribution analysis below and fig. 7a). As expected, the circulating ^{125}I -IgG was subject to dehalogenation which resulted in the accumulation of ^{125}I -associated signal in the stomach and thyroid (fig. 4b, 24 h).

To more accurately describe antibody trafficking at early time points after injection, we performed dynamic SPECT and acquired successive images during the first 10 min after intravenous injection. Analysis of time-activity curves estimated from dynamic SPECT acquisitions showed that the maximum signal of ^{125}I -833c in the lungs was reached within 2 min after injection. Remarkably 78% of this maximum uptake was already reached within the first 30 s (fig. 4e).

Several methods were used to verify that the signal from 833c was specifically localized in the lungs. First, CT-SPECT analysis confirmed localization of ^{125}I -833c related γ -emission to the thoracic cavity (fig. 4c, d), apparently occupying the entire lungs while the heart tissue appeared as a dark shadow. Nuclear signal outside the thoracic cavity reached only background levels.

Secondly, lung vasculature was visualized using an X-ray contrast agent. Rats were injected with ^{125}I -833c; 24 h

Fig. 3. Uptake of 833c in isolated perfused lungs. Isolated, perfused and ventilated rat lungs suspended in water jacketed chamber were perfused with buffer containing $1\text{ }\mu\text{g}$ of ^{125}I -833c or ^{125}I -IgG. Lungs were then perfused with buffer to wash unbound antibody. Activity was measured using a gamma-counter and corresponding uptake is expressed as the % of injected dose (%ID) retained in the lung.

Fig. 4. In vivo imaging of immunotargeting with radiolabeled 833c mAb. Rats were intravenously injected with ^{125}I -833c (30 μCi at 6 $\mu\text{Ci}/\mu\text{g}$). **a, b** Planar γ -scintigraphic images were acquired at 10 min, 1, 4, 8 and 24 h after i.v. injection of ^{125}I -833c (**a**) or control ^{125}I -IgG (**b**). At the first imaged time point (10 min) the 833c antibody already showed specific signal in the lungs. No significant uptake was seen in other organs. **c, d** CT-SPECT images of ^{125}I -833c 24 h after injection. **c** Coronal, sagittal and axial CT-slices and corresponding CT-SPECT fused slices. Specific signal associated with thoracic cavity is apparent. **d** 3D-image of CT-SPECT fusion (left, frontal and right views); presented as skeletal CT isosurface and volumetric SPECT texture. **e** Time-activity curve of ^{125}I -833c antibody uptake in the lungs as estimated from dynamic acquisition.

later the lungs were dissected, perfused with a suspension of barium sulfate through the pulmonary artery and imaged with CT-SPECT. 833c could be seen to fill the lungs (fig. 5a) while the vasculature of the lungs was clearly delineated by CT (fig. 5b). Three dimensional imaging showed that ^{125}I -833c was indeed fully contained within the lungs consistent with ample antibody penetration throughout the entire lungs (fig. 5c).

Finally, macroaggregated albumin labeled with $^{99\text{m}}\text{Tc}$ -Technetium ($^{99\text{m}}\text{Tc}$ -MAA) was used to image lungs. $^{99\text{m}}\text{Tc}$ -MAA is a clinically used lung-perfusion marker which selectively embolizes in lung capillaries [21]. We injected $^{99\text{m}}\text{Tc}$ -MAA 15 min after ^{125}I -833c administration and SPECT images were obtained afterwards. Since ^{125}I and $^{99\text{m}}\text{Tc}$ radioisotopes have substantially different energy spectra, signals emanating from ^{125}I and $^{99\text{m}}\text{Tc}$ can be clearly differentiated (fig. 5d), allowing distinct 3-dimensional images to be discerned when these iso-

topes are co-imaged. Yet clearly, ^{125}I -833c and $^{99\text{m}}\text{Tc}$ -MAA co-localized within the lungs, showing complete overlap (fig. 5e).

Biodistribution Profile of ^{125}I -833c

To quantify specific targeting in vivo, including the time-dependent uptake of 833c antibody in multiple organs, comprehensive biodistribution analysis was performed on a large group of animals (table 1 and fig. 6). Consistent with our results using static planar imaging and dynamic SPECT, ^{125}I -833c uptake was indeed rapid and quite lung specific. As early as 5 min after injection, the first time point measured, ^{125}I -833c activity had already reached its maximum in the lungs and was depleted from the blood. 101.2 %ID/g was found in the lungs (corresponding to the extraction of >70 %ID of 833c) with only 1.8 %ID/g in the blood. The lung-to-blood ratio was 56.2, consistent with strong targeting and pumping

Fig. 5. 833c is targeted specifically to the lungs. ^{125}I -833c was injected intravenously. 24 h later, lungs were isolated, perfused with X-ray contrast agent via pulmonary artery and planar γ -scintigraphic (a), X-ray (b) and tomographic CT-SPECT images (c) were acquired. X-ray and CT scans showed pulmonary arterial tree. 3D visualization after CT and SPECT data sets shown in overlap (c). Co-localization of ^{125}I -833c and $^{99\text{m}}\text{Tc}$ -MAA. 30 μCi of ^{125}I -833c was injected intravenously followed by administration of 30 μCi of $^{99\text{m}}\text{Tc}$ -MAA as a lung perfusion marker 15 min later. CT-SPECT images were acquired 30 min after 833c injection. The corresponding energy spectra with pulse-height analyzer windows for 125-iodine (red) and 99m-technetium (green) are shown (d). ^{125}I -833c signal (red) and $^{99\text{m}}\text{Tc}$ -MAA signal (green) co-localized extensively within the lung (e).

of the antibody to the lungs, as reported [5]. Given that blood volume is approximately 7% of the total body weight (approx. 7–8 ml in the 120 g rat), one can easily estimate that the 13% nonreactive fraction of ^{125}I -833c is most of the 14% ID remaining in the circulation. The non-immunoreactive fraction may also contribute to the low signal detected in the liver (4.4 %ID/g), heart (1.1 %ID/g) and kidney (0.5 %ID/g). Uptake in these tissues decreased over time and displayed only residual values at 24 h or later. On the contrary, lung tissue showed significant uptake of ^{125}I -833c at 24 h after injection. Of course, the ^{125}I -833c signal in the lungs did subsequently decline with time; however, significant uptake was still observed 30 days after injection. Specific activity was still present in the lungs even 60 days later (table 1).

Immunospecificity and localization ratios were used as indexes of specificity of targeting 1 h after injection.

Uptake-based immunospecificity (%ID/g) is defined as the ratio of the uptake of target-specific antibody to control IgG in a given organ. The localization ratio is the ratio of tissue uptake to antibody retained in blood. The ratio of localization ratios for target-specific antibodies and control antibodies is defined as immunospecificity (LR) [13]. These uptake-derived indicators of specificity were calculated from biodistribution data 1 h after injection (fig. 7a–d). Over 90% of 833c uptake was seen in lung tissue. This was highly specific for ^{125}I -833c. Little to no accumulation of the control ^{125}I -IgG was seen in the lung or in any other tissue (fig. 7a). Immunospecificity, based on uptake values, exceeded a score of 400 for lung tissue while all other tissues scored under 10 (fig. 7b). Values of the localization ratio indicate that control ^{125}I -IgG was primarily in the blood pool (all other tissues showed values ≤ 0.2) whereas ^{125}I -833c was quantitatively extracted

Table 1. Biodistribution profile of ^{125}I -labeled 833c mAb

%ID/g	5 min	1 h	4 h	24 h	2 days	3 days	5 days	10 days	20 days	30 days	60 days
Lungs	101.2 $\pm$ 8.4	93.8 $\pm$ 7.6	83.6 $\pm$ 2.6	83.7 $\pm$ 4.6	75.3 $\pm$ 2.3	63.9 $\pm$ 8.5	42.6 $\pm$ 3.3	30.6 $\pm$ 4.1	13.3 $\pm$ 1.1	4.2 $\pm$ 0.8	1.3 $\pm$ 0.2
Blood	1.8 $\pm$ 0.5	0.8 $\pm$ 0.5	0.9 $\pm$ 0.2	0.5 $\pm$ 0.1	0.2 $\pm$ 0.0	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Liver	4.3 $\pm$ 0.5	4.4 $\pm$ 0.7	4.4 $\pm$ 1.3	1.7 $\pm$ 0.6	0.5 $\pm$ 0.1	0.5 $\pm$ 0.0	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Spleen	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Kidney	0.5 $\pm$ 0.1	0.2 $\pm$ 0.2	0.3 $\pm$ 0.0	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Bone	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Stomach	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.3 $\pm$ 0.0	0.6 $\pm$ 0.2	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Esophagus	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.2 $\pm$ 0.0	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Colon	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1	0.5 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Heart	1.1 $\pm$ 0.1	0.7 $\pm$ 0.1	0.9 $\pm$ 0.1	0.9 $\pm$ 0.2	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Pancreas	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Brain	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Muscle	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Skin	0.3 $\pm$ 0.3	0.0 $\pm$ 0.1	0.5 $\pm$ 0.0	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Urinary bl.	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.3 $\pm$ 0.0	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Thymus	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

Table summarizing results of ^{125}I -833c uptake in various tissues of Sprague-Dawley rats over 60 days. Groups of rats ($n = 7-10$) were intravenously injected with $1\text{ }\mu\text{g}$ of ^{125}I -833c, dissected at indicated time points and analyzed for accumulated activity. Uptakes are expressed as %ID/g with corresponding standard deviations.

Table 2. Pharmacokinetic parameters

PK parameters	
$A, \text{ \%ID} \cdot \text{g}^{-1}$	12.45
$B, \text{ \%ID} \cdot \text{g}^{-1}$	1.63
$\alpha, \text{ h}^{-1}$	0.214
$\beta, \text{ h}^{-1}$	0.005
$t_{1/2}(\alpha), \text{ h}$	3.23
$t_{1/2}(\beta), \text{ h}$	152.73
$\text{AUC}, \text{ \%ID} \cdot \text{h} \cdot \text{g}^{-1}$	41.7
$\text{AUC}, \mu\text{g} \cdot \text{h} \cdot \text{g}^{-1}$	0.3
$\text{MRT}, \text{ h}$	158.3
$\text{CL}, \text{ ml} \cdot \text{h}^{-1}$	0.22
$\text{V}_1, \text{ ml}$	6.6
$\text{Vd}(\text{area}), \text{ ml}$	48.8
$\text{Vd}(\text{extrap.}), \text{ ml}$	61.5
$\text{Vss}, \text{ ml}$	34.8

Values were determined to describe time-dependent behavior of ^{125}I -833c activity remaining in blood (beginning 5 min after administration). A_α = Rapid-clearing component of the 2-compartment pharmacokinetic model; B_β = slow-clearing component; %ID/g = injection dose per gram; α = exponential rate constant for the α -phase of plasma decay; β = exponential rate constant for the β -phase of plasma decay; $t_{1/2}(\alpha)$ = half-life of the α -phase of plasma decay; $t_{1/2}(\beta)$ = half-life of the β -phase of plasma decay; AUC_0^∞ = area under the concentration-time curve from zero to infinity; MRT = mean residence time; CL = clearance; V_1 = distribution volume; $\text{Vd}(\text{area})$ = apparent volume of distribution; $\text{Vd}(\text{extrap.})$ = extrapolated volume of distribution; Vss = distribution volume at steady state.

from circulation (124-fold enrichment in lungs over blood; fig. 7c). The immunospecificity (LR) was superior for lung (4,340) compared to all other tissues (≤ 72 ; fig. 7d). Thus, several different methods to quantify degree and specificity of targeting all indicate very specific lung targeting with 833c.

Pharmacokinetic Analysis of 833c

Pharmacokinetic analysis of biodistribution data provides the most comprehensive insight on specificity of targeting. Perhaps the most accurate index of specificity of accumulation and degree of immunotargeting reflecting the whole temporal period is cumulative residence, which is based on the numerical integration of tissue uptake over time with extrapolation to infinity (AUC_0^∞). Cumulative residence of ^{125}I -833c in the lungs was vastly superior to other tissues (fig. 8) The AUC_0^∞ for lungs was 21,000 and ≤ 200 for all other tissues.

We also analyzed the kinetics of the 833c signal in the lungs in order to quantify antibody retention in lung tissue. The lung signal from ^{125}I -833c decreased by first-order kinetics with an elimination rate constant, k , of 0.006 h^{-1} , corresponding to a $t_{1/2}$ of 110 h. This indicates very slow release or catabolism of ^{125}I -833c in the lung tissue.

To move beyond analysis of tissue-specificity of 833c accumulation, we used PK analysis to create a limited de-

Fig. 6. Time dependent biodistribution profile of 833c. Rats were intravenously injected with 1 μ g of ¹²⁵I-833c and uptake in selected tissues (lung, blood, liver, spleen, kidney and brain) was determined at different time points (beginning at 5 min and continuing through 30 days). Uptakes are expressed as mean values (n = 7–10) with corresponding SD bars.

scription of time-dependent profile of 833c in the blood. As seen from dynamic acquisition (fig. 4e), initial lung uptake of ¹²⁵I-833c reached a maximum within the first 2 min. Unfortunately, at the first time point adequate for biodistribution analysis (5 min after injection, assuming complete mixing of injected bolus with blood), the vast majority of the antibody (immunoreactive fraction of ID) had already been extracted from the blood. Any activity of ¹²⁵I-833c in the blood after 5 min thus mostly reflects the signal from the non-immunoreactive fraction of antibody and relates little to any further lung targeting and uptake. Remaining antibody available in the blood pool

showed an extensive distribution phase, $t_{1/2}(\alpha) = 3.2$ h, related to extravasation of 833c to the interstitium of internal organs and a prolonged elimination phase, $t_{1/2}(\beta) = 152.7$ h, which is related to the clearance of ¹²⁵I-833c from blood to the nonextravascular space or excretion from the body. The uptake-time profile of 833c in the blood followed the kinetics of a 2-compartment pharmacokinetic model described by a bi-exponential equation with a 4-parameter fit: $\%ID/g(t) = 12.45 [\exp(-0.214t)] + 1.63[\exp(-0.005t)]$.

The pharmacokinetic parameters are summarized in table 2. The distribution volume of the central compart-

Fig. 8. Cumulative residence of ^{125}I -833c in different tissues. Cumulative residence was calculated by numerical integration of fraction of injected dose of ^{125}I -833c in a particular tissue ($n = 7-10$) over time with extrapolation to infinity (AUC_0^∞). Cumulative residence of 833c in the lungs is substantially superior compared to all other tissues.

Fig. 7. Specificity of ^{125}I -833c targeting quantified by immunospecificity and localization ratio indexes. The uptake of ^{125}I -833c and ^{125}I -IgG were determined 1h after administration (**a**). The uptake-based immunospecificity index (%ID/g; **b**) was calculated as the ratio of ^{125}I -833c to ^{125}I -IgG found in each tissue. The localization ratio was based on the tissue-to-blood ratios (**c**), then localization ratio-based immunospecificity (LR) was calculated from localization ratios of ^{125}I -833c to ^{125}I -IgG (**d**).

ment V1 was 6.6 ml corresponding with the expected plasma volume of a rat. The distribution volume at steady state, V_{ss} , was 34.8 ml and corresponds with distribution of ^{125}I -833c outside of the circulation. Total blood clearance, CL, was 0.22 ml/h. These pharmacokinetic parameters are not corrected for the immunoreactive fraction; thus, they reflect a complex pattern of rapid uptake of completely immunoreactive 833c antibody and relatively slow clearance of the non-immunoreactive portion of ^{125}I -833c antibody.

Discussion

Antibodies against the membrane-bound form of APP can target lung vasculature and are rapidly transcytosed into the underlying tissue by caveolae [3, 5]. Here, we expand on these findings by performing, for the first time, a detailed biodistribution and pharmacokinetic analysis to describe the processing of 833c *in vivo*. We show that 833c is very rapidly and efficiently extracted from the circulation by lung endothelium, both *in situ* and *in vivo*. Almost all active 833c (91.9 % of the immunoreactive fraction) binds to the lung vasculature during the first pass through isolated perfused lung. This binding is much higher than that achieved with other antibodies recently used to target lung vasculature. Only 20–45 %ID of antibodies against endothelial specific proteins localized outside of caveolae such as ICAM-1, Thy1.1, ACE and OX-43 accumulate in lung tissue when perfused through isolated lungs [22]. Similar binding of 833c could be seen *in vivo*. Dynamic acquisition showed that maximum uptake of 833c in lung was reached within 2 min after injection with little further lung uptake and little specific accumulation in other organs.

The immunotargeting efficacy and specificity of intravenously injected 833c antibodies seen here likely depends on a number of factors. APP is expressed at high levels on lung vasculature [5] and the pulmonary endothelium comprises approximately 30% of the vascular surface area of the body, providing a great deal of surface area to bind antibody. Also, the lungs are the only organ to receive the entire cardiac output. The *in situ* experiments suggest that most of 833c may actually be cleared from the blood during the first pass through the lungs, before the injected antibody even has a chance to equilibrate in the full blood volume after injection. This rapid clearance likely also limits potential non-specific uptake by other organs, as observed *in vivo*.

Efficient targeting can be prevented *in vivo* if antibodies bind to non-target tissue or if they are scavenged by the reticuloendothelial system (i.e. liver, spleen). APP expression has been reported in other cell types located within the tissue, such as epithelium in renal proximal tubules; however, extravascular localization renders renal APP quite inaccessible to circulating anti-APP antibodies, especially within minutes after injection. APP may also be expressed on white blood cells [23]. However, since 833c was rapidly cleared from the blood and showed no specific blood binding, it is unlikely that 833c binds to white blood cells. An antibody to OX-45, a protein found on lung endothelial as well as white blood cells, is retained in the buffy coat subfraction of the blood and can only target the lung when blood cells are removed [2].

Other proteins, including PECAM-1, podocalyxin, CD34 and ACE showed moderate lung-specificity of expression and antibodies against these proteins have been used to target the lungs *in vivo* [24]. Likely due, in part, to expression in multiple tissues, antibody uptake was lower than what we observed with 833c. Podocalyxin is widely expressed in endothelial cells and antibodies against podocalyxin accumulate in several tissues [24]. PECAM antibodies show targeting primarily in liver and lung, CD34 and ACE antibody targets lungs, heart and possibly liver. In mice and humans, CD34 is found in all endothelial cells but is a restricted marker of lung endothelium in the rat [25]. Overall, the *in vivo* distribution of each antibody, including APP antibodies, correlates well with the Western analysis of protein expression at the luminal surface of vascular endothelial cells from multiple tissues [5].

Whole-animal imaging and biodistribution analysis shows that ^{125}I -833c specifically accumulates in the lungs with little to no significant uptake in other tissues. Control ^{125}I -IgG shows a markedly different pattern. The signal spread diffusely throughout the body and began to accrete in the thyroid by 24 h, suggesting that the ^{125}I -IgG antibody is dehalogenated. Rapid extraction of ^{125}I -833c from circulation by pulmonary endothelium and overall persistent uptake of ^{125}I -833c in lung tissue suggests slow catabolism and protection from the dehalogenation that readily occurred with control ^{125}I -IgG.

APP is highly enriched in endothelial cell caveolae [4, 5, 26]. Thus, whether an antibody binds within caveolae or elsewhere on the endothelial cell surface is likely a key factor determining how the endothelium processes each antibody. The pumping capability of caveolae may contribute significantly to the extraordinary degree and rapidity of antibody uptake in the lung [5]. Unlike the APP antibodies, ^{125}I -labeled antibodies that bind at the endo-

thelial cell surface (but not in caveolae) can rapidly target lung but then undergo dehalogenation by exposure to the blood, resulting in rapid loss of signal [5].

It is necessary to take into consideration the impact of the radiochemistry of antibody labeling on the uptake, metabolism and subsequent clearance of antibody. After cellular internalization, ^{125}I -radiolabeled antibodies are processed in the lysosomal compartments and iodotyrosine catabolites are rapidly excreted from the cells [27]. This might reduce mean residence time of antibody radiolabel in target tissue. This problem can be partially overcome by alternative labeling methods of antibodies with radioiodinated derivatives of non-metabolizable entities containing unnatural D-amino acids [28] or with inert disaccharides, such as tyramine-cellobiose [29]. The significantly extended half-life of 833c antibody signal in lung tissue indicates a different route of processing than internalization by endothelial cells and trafficking into the lysosomal compartment with subsequent antibody degradation. Instead, it correlates with rapid transcytosis of APP-targeted probes across the endothelium into the lung interstitial space [5] and suggests that the antibody remains in the interstitium for an extended period of time without extensive uptake by surrounding pulmonary cells and subsequent dehalogenation.

Although specific, non-caveolar plasma membrane targets may be useful for some applications, such as limiting or promoting local coagulation, inflammation, or even tissue destruction, the ability of APP antibodies to target caveolae and penetrate into lung tissue may provide a pathway to bypass the normally restrictive endothelial cell barrier. This can direct drug, nanoparticles and even gene delivery systems deep within a tissue to the actual cells where these agents can be rendered effective in improving the diagnosis, functional imaging and treatment of lung diseases. By targeting imaging agents to the lungs, 833c can facilitate the high-resolution, in vivo imaging of molecular events that occur at the endothelial cell surface or even, and perhaps more important-

ly, inside the tissue without the need for more invasive techniques, such as surgery or biopsy. Recent probes have been developed that dynamically follow changes in oxygen levels, pH [30], enzyme activity [31, 32], gene expression [33, 34], and apoptosis [35, 36]. Coupled with such reporters, 833c could target labeled therapeutics to the lungs where drug processing and efficacy could be followed over time [33, 36–39].

Targeting caveolar proteins such as APP provides a unique means to actively pump antibody across the endothelium and into underlying tissue. For basic research, this tissue penetrating may allow the structure and function of the lungs to be explored dynamically in vivo. The applications for clinical discoveries are particularly profound, especially for genetic and acquired diseases of the lung, such as acute lung injury, cystic fibrosis, chronic obstructive pulmonary disease, pneumonia, pulmonary fibrosis, pulmonary hypertension, and acute respiratory distress syndrome. By targeting and concentrating imaging agents or therapeutics to the lungs, non-specific side effects can be virtually eliminated. Pathological changes can be identified earlier and followed over time. Additionally, therapeutics that have been abandoned due to toxic side effects may find renewed utility when coupled with targeting antibodies such as 833c. The efficacy of antibody-targeted delivery depends vitally on the pharmacokinetics of antibody processing. As we show here, APP antibodies are rapidly removed from the blood within 2 min, concentrated in the lungs where they are protected from further degradation, and maintained in tissue for up to 30 days. Targeting tissue-specific caveolar proteins may therefore provide a pathway to rapidly and specifically concentrate molecules in a chosen tissue.

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