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SPECT imaging of peripheral amyloid in mice by targeting hyper-sulfated heparan sulfate proteoglycans with specific scFv antibodies

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Abstract

Introduction—Amyloid deposits are associated with a broad spectrum of disorders including monoclonal gammopathies, chronic inflammation, and Alzheimer's disease. In all cases, the amyloid pathology contains, in addition to protein fibrils, a plethora of associated molecules, including high concentrations of heparan sulfate proteoglycans (HSPGs).

Methods—We have evaluated radioiodinated scFvs that bind HS for their ability to image amyloid deposits *in vivo*. scFv's with different binding characteristics were isolated by phage display using HS extracted from bovine kidney or mouse and human skeletal muscle glycosaminoglycans (GAGs). Following purification and radioiodination, the biodistribution of ¹²⁵I-scFv's was assessed in mice with inflammation-associated AA amyloidosis or in amyloid-free mice by using SPECT imaging, biodistribution measurements and tissue autoradiography.

Results—Four different scFv's all showed binding *in vivo* to amyloid in the spleen, liver and kidney of diseased mice; however, three of the scFv's also bound to sites within these organs in disease free mice. One scFv specific for hypersulfated HSPGs preferentially bound amyloid and did not accumulate in healthy tissues.

Conclusions—These data indicate that HS expressed in amyloid deposits has unique qualities that can be distinguished from HS in normal tissues. A scFv specific for rare hypersulfated HS was used to selectively image AA amyloid in mice with minimal retention in normal tissue.

Keywords

AA Amyloid; Mice; scFv; SPECT imaging; heparan sulfate

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1. Introduction

Amyloid diseases are characterized by organ deposition of fibrillar forms of several different proteins [1, 2]. The peripheral amyloids, most cases of which are associated with the deposition of immunoglobulin light chains, serum amyloid protein A (sAA) or transthyretin, can involve a single or many different organs depending on the patient. The ability to image the distribution and extent of disease will contribute not only to diagnostic and prognostic staging but also to planning and evaluation of treatment efficacy. Very early during the course of the disease progression, amyloid deposits acquire accessory molecules including SAP [3] and heparan sulfate proteoglycans (HSPG) [4–6]. HSPGs are a heterogeneous group of glycoproteins in which core proteins are decorated with heparan sulfate-containing oligosaccharides (HS). These HS molecules are a group of related linear polysaccharides that vary in molecular weight and have a high degree of molecular variability that is largely due to differences in epimerization and the extent and pattern of sulfation of hydroxyl groups within the sugars comprising the linear backbone [7]. There is some evidence that hypersulfated HS units are selectively found in amyloid deposits [8]. While it is clear that different forms of HS are expressed at different anatomical sites in the body, determining the exact chemical structures has been challenging [9, 10]. Thus, understanding the slight differences in structure has been largely empirical. We have identified a number of scFv's that bind to HS from various sources with different avidities [11–14]. In an effort to develop imaging agents that are diagnostic for amyloid deposits, we have tested four of these scFv's in mice for their biodistribution as radioiodinated tracers. The fact that HSPGs are ubiquitously distributed in the plasma and basement membranes of normal tissue complicates the use of many anti-HS reagents for the specific detection of amyloid deposits [15]. We hypothesized that at least three factors would allow us to distinguish HS associated with amyloid from that expressed in normal tissues: 1) very high concentrations of HS are present in the amyloid, 2) unique bio- or electrochemical features of the amyloid-associated HS might be preferentially recognized by an scFv with appropriate specificity, 3) enhanced exposure of the amyloid to the circulation promotes first or early pass association of the targeting molecule to the *in vivo* injected tracer.

An experimental murine model of systemic AA amyloidosis served as our test system. The transgenic mouse strain, H2-L^d-huIL-6 Tg Balb/c (C) (H2/huIL-6), expresses the human interleukin 6 (hIL-6) pro-inflammatory cytokine constitutively under the control of the murine major histocompatibility complex H2-L^d promoter [16–18]. Consequently, these animals express high levels of acute phase proteins, notably sAA, the precursor of AA amyloid. This results in the natural onset of disease by ~ 4 – 5 months of age. Amyloid appears initially in the spleen; however, fueled by circulating sAA levels of > 1 mg/mL, it progresses rapidly to involve the liver, pancreas, adrenal, heart, vasculature and kidneys. By ~8 months of age the mice become moribund due to failure of involved organs. The onset of AA in IL6 mice can be induced or accelerated by injection of amyloid enhancing factor (AEF), a splenic extract containing pre-formed fibrils isolated from mice with AA [16]. This model of inducible AA has been used repeatedly as a robust, predictable system for *in vivo* evaluation of the efficacy of amyloid-binding reagents using small animal imaging [17, 18].

In this report, we demonstrate that all four anti-HS scFv's localize specifically at the sites of amyloid deposits in mice with heavy amyloid burden and that patterns of deposition can be clearly differentiated from those in normal mice. NS4F5, an scFv recently shown to bind hypersulfated HS moieties [8], proved to be unique in that it did not bind to HS in normal mice, enhancing its value as an imaging agent for diseased tissue. This scFv, or improved molecular forms, may prove useful for imaging peripheral amyloidosis.

2. Materials and Methods

2.1 ScFv purification and radiolabeling

Heparan sulfate (HS) reactive scFv's HS4C3 [13], NS4F5 [14] and HS4E4 [19], as well as the chondroitin sulfate (CS)-binding scFv, GD3G7, [20] were derived by panning the phagemid synthetic scFv display Library No. 1 (from Dr. G. Winters, Cambridge University, Cambridge, UK) on HS from bovine kidney (Sigma) or human lung, as well as rat embryo-derived CS, using established techniques [11, 21] (Table 1). Antibodies were isolated from periplasmic extracts of phagemid infected HB2151 *E. coli* using nickel affinity chromatography as described previously [11]. Purified scFv's were analyzed on SDS-PAGE and judged to be >95% pure by observation of the amount of CBB stain located in the gel region between 27 and 30 kDa (data not shown). Some aggregation was noted in samples stored for over 1 month or those that were frozen and thawed frequently; therefore, purifications were conducted within about 1 week of radiolabeling. ScFv's (20–100 µg) were radiolabeled with 0.5–2.5 mCi ¹²⁵I (Perkin Elmer) using 5 µg of chloramine T from a freshly prepared water stock solution of 1 mg/mL. The reactions were performed in a final volume of <500 µL of 0.1 M sodium phosphate buffer, pH 7.6 for 1 min at RT. The reaction was quenched by addition of 5 µL of 2 mg/mL sodium metabisulfite freshly prepared in water, and the radiolabeled scFv's were purified by gel filtration on Sepharose Aca 44 gel in 0.01 M sodium phosphate (pH 7.6) and 0.15 M NaCl (PBS) containing 5 mg/mL bovine serum albumin (PBS-BSA) as described previously [22]. One µL aliquots were taken from each eluted fraction and analyzed for ¹²⁵I to determine the elution profile. Fractions of radiolabeled scFv (running in the elution profile region corresponding to 25–30kDa) were pooled and were evaluated for radiochemical purity by SDS-PAGE and phosphorimaging [22]. The radiochemical yield was estimated by summing radioactivity in the total column eluate and calculating the % ¹²⁵I recovered in the pooled scFv fractions. In all, 9 radiolabelings were performed on 8 different purified samples of the scFv's. The radiochemical yield was 50.9±10.0%, and the radiochemical purity was 96.6±2.8%. Specific activities ranged from 25–40 µCi/µg protein.

2.2 AA amyloid mouse model

The binding of ¹²⁵I-labeled scFv with AA amyloid was studied in H2/huIL-6 transgenic mice [18, 23]. Amyloidosis was induced in groups of 8-week-old female H2/hIL-6 mice by injecting 100 µg of murine AA-derived AEF [16] suspended in 100 µL of PBS in the lateral tail vein. Non-transgenic mice from the H2/hIL-6 colony (WT), identified by genotyping, served as controls. All *in vivo* studies were performed using mice at 6 wk post-AEF injection, at which time extensive hepatosplenic and renal AA amyloidosis was present (AA mice) [16]. All studies were performed in accordance with acceptable guidelines for the ethical care and humane use of experimental animals and with approval by the University of Tennessee's Institutional Animal Care and Use Committee (IACUC).

2.3 microSPECT/CT Imaging

Groups of 3 AA or WT mice were injected with ~200 µCi (~ 25 µg) of ¹²⁵I-labeled scFv's diluted in a volume of 200 µL of 5 mg/mL BSA in PBS in the lateral tail vein, and then they were housed in a satellite facility with food and water provided *ad libitum* for 1 h, after which time they were sacrificed by an isoflurane inhalation overdose using the drop chamber method. SPECT/CT images as well as tissue biodistribution measurements and microautoradiography were then performed [24].

SPECT and CT postmortem images were acquired using a microCAT II + SPECT imaging platform (Siemens Preclinical Solution, Knoxville, TN). Mice were positioned prone on a cardboard imaging platform on the scanner bed. Three 70 µCi Co-57 point sources were

secured to the underside of the platform to facilitate co-registration of the CT and SPECT images. The CT data were acquired using an X-ray voltage biased to 70 kVp with a 500 μ A anode current; the full 90 mm $\times$ 60 mm field of view was used, and the data were binned 4 $\times$ 4. A series of 360, 1-degree projections were collected using an exposure of 475 msec per projection. The data were reconstructed in real-time using an implementation of the Feldkamp filtered back-projection algorithm onto a 512 $\times$ 512 $\times$ 768 matrix with isotropic 0.077 mm voxels [24].

SPECT images were collected using a 2 mm-diameter single pinhole collimator positioned ~60 mm from the center of the axis of rotation. Each dataset comprised 40 projections with a 60 sec exposure at each azimuth. Data from each SPECT detector were reconstructed independently post hoc onto an 86 $\times$ 86 $\times$ 114 matrix of 1 mm isotropic voxels using a 3D ordered subset expectation maximization algorithm (OSEM) with 8 iterations and 4 subsets. SPECT and CT datasets were imported into the Amira 3D visualization software package (Mercury Computer Systems) and co-registered manually.

SPECT/CT images of live animals under isoflurane anesthesia in a dedicated imaging chamber (M2M Imaging© Cleveland, OH) were collected by using an Inveon trimodality PET/SPECT/CT scanner (Siemens Preclinical Solutions, Knoxville, TN). Mice were maintained under anesthesia for 45 min, while CT and then SPECT image data were collected. An internal heating pad was used to keep the chamber at 39°C. CT images consisting of 360 $\times$ 1° projections at each of 2 bed positions were acquired first. The exposure time was 225 msec with a 32.3% FOV and detector settings at 80 kVp and 500 μ A. CT beam hardening correction was applied during data reconstruction onto a 512 $\times$ 512 $\times$ 768 image matrix using the COBRA software package (Exxim Computing Corporation, Pleasanton, CA). The effective image pixel size was 106 μ m. SPECT data were acquired immediately following the CT using a 5-pinhole collimator with a detector radius of rotation at 30 mm. Images were acquired over 1.5 detector revolutions with 6° between each of 90 projections. A 90 mm bed travel was used. Each projection was acquired for 16 sec, and the data were histogrammed with a window setting of 25 – 45 KeV. Reconstruction was performed using ordered subset expectation maximization 3D (OSEM3D) with 8 iterations and 6 subsets.

2.4 Biodistribution

After SPECT/CT imaging, mice were processed immediately or frozen for processing at later times. Samples of tissues (muscle, skin, liver, pancreas, spleen, kidney, stomach, upper and lower intestines, lung and heart) were placed in tarred vials, weighed and the 125 I radioactivity measured using an automated Wizard 3 gamma counter (1480 Wallac Gamma Counter, Perkin Elmer). Biodistribution data are expressed as the % injected dose/gram (%ID/g). Biodistribution data were also normalized to the radioiodine content of muscle, an organ that contains no detectable amyloid. In addition, tissue samples were fixed in buffered formalin for ~24 h for histological and microautoradiographic analyses.

Approximately 10 mg samples of liver, kidney, stomach and blood were snap frozen and stored on dry ice until processed for metabolite studies. Samples were thawed and dissolved in 1% SDS and 8 M deionized urea. Material extracted from each sample was adjusted such that approximately equivalent concentrations of 125 I were loaded onto 4–12% SDS-polyacrylamide electrophoresis (SDS_PAGE) gels (NuPAGE gels and Novex mini gel apparatus; Invitrogen). For visualization of the radiolabeled products, the gels were exposed to phosphorimager screens for 2 or 4 h before analysis using the Cyclone imager. Events occurring in regions of interest were quantified using OptiQuant Cyclone-Plus software (ver 5.0.0.2; Perkin Elmer).

2.5 Autoradiography and Immunohistochemistry

For autoradiography, 4–6 μm -thick sections were cut from formalin-fixed, paraffin-embedded blocks onto Plus microscope slides (Fisher Scientific), dipped in NTB-2 emulsion (Eastman Kodak), stored in the dark and developed after a 4 d exposure. Tissue sections were then counter-stained with hematoxylin and eosin. Tissue amyloid deposits were identified microscopically on slides viewed under cross-polarized illumination after staining with alkaline Congo red [17]. The HSPG was immunostained following incubation of the tissue in citrate antigen-retrieval solution (BioGenex, San Ramon, CA) at 90°C for 30 min, using a core protein-reactive rat polyclonal antibody (clone HK-102; Seikagaku Corp., Tokyo, Japan) added at a dilution of 1:400. The slides were developed using a biotinylated goat anti-rabbit secondary provided in the IHC DAB kit (InnoGenex, San Ramon, CA). All tissue sections were examined using a Leica DM500 light microscope fitted with cross-polarizing filters (for the detection of Congo red birefringence). Digital microscopic images were acquired using a cooled CCD camera (SPOT, Diagnostic Instruments, Sterling Heights, MI).

3. Results

3.1. Heparan sulfate proteoglycan immunohistochemistry

HSPG has been shown to be a component of amyloid deposits in experimentally-induced murine models of AA amyloidosis [25, 26]. In our model, progressive and terminal AA can be induced within a few weeks in H2/hIL-6 transgenic mice by injection of AEF [17]. Antibody to perlecan HSPG was used to immunostain tissues from diseased or normal mice. Serial sections were stained with alkaline Congo red to visualize the amyloid deposits or with anti-perlecan core protein polyclonal antibody (Fig. 1). There was significant immunoreactivity with the anti-perlecan antibody in all tissues that correlated well with the location of the AA amyloid deposits, for example in the spleen, kidney, pancreas and liver (Fig. 1). Perlecan staining in WT mice was not seen in normal spleen and kidney; however, minor deposits were visible within the Islets of Langerhans in the pancreas and lining the sinusoids of the liver (Fig. 1). These data indicate that enhanced, amyloid-associated deposition of perlecan HSPG occurs in the H2/hIL-6 mice during AA amyloidogenesis.

3.2. Biodistribution of scFv antibodies in mice

Four scFv's with different binding specificities for HS were purified and radioiodinated. Gel filtration-purified probes were demonstrated to be free of iodide and other labeled proteins (>90% pure) using phosphorimaging of SDS-PAGE gel electrophoresis (Fig. 2). Radioiodinated scFv's were injected in AA-laden or WT control mice for analysis by SPECT/CT imaging, biodistribution and microautoradiography. The major sites of amyloid deposition in the H2/hIL-6 mice are the spleen, liver, and pancreas. Biodistribution data, which tracks only the total amount of radioiodide retained in a tissue sample, showed that retention of all four radiolabeled scFv's at 1 h in AA mice was greater or equivalent to that seen in the WT mice (Fig. 3 and Table 2). At 1 h post-injection, retention of each of the scFvs in the pancreas was similar in AA and WT demonstrating no specific uptake of any reagent in this organ. To correct for a decrease in renal clearance of the scFv (the major site of scFv catabolism in mice) in H2/hIL-6 mice due to renal amyloid pathology, we calculated tissue:muscle ratios for each data set (Fig. 3). Notably, the scFv NS4F5 produced at least a 2-fold increase in the tissue:muscle ratio in the liver, spleen, and kidney of AA mice as compared to WT animals. In contrast, the ratios calculated for scFv's GD3G7 and HS4C3 were not greater than 2 in any tissue studied. The scFv HS4E4 tissue:muscle ratio in the AA mouse kidney was more than twice that seen in WT animals; however, no increase in other amyloid-laden organs was observed. Even though all four scFv's that bind HSPG variants

had enhanced deposition in AA mouse tissues, the scFv NS4F5 demonstrated the best discrimination between tissues of AA and WT mice.

In general, the distribution of radiotracer in amyloid-free WT mice was characterized by relatively high concentrations in the kidney (~ 9 – 13 % ID/g) and stomach (~10 – 22 % ID/g) which is indicative of renal catabolism and dehalogenation. This was accompanied by sequestration of the free iodide by the stomach and thyroid. Additionally, since the kidney is a rich source of HSPGs, it is likely that certain of these reagents reacted specifically with structures within the healthy kidney; although, this cannot be discerned from biodistribution or imaging techniques alone.

To support the contention that loss of free iodide from the non-targeting scFvs had occurred, samples of blood, liver, stomach, and kidney were taken and subjected to SDS-PAGE followed by quantitative detection of ^{125}I using phosphor imaging of unstained gels. Representative autoradiograms from tissue from either AA or WT mice at 1 h post-injection are shown in Fig 4. There is a clear separation of the scFv band from the free iodide. Scant radioiodine was observed at other positions within the gel profile. Gels of samples from duplicate mice either AA or WT at 1 or 4 h post-injection were analyzed using phosphorimaging. Samples taken 24 h post-injection contained too little radioactivity to be analyzed effectively. Regions of interest comprising the scFv or free iodide areas were quantified and the results presented in Table 3. At 1 h post-injection the majority of ^{125}I remained associated with scFv in the liver, kidney, and blood, while that seen in stomach was largely free iodide, presumably associated with iodide symporters. In contrast, in WT mice, where the scFv remained unbound due to a lack of target, the majority of ^{125}I was present as free iodide. In AA mice, at 4 h post-injection, most of the ^{125}I from organs that contain significant amounts of amyloid-associated HS (e.g., liver, kidney, and to a lesser degree, the stomach) remained bound to the scFv protein. In the WT mice, the detectable ^{125}I was largely free iodide with some intact iodinated scFv remaining in kidney, either associated with amyloid or undergoing catabolism. These data support the contention that iodinated scFv bound to target HS was protected from dehalogenation, whereas material catabolized within the kidney was prone to dehalogenation releasing free iodide into the circulation to be taken up in the stomach and thyroid. We were unable to consistently collect thyroid samples free of adjacent tissue; therefore, that organ was not analyzed.

3.3.1 Micro-distribution of scFv in AA and WT mice—Autoradiography confirmed that each of the scFv's was capable of binding to HS in AA amyloid, but the binding to healthy, amyloid-free tissue varied widely among them. All four scFv's showed some retention of radiiodine in kidney in the tubular lumens and, to a lesser degree, in the Bowman's space circumscribing the glomerulae consistent with the mode of excretion (data not shown). In addition, three of the scFv's, especially HS4C3, showed binding to tissues of WT mice. Autoradiography demonstrated that scFv NS4F5 localized at sites of amyloid in liver and spleen of mice with AA amyloidosis. (Fig. 5). The silver grains, indicating positions of ^{125}I , corresponded to position of amyloid (stained with Congo red in serial sections) around vessels of portal triads of the liver and in perifollicular areas of the spleen. Accumulation of NS4F5 in hepatic amyloid was consistently greater than that associated with equally dense Congo Red stained splenic deposits of amyloid as evidenced by the increased density of silver grains in the liver as compared to the spleen.

3.4. In vivo SPECT/CT imaging of AA amyloid

To perform meaningful, non-invasive molecular imaging of amyloidosis *in vivo*, it is necessary to utilize amyloid-specific radiotracers that distribute differentially in AA and WT mice and thereby discriminate healthy subjects from those with amyloid disease.

Furthermore, deposition in non-diseased tissue should be minimal so as to not obscure detection of AA in those organs. We compared the ability of each scFv with SPECT/CT imaging to differentiate between mice with AA from those with no amyloid disease (Fig. 6). At 1 h post-injection of all four ^{125}I scFv's in WT mice, most of the radioactivity was observed in the stomach with traces remaining in the kidneys. This pattern corroborates the tissue biodistribution data (Fig. 3) and is consistent with renal catabolism and dehalogenation of the proteins followed by sequestration of the released radioiodide in the stomach (iodide symporters) and thyroid (iodination of thyroxine) [27]. Metabolite studies presented in Fig. 4 and Table 3 support these contentions. There was little or no hepatosplenic accumulation of scFv HS4C3 visible in the SPECT images of the WT mice even though some binding was evidenced autoradiographically. GD3G7 scFv showed more residual activity in kidneys than did the other scFv's indicating slower clearance or actual low amounts of specific binding. The SPECT images of radiolabeled GD3G7, HS4E4, and HS4C3 scFv's in mice with AA were dominated also by activity in the kidneys, stomach and thyroid with some activity in the liver (especially HS4E4). In contrast, the distribution of the scFv NS4F5 in the AA mice was visually distinct from that seen in the WT animal as evidenced by increased uptake in the amyloid-laden liver and spleen. This reagent alone was capable of readily discriminating amyloidotic mice from the WT controls by using SPECT imaging.

Since scFv NS4F5 was clearly the best candidate for AA imaging, a second set of experiments was conducted to define optimal imaging parameters. Both AA and WT mice were injected with ^{125}I -labeled NS4F5 and were imaged live under isoflurane anesthesia at 1 h, 4 h and at 24 h. Fig. 7 presents images of representative mice at 1 and 4 h. Too little radioisotope remained at 24 h to obtain sufficient counts for imaging using the same acquisition parameters. At 1 h post-injection, uptake was predominantly observed in the amyloid-rich liver and spleen of AA mice. In contrast, the stomach and thyroid (sites of free iodide sequestration following dehalogenation of the scFv) dominated the WT mouse image. Images of both AA and WT animals acquired at 4 h post-injection were similar – mostly stomach and thyroid. These data confirmed that specific amyloid imaging could be performed at 1 h post injection, but that the scFv was lost from the amyloid site by 4 h.

Imaging data were supported by biodistribution measurements at 1, 4, and 24 h post-injection of ^{125}I -NS4F5 (Fig. 7). At 1 h post-injection, the AA mice had high levels of ^{125}I in liver, spleen and kidney relative to WT mice. The ratios of AA to WT were >2 at 1 h and approximately 4 at 4 h post-injection. By 24 h most of the ^{125}I was cleared (note different scales), and the ratio of ^{125}I in each organ of AA and WT mice was similar but remained >1 which reflects the slower clearance of scFv from AA animals, likely due to defective kidney function in the diseased mice.

4. Discussion

Amyloidosis is a protein aggregation process associated with an increasing number of diseases and normal physiological processes [28]. In disease, the extent and distribution of the amyloid with organs and tissues is of prognostic and diagnostic importance. In this regard, it is desirable to be able to non-invasively and quantitatively image the biodistribution of amyloid in patients and thereby also monitor changes in amyloid burden in response to therapy. Presently, radioiodinated serum amyloid P component (SAP), a pentraxin protein of $>250,000$ Mw which binds all amyloid fibrils, is the most widely utilized clinical radiotracer for peripheral amyloid, but it is available only in the United Kingdom [29–33]. Thus, alternative reagents that can be more widely employed would be desirable.

Although large biological radiotracers such as SAP and monoclonal antibodies [34] can be used to image some peripheral amyloid deposits (because the target is macroscopic and readily accessed from the circulation), for several reasons, we have focused on developing relatively small molecular probes for amyloid imaging. Smaller probes have the potential advantages of better penetration of tissues and of faster clearance from circulation promoting imaging with short-lived radioisotopes, such as ^{18}F . Since good imaging agents are generally those that bind avidly and quickly to targets and are rapidly cleared from the normal tissue, an optimal radiotracer for amyloid might be a relatively low molecular weight, easily diffusible, biological reagent with high specificity for the target. The scFv's described herein meet these criteria since they were cleared rapidly (1–2 h) from normal tissue and bound tightly to target amyloid deposits in the liver and spleen. By using radiolabeled NS4F5, AA and WT mice could be differentiated by SPECT/CT imaging as early as 1 h post-injection; although, by 4 h post-injection, scFv NS4F5 apparently was mostly released from amyloid (see Fig. 7) which complicates analysis of the biodistribution data and leads to a decreased binding to hepatosplenic pathology as seen in SPECT images (Fig. 7). By way of comparison, imaging similar mice with ^{125}I -SAP provided a clear definition of AA amyloid in liver and spleen, as did the NS4F5 scFv probe although accumulation of scFv's in amyloid was somewhat less intense than that of SAP [17, 18]. Notably, for reasons yet to be determined, neither the NS4F5 scFv nor SAP were able to bind pancreatic AA amyloid deposits in sufficient amounts to be detected in biodistribution or SPECT images [17, 18]. The scFv probes used in this study have not been optimized for stability of folding and valency of interaction. In particular, valency is associated with retention at the target site more than initial accumulation, and it is possible that formation of a diabody of NS4F5 might result in an imaging agent that could be easily seen at later time points and within the pancreas.

HSPGs are universal components of all amyloid deposits irrespective of the amyloid precursor proteins that comprise the fibrils or whether they are localized in the peripheral organs, as in patients with light chain (AL) or AA amyloidosis, or in the brains of patients with Alzheimer's disease. In the experimental model of murine AA amyloidosis that relies on injection of pre-inflammatory agents such as AgNO_3 solution or lipopolysaccharide, the up-regulation and synthesis of the perlecan HSPG precedes the appearance of Congo red-birefringent fibrils [5]. Indeed, in this AgNO_3 model, mice incapable of producing extended HS chains due to the over expression of the human heparanase transgene do not develop AA amyloidosis in spite of pro-inflammatory stimulation and AEF injections [35]. The early appearance of HSPGs in amyloid-prone tissues makes them a prime target for early disease detection. Although HSPG deposition appears essential in amyloidogenesis, its roles as a biomarker of the disease and a target for therapy and diagnostic imaging are less well studied.

HSPGs are widely distributed throughout the body as components of cell-anchored receptors, the extracellular matrix, and as free GAGs in the circulation, and they interact with an ever-growing panel of growth factors, chemokines and viruses [7]. Thus, targeting HSPGs in amyloid as a means of imaging the deposits requires that either the HS in amyloid be highly concentrated relative to the background healthy tissues or that it is biochemically unique. We have now shown immunohistochemically that the perlecan HSPG is associated with amyloid in the H2/hIL-6 mice and that, based on the intensity of tissue immunostaining, there is a significantly higher concentration in the amyloid as compared to healthy tissues. This agrees with observations that the onset of amyloidosis in CBA/J mice caused an ~8 to 12-fold increase in the total amount of newly synthesized splenic HS [36, 37].

The distribution of HS in healthy tissues, although a complicating variable, may be overshadowed due to the very high concentration in amyloid. Indeed, all four of the scFv's tested exhibited preferential binding to the liver and spleen in AA mice as compared to the WT mice implying specific uptake in the amyloid within these organs; however, with the exception of scFv NS4F5, the tissue:muscle ratios for these organs in the AA and WT were indistinguishable (Fig. 3). In addition, ^{125}I scFv NS4F5 gave superior SPECT/CT discrimination between AA and WT mice (Figures 5 and 7) supporting the biodistribution data. Thus, in the model system, the amount of amyloid disease dominated the deposition pattern of the scFv's. In human disease, patients can present with lower tissue amyloid loads and any residual reaction with normal tissue would complicate interpretation of the image.

A second reason for probe localization is the binding specificity of the scFv. To avoid even minor reaction with HS in normal tissue, the selectivity of the scFv for HS structures unique to amyloid deposits is important. It is likely that slow clearance from the AA mice contributes to the retention of probes in these tissues, but another factor is the latent reaction of some of the scFv's with normal tissue HS. Of note, the hepatic sinusoids of WT mice were particularly well stained with scFv HS4C3.

The production and biochemical structure of tissue HSPGs has been shown to change in response to inflammation [38, 39]. In organs with naturally higher concentrations of HS (e.g., the liver and kidney), specific visualization of amyloid might be hindered to a greater degree than in other tissues. Amyloid-associated HS is structurally distinct from that found in amyloid-free tissues, which provides another opportunity for specific targeting of amyloid. In 1997, Lindahl and Lindahl noted that human AA amyloid-derived HS was structurally distinct from tissue HS based on a shift in the ratio of disaccharides following deamination [40]. Later, it was shown that HS derived from amyloidotic spleens of CBA/J mice similarly contained a disaccharide profile that was clearly distinct from that obtained from amyloid-free tissue by using capillary chromatography and time-of-flight mass spectrometry [37].

Based on our SPECT/CT imaging and autoradiographic analyses, we have identified the scFv NS4F5 as one of a panel of 4 that can specifically bind amyloid-associated HS *in vivo* with an amyloid-tissue:muscle ratio greater than 2. This reagent, isolated using human lung HS extract as the target, has been shown by *in vitro* characterization to preferentially bind HS with *N*-sulfation, C5-epimerization and high degrees of 2-*O*- and 6-*O*-sulfation [14]. This hypersulfated form of HS appears to be rare in normal animals, based on a lack of binding of NS4F5 with healthy tissues in the WT mouse, notably the kidney and liver [8]. These data indicate that, in addition to an increase in concentration, the HS in amyloid is hypersulfated relative to the ubiquitous tissue HS and that the combination of these renders HS an important new biomarker for targeting amyloid using reagents such as scFv NS4F5. Specific accumulation of as little as 2 fold over control tissues is often sufficient for images obtained with other probes such as ^{18}F -fluoro-deoxyglucose for tumor detection. Although scFv NS4F5 demonstrated 2 fold or greater accumulation at disease sites and thus has potential as a specific amyloid imaging agent, its performance may be enhanced by production of multivalent forms of the scFv such as diabodies that would enhance retention at sites of amyloid deposition *in vivo*. This modification, coupled with optimization of imaging times, could produce a probe with high AA affinity that would combine sensitivity with selectivity for AA amyloid deposits especially when subjects are imaged at later times.

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Fig. 1.

Staining for AA amyloid in tissue sections of AA and WT mice. Tissue sections from formalin-fixed paraffin-embedded samples were stained with the amyloid binding dye Congo red (left hand panels) or were treated for antigen retrieval and stained with antibody to the protein portion of perlecan HSPG (middle and right hand panels) as described in methods.

Fig. 2. Radioiodinated preparations of scFv that had been partially purified by gel filtration chromatography were separated on SDS-PAGE gels under reducing conditions. The gels were exposed to a phosphorimager. ScFv's of ~28 KDa are designated by full arrows and free iodide is indicated by the arrowhead.

Fig. 3. Biodistribution of radioiodinated scFv's in AA mice (dark bars) and WT mice (white bars) 1 h post injection for 3 mice per group. Data are expressed as % injected dose per gram of tissue (%ID/g) with error bars indicating SD (left panels) or the values normalized to radioisotope content of muscle (right panels).

Fig. 4. Phosphorimage of SDS-PAGE gels of tissue extractions from either AA (left) or WT (right) mice that had been injected with ^{125}I -labeled NS4F5 1 h previously. Total protein was extracted from the tissues with SDS-urea solution and molecular sizes separated on the gel. ScFv runs midway in the gel (asterisk) and radio iodide runs slightly ahead of the dye front (arrow). Lanes: 1, blood; 2, liver; 3, stomach; 4, kidney; 5, standard

Fig 5.

Microdistribution of radioiodinated scFv NS4F5 in liver and spleen 1 h post-injection as visualized by microautoradiography (mag ~200X). Sections of tissue samples from AA or WT mice injected with radioiodinated scFv were exposed to photographic emulsion for 4 d before development. Slides were developed exposing the position of ^{125}I as black silver grains. Accumulation of radiolabeled scFv coincident with amyloid deposition is indicated with arrows. Serial sections of the AA tissues were stained with Congo red to visualize amyloid deposits (green birefringence).

Fig. 6. SPECT/CT imaging of radioiodinated scFv ($\sim 200 \mu\text{Ci}$, $5 \mu\text{g}$) in AA or WT mice 1 h post-injection. Panels are designated as to the scFv and AA or WT animal used. The upper left panel of the planer image indicates the positions of thyroid (T), liver (L), stomach (S), or kidney (K). SPECT/CT scans were done on all animals ($n=3/\text{grp}$) resulting in nearly identical images within a group.

Fig 7. SPECT/CT imaging and biodistribution data for AA and WT mice injected with 120 μCi ^{125}I -labeled NS4F5. Upper panels are SPECT/CT images of representative AA and WT mice imaged under anesthesia at 1 h and then again at 4 h. Similarly injected cohorts ($n=3$) of AA (red) and WT (blue) mice were euthanized at 1, 4, and 24 h and the tissues collected for biodistribution calculations and for metabolite studies (see Table 3).

Table 1

Binding characteristics of scFv's reactive with HS

scFv Antibody	Immunogen	VH CDR3 Sequence	GAG reactivity	Preferred chemical group	Ref
GD3G7	Rat embryo: glycosaminoglycan extract	GRWTQMT	CS-E	GalNAc4S6S	[20]
HS4E4	Bovine Kidney: HS	HAPLRNTRTNT	HS	N-acetylation N-sulfation C5-epimerization	[19]
HS4C3	Bovine Kidney: HS	GRRLKD	HS	IdoA2S-GlcNS3S6S N-sulfation 2-O-sulfation 6-O-sulfation 3-O-sulfation	[13]
NS4F5	Human lung: HS extract	SGRKGRMR	HS	N-sulfation C5-epimerization 2-O-sulfation (high) 6-O-sulfation (high)	[14]

Table 2
Biodistribution of radioiodinated scFv's in AA and WT mice (mean %ID/g $\pm$ SD)

Tissue	GD3G7		HS4E4		HS4C3		NS4F5	
	AA	WT	AA	WT	AA	WT	AA	WT
skin	1.3 $\pm$ 0.3	1.0 $\pm$ 0.4	1.0 $\pm$ 0.3	1.0 $\pm$ 0.3	1.0 $\pm$ 0.1	0.9 $\pm$ 0.1	0.6 $\pm$ 0.1	0.9 $\pm$ 0.1
muscle	1.8 $\pm$ 0.2	1.7 $\pm$ 0.4	1.6 $\pm$ 0.2	1.2 $\pm$ 0.2	1.1 $\pm$ 0.0	1.3 $\pm$ 0.1	1.2 $\pm$ 0.2	1.7 $\pm$ 0.3
liver	5.5 $\pm$ 2.1	5.0 $\pm$ 1.1	5.5 $\pm$ 1.4	4.6 $\pm$ 1.5	7.7 $\pm$ 0.0	6.4 $\pm$ 0.9	10.9 $\pm$ 1.6	6.7 $\pm$ 1.2
spleen	5.6 $\pm$ 1.7	5.6 $\pm$ 1.0	4.9 $\pm$ 2.4	3.9 $\pm$ 1.9	7.8 $\pm$ 0.6	5.2 $\pm$ 1.3	7.8 $\pm$ 3.5	3.8 $\pm$ 0.5
pancreas	5.7 $\pm$ 2.2	5.2 $\pm$ 0.6	3.7 $\pm$ 0.8	3.2 $\pm$ 1.1	5.0 $\pm$ 0.2	4.4 $\pm$ 0.5	3.9 $\pm$ 0.7	4.9 $\pm$ 1.7
kidney	15.3 $\pm$ 2.5	9.4 $\pm$ 2.3	29.5 $\pm$ 18.2	9.8 $\pm$ 1.2	12.2 $\pm$ 0.6	12.7 $\pm$ 1.6	11.8 $\pm$ 0.5	9.4 $\pm$ 1.6
stomach	9.8 $\pm$ 8.0	12.5 $\pm$ 4.2	10.9 $\pm$ 6.3	21.2 $\pm$ 2.4	7.9 $\pm$ 1.0	10.8 $\pm$ 1.2	10.7 $\pm$ 8.2	22.2 $\pm$ 4.1
up intestine	4.7 $\pm$ 0.9	3.3 $\pm$ 0.5	4.1 $\pm$ 1.2	3.0 $\pm$ 0.5	4.3 $\pm$ 0.0	3.5 $\pm$ 0.5	3.7 $\pm$ 0.2	4.0 $\pm$ 1.6
low intestines	4.6 $\pm$ 0.9	3.3 $\pm$ 0.7	3.2 $\pm$ 0.5	2.3 $\pm$ 0.3	3.2 $\pm$ 0.4	2.8 $\pm$ 0.3	2.8 $\pm$ 0.1	3.3 $\pm$ 0.7
heart	3.6 $\pm$ 0.6	2.9 $\pm$ 0.5	3.6 $\pm$ 1.0	2.1 $\pm$ 0.2	3.7 $\pm$ 0.4	3.3 $\pm$ 0.3	3.5 $\pm$ 0.2	3.2 $\pm$ 0.4
tongue	4.4 $\pm$ 0.9	4.6 $\pm$ 0.6	3.8 $\pm$ 0.8	3.5 $\pm$ 0.3	3.5 $\pm$ 1.5	2.8 $\pm$ 0.1	3.3 $\pm$ 0.4	4.6 $\pm$ 0.3

Table 3

Quantitation by autoradiography of ^{125}I -NS4F5 scFv in organs and tissues (% ^{125}I in scFv band)

	Activity 1 h pi (mean $\pm$ SD)		Activity 4 h pi (mean $\pm$ SD)	
	AA mice	WT mice	AA mice	WT mice
Blood	79 $\pm$ 7.0	6.0 $\pm$ 3.1	62.0 $\pm$ 15.2	8.0 $\pm$ 10.9
Liver	78 $\pm$ 1.0	44.0 $\pm$ 14.0	72.0 $\pm$ 11.1	69 $\pm$ 17.5
Stomach	9.5 $\pm$ 1.2	5.0 $\pm$ 5.6	37.0 $\pm$ 12.0	4.0 $\pm$ 2.9
Kidney	82 $\pm$ 0.9	44 $\pm$ 0.0	73 $\pm$ 8.3	31 $\pm$ 15.1
Standard	88 $\pm$ 3.2	89 $\pm$ 4.5	85 $\pm$ 4.0	85 $\pm$ 6.0