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Comparative analysis of peptide p5 and serum amyloid P component for imaging AA amyloid in mice using dual-isotope SPECT

Jonathan S. Wall^{1,2}, Tina Richey², Angela Williams², Alan Stuckey¹, Dustin Osborne¹, Emily Martin², and Stephen J. Kennel^{1,2}

¹Department of Radiology, University of Tennessee Graduate School of Medicine, Knoxville, TN 37920

²Department of Medicine, University of Tennessee Graduate School of Medicine, Knoxville, TN 37920

Abstract

Purpose—¹²³I-labeled human serum amyloid P component (SAP) is used clinically only in the UK for imaging visceral amyloidosis to assist with diagnosis, disease staging and monitoring response to therapy. We compare a new amyloid-reactive probe, peptide p5, with SAP for imaging amyloidosis.

Procedures—Dual-energy SPECT/CT images were acquired of ¹²⁵I-labeled SAP and ^{99m}Tc-labeled p5 in mice with systemic AA amyloidosis (n = 3). Twelve organs and tissues were harvested for radiotracer biodistribution assessment and for micro-autoradiographic analysis.

Results—¹²⁵I-SAP and ^{99m}Tc-p5 localized equivalently in amyloid deposits in liver (~10 %ID/g) whereas, ¹²⁵I-SAP was 2 fold higher in the spleen (~20 %ID/g; ^{99m}Tc-p5, ~10 %ID/g). In contrast, ^{99m}Tc-p5 was bound to pancreatic and intestinal amyloid ~5-fold more efficiently as evidenced in biodistribution data.

Conclusions—Radiolabeled p5 is an effective amyloid-imaging radiotracer as compared to SAP in the murine model of amyloidosis and may be rapidly translated for imaging patients with visceral amyloidosis in the USA.

Keywords

Visceral amyloidosis; peptide imaging; dual isotope SPECT; serum amyloid P component; AA amyloid

Introduction

Amyloidosis is a complex pathology associated with the deposition of protein fibrils, heparan sulfate proteoglycans (HSPG) and serum amyloid P component (SAP) in various organs and tissues causing dysfunction and death. Amyloid, composed of different protein fibrils, deposits in visceral organs leading to a number of disorders including light chain (AL), reactive (AA), or senile systemic (ATTR) amyloidosis [1, 2]. Radioiodinated serum amyloid P component is the only routinely used radiotracer to detect the whole body burden of visceral amyloid in patients [3–5]; however, due to its human origin, it is not approved for

use in the USA. As an alternative, we have identified a 31-mer heparin-binding peptide that accumulates rapidly and specifically in visceral AA amyloid deposits in a preclinical murine model of the disease. The peptide, designated p5, bound amyloid within 1 h pi and remained associated with the deposits for up to 24 h, as evidenced in single photon emission computed tomographic (SPECT) images, by measurements of radioactivity biodistribution, and in micro-autoradiographs [6].

The p5 peptide is a chemically-synthesized 31-amino acid reagent [7] which allows for rapid imaging and may have advantages for approval for clinical trials in the USA. Such a small peptide is able to penetrate vascular endothelium, be rapidly cleared from the circulation if not bound to target and is likely to be non-immunogenic [8–10].

The aim of this study was to validate the efficacy of radiolabeled p5 peptide as an alternative tracer for imaging visceral amyloid *in vivo*. Amyloid deposits, even in experimental animals, can vary between individuals. For this reason, we chose to compare the p5 peptide and SAP by using dual energy SPECT imaging and biodistribution measurements in the same animals (see Table 1 for physical comparison). Both tracers were selectively retained by amyloid in the visceral organs of mice with AA amyloidosis; however, ^{99m}Tc -p5 peptide accumulated significantly better in pancreatic and intestinal amyloid relative to SAP. These data indicate that imaging with p5 affords a viable alternative to SAP for detecting deposits in patients with visceral amyloidosis.

Materials and Methods

Animals

AA amyloidosis was studied in H2-L^d-huIL-6 Tg Balb/c (C) transgenic mice that constitutively express the human interleukin-6 transgene resulting in an acute “inflammatory” state with 1 – 4 mg/mL circulating serum amyloid protein A, the AA amyloid precursor protein [11, 12]. Amyloid was induced in 8 wk old female mice by iv administration of 100 μg of isolated AA amyloid fibrils (AEF) in 100 μL of sterile phosphate-buffered saline (PBS). Mice used in these experiments were 6 wk post induction. All animal procedures were performed under the auspices of protocols approved by the University of Tennessee Animal Care and Use Committee. The University of Tennessee is an AAALAC-I-accredited Institution.

Radiotracer preparation

Human SAP was isolated from autopsy-derived amyloid-containing spleen by first washing homogenized tissue in Tris-buffered saline containing 2 mM CaCl_2 . SAP was eluted from washed residue by addition of buffered saline containing 10 mM EDTA and was further purified by affinity chromatography using O-phosphoryl ethanol Sepharose. Purified SAP was radioiodinated with ^{125}I (Perkin Elmer, Waltham, MA), using chloramine T and purified by gel filtration chromatography, as previously described [13]. Peptide p5 was synthesized (Keck Laboratories, New Haven, CT) and then purified by reverse phase HPLC before being labeled with ^{99m}Tc [14], or ^{125}I [6]. ^{99m}Tc -labeled p5 peptide for imaging and biodistribution studies was diluted into 0.1% sterile gelatin in PBS and purified by gel filtration on a 5 mL PD10 column equilibrated in gelatin/PBS. Radioiodinated p5 was similarly prepared for micro-autoradiographic studies. Peak fractions of radiotracer were pooled and the product’s radiochemical purity was established by SDS polyacrylamide gel electrophoresis (PAGE) analysed by phosphor imaging (Cyclone Storage Phosphor System, Perkin Elmer, Shelton, CT).

SPECT/CT imaging

Each of 3 AA mice was injected with 6.5 μg of ^{125}I -SAP (5.1 ± 0.3 MBq) and 22 h thereafter 7.5 μg of $^{99\text{m}}\text{Tc}$ -p5 (36.6 ± 3.5 MBq – both injected doses were estimated based on measuring the remaining activity in the syringe after each injection) in the lateral tail vein. After a further 2 h, the mice were given ~ 500 μL of a 1:1 dilution of Iohexol in PBS ip 1 min before being euthanized by an isoflurane inhalation overdose and image data collected.

Dual energy SPECT images were acquired sequentially using an Inveon trimodality imaging platform (Siemens Preclinical Solution, Knoxville, TN; [15]). Low (^{125}I ; 25 – 45 keV: calculated acquisition sensitivity = 244 cps/MBq) then high ($^{99\text{m}}\text{Tc}$; 126 – 154 keV: calculated acquisition sensitivity = 286 cps/MBq) energy gamma photons were acquired using a helical SPECT acquisition protocol with sixty 16-sec projections and 90 mm of bed travel. A 0.5 mm-diameter 5-pinhole (Mouse Whole Body) collimator was used at 30 mm from the center of the field of view. Data were reconstructed using a 3D maximisation a posteriori (MAP) algorithm (zoom = 1; β = 1; 16 iterations; 3 subsets) with 0.5 mm isotropic voxels and with x and y dimensions of 88 and z dimension of 312, which yielded a reconstructed FOV of 4.4 cm $\times$ 4.4 cm $\times$ 15.6 cm. CT data were acquired using an x-ray voltage biased to 80 kVp with a 500 μA anode current and the data were binned by a factor of 4 resulting in a projection size of 512 $\times$ 768. A 225 msec exposure was used and 360, 1-degree projections were collected. The data were reconstructed using an implementation of the Feldkamp filtered back-projection algorithm [16] onto a 512 $\times$ 512 $\times$ 1296 matrix with isotropic 0.11 mm voxels.

SPECT and CT datasets were visualized using the Inveon Research Workplace 3D visualisation software package (Siemens Preclinical Solution, Knoxville, TN).

Micro-autoradiography study

Two groups of three 8 wk-old H2-Ld-huIL-6 Tg Balb/c (C) female mice, 6 wk post-AEF injection were injected iv with 3.7 MBq of ^{125}I -SAP or ^{125}I -p5 peptide. The mice were sacrificed at 24 h and 2 h post SAP and p5 injection, respectively, as described above and organs harvested for micro-autoradiographic comparison as described below.

Biodistribution and autoradiography

Samples of 12 tissues were harvested post mortem from the 3 mice [17]. Each tissue was placed into a tarred, plastic vial and weighed. The radioactivity in the ^{125}I and $^{99\text{m}}\text{Tc}$ energy windows was measured using an automated Wizard 3 gamma counter (1480 Wallac Gamma Counter, Perkin Elmer) using a crossover from high to low energy channel of 4.6% that was determined empirically using a pure sample of $^{99\text{m}}\text{Tc}$ counted using the dual energy windows. The calculated percent injected dose values were decay corrected to the time of injection by counting samples of the original injected material. Additional samples of tissue were fixed in 10% buffered-formalin for 24 h and embedded in paraffin, and 6 μm -thick sections were cut onto Plus^{TM} microscope slides (Fisher Scientific), dipped in NTB-2 emulsion (Eastman Kodak), stored in the dark and developed after a 96 h exposure. Each section was counter-stained with hematoxylin. The presence of amyloid deposits was microscopically confirmed in consecutive tissue sections viewed under cross-polarized illumination after staining with alkaline Congo red. All tissues were examined using a Leica DM500 light microscope fitted with cross-polarizing filters. Digital microscopic images were acquired using a cooled CCD camera (SPOT, Diagnostic Instruments, Sterling Heights, MI).

Specific Binding studies

Cohorts of three 8 wk-old H2-Ld-huIL-6 Tg Balb/c (C) transgenic mice at 6 wk post-AEF injection received either 2 μ g ($\sim 100 \mu$ Ci) of ^{125} I-p5 peptide alone or mixed with 500 μ g of unlabeled peptide. At 2 h post-injection the mice were euthanized by isoflurane inhalation, the organs harvested and biodistribution of the radiotracer was determined as described above.

Results

SAP was radioiodinated with a radiochemical yield of $\sim 50\%$ and purified to a radiochemical purity of $>95\%$. Peptide p5 was coupled with ^{99m} Tc with a radiochemical yield (decay corrected) of $\sim 75\%$ and purified to a radiochemical purity of $>90\%$ (data not shown). Previous imaging experiments with ^{125} I-SAP and ^{125} I-p5 peptide had demonstrated that both agents imaged amyloid deposits, but there were differences in the relative uptake of each tracer, possibly resulting from differences in tracer affinity or possibly due to different amyloid burdens of the mice used. For a direct comparison of amyloid-binding efficacy, ^{125} I-SAP and ^{99m} Tc-p5 peptide were both injected into three mice with AA amyloidosis. Conditions for imaging AA amyloid in this mouse model using ^{125} I-SAP have been established and were adapted from previous studies in humans where images were acquired at 24 h pi [13, 12, 18]. The optimal time for imaging ^{125} I-p5 was previously established [6]. We have demonstrated with dual energy biodistribution experiments that ^{99m} Tc- and ^{125} I-p5 peptide bind comparably ($< 30\%$ difference) in the liver, spleen, and pancreas of mice with AA amyloidosis (data not shown). The distribution of radioactivity at 24 h pi of SAP and 2 h pi of peptide p5 was quantified by dual energy gamma counting of tissue samples harvested at necropsy. Both radiotracers were readily detected in the liver, spleen, pancreas and intestines, which are the major sites of amyloid deposition in these mice (Table 2, Fig. 1). In the liver, the radioactivity associated with SAP and p5 was equivalent ($\sim 11\%$ ID/g). In the spleen, however, ^{125} I-SAP was present at ~ 2 -fold greater amounts relative to ^{99m} Tc-p5 peptide ($p = 0.006$), likely due to variable concentrations of SAP and p5 binding sites, or steric inhibition of the p5-amyloid interaction due to the extensive amyloid fibril deposits. In contrast, there was significantly more ^{99m} Tc-p5 relative to SAP in the pancreas ($p = 0.006$), small intestine ($p = 0.002$) and heart ($p = 0.015$). Although there was also > 3 -fold more ^{99m} Tc-p5 in the large intestine and cecum, the difference was not statistically significant ($p = 0.075$, for both tissues). Specific inhibition of ^{125} I-p5 binding to the liver, pancreas, and upper intestines was achieved with co-injection of 500 μ g of unlabeled peptide, which resulted in a greater than 2-fold decrease in each tissue (P values of 0.04, 0.06, and 0.01, respectively).

The quantitative biodistribution data were confirmed in the dual energy SPECT/CT images of each of the mice (Figs. 2 & 3). The ip injection of CT contrast agent facilitated visualisation of the organ and tissue boundaries. In mouse #1, retention of ^{99m} Tc-p5 peptide was observed in the liver, spleen, intestines and pancreas (Figs. 2A, C, and E). No activity was observed in the stomach or thyroid – sites of ^{99m} Tc and radioiodide accumulation in mammals [19]. In contrast, free ^{125} I-iodide, liberated during the hepatic catabolism of ^{125} I-SAP, was sequestered by the thyroid. Amyloid-associated ^{125} I-SAP was observed solely in the spleen and liver but notably in highest concentrations within the spleen (Figs. 2B, D, and F). This pattern of reactivity was similarly observed in the fused dual energy SPECT images of each of the 3 mice studied (Fig. 3).

By virtue of the ip contrast CT injection, the pancreas was identified in all 3 mice in the CT images and when co-registered with the ^{99m} Tc-p5 data, the amyloid-bound peptide was evidenced in this organ (Figs. 4A–C). This was not observed in the ^{125} I-SAP SPECT/CT images. The significant increase in peptide p5 accumulation in the pancreas relative to SAP

and the specific binding of both tracers to the amyloid deposits was confirmed by micro-autoradiography (Fig. 4D & E). For these experiments, it was necessary to use ^{125}I -labeled probes in separate mice with similar (4+) amyloid burden as evidenced by comparing Congo red-stained adjacent tissue sections since it was not possible to obtain high resolution micro-autoradiographs with $^{99\text{m}}\text{Tc}$ -p5. These results verified the organ amyloid distribution determined by dual energy imaging.

Discussion

Visceral amyloidosis is an orphan condition with an incidence of ~ 5000 new cases per year in the USA. The clinical standard for the non-invasive, whole body detection of visceral amyloid is planar gamma scintigraphy using ^{123}I -SAP, which is available only in Europe [20]. The importance of this technique is supported by the fact that over ~1500 scans are performed annually in the UK alone, with numerous patients traveling from other countries to England to access this procedure. The image data is used not only to supplement laboratory diagnostic tests performed on tissue biopsies, but also to provide a picture of the whole body amyloid burden, which may influence treatment options and prognosis. The ability to non-invasively and repeatedly image a patients amyloid load can be used to document response to therapy or relapse – a procedure that would be of benefit not only in routine clinical management but also during clinical trial evaluation of novel anti-amyloid therapeutics.

As an alternative to this technique, we have developed a radiolabeled heparin-binding peptide, designated p5, and evaluated it in mice with systemic AA amyloidosis. The peptide was shown by SPECT imaging, biodistribution, and micro-autoradiography to accumulate rapidly within amyloid-laden, but not healthy organs and tissues [6]. To eliminate the effect of variation in amyloid deposition within individual mice, we used dual-isotope SPECT imaging to compare the efficacy of SAP and peptide p5 in individual animals. Dual isotope SPECT imaging and tissue analysis afford a direct quantitative evaluation of the comparative efficacy of radiotracers *in vivo* and limits the confounding effects of heterogeneity in pathology and physiology in animal models of disease.

SPECT images were consistent with previous findings using radio-iodinated SAP and p5 separately in different mice [7, 13, 12, 6]. The dual energy approach demonstrated directly that peptide p5 imaged amyloid in organs not visualized by SAP in the same mouse, notably the pancreas and intestines. This may have resulted from the enhanced detection of high energy $^{99\text{m}}\text{Tc}$ photons due to the decreased scatter and attenuation relative to the ^{125}I -SAP-derived photons [21]; however, biodistribution and autoradiography of excised, isolated, small tissue samples confirmed that peptide p5 was sequestered preferentially in the pancreas and intestines relative to SAP. This advantage may be due to the fact that the peptide is much smaller than SAP, facilitating trans-vascular wall migration and thus enhanced access to extravascular amyloid deposits. One apparent limitation evident from this study is the inability to specifically image renal amyloid using $^{99\text{m}}\text{Tc}$ -p5 because it is cleared through the kidneys. To circumvent this we anticipate using radioiodinated (^{124}I) p5 peptide for clinical imaging by using PET/CT. We have demonstrated that the p5 peptide not bound to amyloid deposits is dehalogenated rapidly within the kidneys during catabolism and that the free radioiodide is sequestered by the stomach and thyroid [6]. The amyloid-bound peptide is dehalogenated at a much slower rate (if at all). Thus, renal amyloid deposits may be visualized in patients by PET imaging using ^{124}I -p5 at time points that allow the clearance or dehalogenation of unbound peptide to occur.

Therefore, in addition to being chemically synthesized and rapidly cleared from the circulation, the increased tissue penetration and therefore enhanced amyloid labeling, afford

p5 certain advantages over SAP that may facilitate its translation to the clinic in the USA for imaging patients with visceral amyloidosis.

Conclusions

Radiolabeled p5 is capable of imaging amyloid deposits in liver and spleen of AA mice nearly as efficiently as SAP. In addition, images of p5 demonstrated amyloid in both pancreas and intestines providing a more complete picture of amyloid organ distribution in this animal model. Efforts are in progress to obtain FDA approval for clinical translation of p5 as a visceral amyloid imaging agent in the USA.

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Figure 1. Mean biodistribution of ¹²⁵I-SAP and ^{99m}Tc-p5 peptide in mice with AA amyloidosis. Significantly more ¹²⁵I-SAP (blue) was retained by amyloid in the spleen as compared to ^{99m}Tc-p5 (red); whereas more ^{99m}Tc-p5 was observed in the pancreas, small intestines, and heart. * $p < 0.05$, ** $p < 0.01$.

Figure 2.

Co-localization of ^{125}I -SAP and $^{99\text{m}}\text{Tc}$ -p5 peptide in the AA mouse was evidenced by SPECT/CT imaging. SPECT images of $^{99\text{m}}\text{Tc}$ -p5 peptide (A, C, & E) and ^{125}I -SAP (B, D, & F) in the same coronal, sagittal, or transverse plane in mouse 1. Activity in the liver, (L), spleen (Sp), pancreas (P), intestines (I), thyroid (T) was evident. The stomach (St) was devoid of activity. Note the intense splenic uptake of ^{125}I -SAP (B & F) as compared to $^{99\text{m}}\text{Tc}$ -p5 (A & E) consistent with the biodistribution data. The kidneys are not visible in any of the planes presented here.

Figure 3. Enhanced uptake of $^{99\text{m}}\text{Tc}$ -p5 relative to ^{125}I -SAP was observed in the intestines and pancreas of mice with AA amyloidosis. SPECT (A & C) and SPECT/CT images (B & D) showing the distribution of ^{125}I -SAP (A & B) and $^{99\text{m}}\text{Tc}$ -p5 (C & D) peptide in mouse 1.

Figure 4. ^{99m}Tc -p5 imaging provides enhanced visualization of pancreatic AA amyloid relative to ^{125}I -SAP. (A & B) Contrast enhanced CT images of the liver (L), stomach (St), intestine (I) and pancreas (arrows) in mouse 1. (C) ^{99m}Tc -p5 SPECT overlay co-registers activity with the pancreas seen in the CT data. (D) Micro-autoradiograph of pancreas from AA mouse administered ^{125}I -SAP (D) or ^{125}I -p5 (E) – exposure time was 4 d for each sample; original mag. 40 $\times$; a light H&E stain was used in (D) to assist in visualizing the weak radiograph signal (black deposits).

Table 1

Comparison of peptide p5 and serum amyloid P component

	Peptide p5	Serum amyloid P component (SAP)
Molecular state	monomer	pentamer (A-E subunits)
MW ¹	3,303.7	23,258.5 ²
Theoretical pI ¹	10.31	6.12
Net charge	+8	-2 ²
Crystal structure	nd	PDB ID - 1SAC
Amino acid sequence	CGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK Q	PDB ID - 1SAC_(A-E)
Source	chemically synthesized	human plasma
Target in amyloid ³	heparan sulfate proteoglycan	amyloid fibrils
Catabolism	kidney	liver

¹ Calculated using, www.expasy.org/cgi-bin/protparam;

² per monomer;

³ predicted target; nd, not determined.

Table 2

Biodistribution of radiolabeled SAP and p5 peptide in mice with AA amyloidosis

Tissue	Biodistribution (%ID/g)*					
	Mouse 1		Mouse 2		Mouse 3	
	¹²⁵ I-SAP	^{99m} Tc-p5	¹²⁵ I-SAP	^{99m} Tc-p5	¹²⁵ I-SAP	^{99m} Tc-p5
Muscle	0.1	0.2	0.1	0.3	0.1	0.3
Liver	7.5	9.2	10.0	12.0	12.0	12.9
Pancreas	0.3	9.1	0.3	8.6	0.5	11.1
Spleen	17.7	9.3	20.3	10.2	21.7	10.7
L kidney	0.5	14.9	0.6	16.0	0.5	14.4
R kidney	0.5	11.9	0.7	17.6	0.4	14.4
Stomach	0.7	3.6	1.4	4.5	1.7	5.1
Sm. intestine	0.6	4.5	0.5	4.0	0.9	4.3
Lg. intestine	0.3	4.6	0.2	1.9	0.2	2.4
Heart	0.3	0.7	0.4	1.0	0.5	1.1
Lung	0.4	0.6	0.4	0.7	0.4	0.7
Cecum	0.3	2.3	0.5	3.9	0.8	6.5

* Data for SAP and p5 were collected at 24 h and 2 h pi, respectively.