Published in final edited form as:

Magn Reson Med. 2008 September ; 60(3): 564–574. doi:10.1002/mrm.21684.

MRI of the basement membrane using charged nanoparticles as contrast agents

Kevin M. Bennett¹, Hua Zhou², James P. Sumner¹, Stephen J. Dodd¹, Nadia Bouraoud¹, Kent Doi², Robert A. Star², and Alan P. Koretsky¹

¹Laboratory of Functional and Molecular Imaging, National Institute of Neurological Disease and Stroke, National Institutes of Health, Bethesda, MD

²Renal Diagnostics and Therapeutics Unit, National Institute of Diabetes and Digestive and Kidney Disease, National Institutes of Health, Bethesda, MD

Abstract

The integrity of the basement membrane is essential for cellular growth, and is often altered in disease. In this work, a method for noninvasively detecting the structural integrity of the basement membrane, based on the delivery of cationic iron-oxide nanoparticles, was developed. Cationic particles accumulate due to the highly negative charge of proteoglycans in the basement membrane. The kidney was used to test this technique because of its highly fenestrated endothelia and well established disease models to manipulate the basement membrane charge barrier. After systemic injection of cationic or native ferritin (CF or NF) in rats, *ex vivo* and *in vivo* MRI showed selective accumulation of CF, but not NF, causing a 60% reduction in signal intensity in cortex at the location of individual glomeruli. Immuno-fluorescence and electron microscopy demonstrated that this CF accumulation was localized to the glomerular basement membrane (GBM). In a model of GBM breakdown during focal and segmental glomerulosclerosis, MRI showed reduced single glomerular accumulation of CF, but a diffuse accumulation of CF in the kidney tubules caused by leakage of CF through the glomerulus. Cationic contrast agents can be used to target the basement membrane and detect the breakdown of the basement membrane in disease.

Introduction

The basement membrane (BM) is a major functional component of the extracellular matrix. Endothelial and epithelial cells are lined by a three-dimensional matrix of proteins and proteoglycans comprising and surrounding the BM. The BM is structurally remodeled by cells during movement and proliferation. Remodeling of the BM is a normal process that occurs throughout development [1], and breach of the BM is a critical step for malignant cells to invade locally and metastasize [2,3]. The BM plays a formative role in development, and synthetic matrices are being developed to support tissue regeneration after tissue injury [4]. In several organs, the basement membrane is covered by a fenestrated endothelium which directly exposes the BM to the circulation. The fenestrations allow molecules to be selectively filtered through the BM or retained in the bloodstream [5-7]. Due to the functional importance of the basement membrane and its role in many cellular processes and organ functions, a method for detecting the integrity of the BM may allow noninvasive and early detection of developmental and disease processes. Noninvasive magnetic resonance imaging (MRI) is well suited to detect basement membrane alterations because MRI can be tuned to detect small differences in contrast, allowing visualization of soft tissues.

Functional and targeted contrast agents can be developed to spatially localize molecular targets in the body [8-12].

The goal of this work was to develop an MRI-visible contrast agent that is specifically targeted to the BM. The kidney is an excellent organ in which to study BM-targeted contrast agents, since 150 L of filtrate must pass through the glomerular basement membrane (GBM) in humans each day. The kidney contains $\sim 10^4$ (rat) to 10^6 (human) nephrons, each comprised of a single glomerulus and tubule. Each glomerulus contains a capillary tuft providing a high surface area for filtration. The functional filtration unit consists of fenestrated endothelial cells, a surrounding GBM, and specialized epithelial cells (podocytes). The glomerulus filters based on charge and size, and retains large or anionic proteins in the blood [6,13]. Endothelial cells lining the glomerular capillaries are fenestrated by 30-100 nm openings into the GBM, and GBM contains proteoglycans which form an anionic charge barrier. The fenestrations are large enough to allow nanoparticles, useful as MRI contrast agents, access to the GBM. Podocyte foot processes attached to the external GBM surface form inter-cellular filtration slits, which form a secondary site to filter proteins and solutes [14].

The loss of glomerular integrity is a common feature of renal disease in humans and animals. The breakdown of the filtration barrier leads to proteinuria, which can cause renal tubular damage and progression of kidney disease [15]. However, direct examination of human glomerular structure and disease is impractical without biopsy. One method for assessing GBM integrity in animals relies on the accumulation of cationic tracer molecules in the GBM, which is detected *ex-vivo* using immunohistochemistry or electron microscopy. Obviously, this is impractical to use in humans. A noninvasive method to detect and identify mechanisms of GBM and glomerular disorders is greatly needed [16-20].

Animal models have been developed to study glomerular disease. Injection of puromycin amnionucleoside (PAN) induces focal (only some glomeruli involved) and segmental (involving only a portion of a glomerulus) glomerular sclerosis and massive proteinuria. This is a good model of human focal and segmental glomerulosclerosis (FSGS), which is a major cause of end-stage renal failure in children and young adults [21,22]. Early diagnosis of human FSGS is difficult because sclerosis is heterogeneous and renal biopsy might not be able to detect the lesion, especially at the early stage. In the FSGS model, it is suggested that glomerular integrity is compromised by a breakdown of the glomerular and podocyte charge barrier, and repeated injection of PAN causes pathological damage similar to human FSGS.

In this work, the GBM of rat kidney glomeruli was labeled *in vivo* by systemic injection of cationic iron oxide nanoparticles (ferritin) which accumulate by electrostatic interaction at sites of negative charge. Small cationic particles are known to accumulate in the BM of the kidney, pancreas, brain, and other organs, and have been used for electron microscopy [23-30]. Ferritin is a protein which oxidizes and stores iron. Typically 1500-3000 irons are sequestered in the ferritin core, enabling ferritin to be used as an endogenous and exogenously delivered contrast agent for MRI [31]. Ferritin nanoparticles were used in this work because of their small size (13 nm) relative to the endothelial fenestrations (80 nm). Cationic ferritin [32] is created by the attachment of positively charged amine groups, and is used as a probe for electron microscopy to detect charged molecules in cells and tissue. Here we show that the selective accumulation of CF in the GBM can be detected with MRI, and a change in CF accumulation can be used to detect glomerular dysfunction *in vivo*. This raises the possibility of non-invasively detecting molecular changes in the basement membrane throughout the body.

Materials and Methods

Native horse spleen ferritin (NF) and cationized horse spleen ferritin were purchased from Sigma Aldrich (Sigma, St. Louis). CF was developed by the method of Danon et al. [32] Iron concentrations for the ferritin preparations were obtained by atomic absorption (West Coast Analytical Service, Inc., Santa Fe Springs, CA). The isoelectric point (PI) of ferritin was measured by isoelectric focusing in a polyacrylamide gel. The PI of CF was greater than 9.5, compared to a PI of 3.5 for NF.

In Vitro Preparation and Imaging

For the experiments, eight rats were anesthetized with a 30% oxygen/70% nitrogen (oxygen-enhanced air) gas mixture containing 5% isoflurane by nose-cone and given an injection of 3.3mg/100 g of either native horse spleen ferritin (NF, Sigma, St. Louis) or cationized horse spleen ferritin, in physiological saline. Because NF- and CF- inoculated rats were matched for weight in each experiment (to 5 g accuracy), the same volume was given to each rat. A single bolus of CF was intravenously injected over 1 minute to animals. The animals were allowed to recover, and the procedure was repeated one to five more times in 1.5 hour intervals. The animals were sacrificed 1.5 hours after the final injection by perfusion with saline for approximately 30 minutes, and then with 4% paraformaldehyde for ten minutes.

The chosen dose of CF and NF was discovered in a pilot study. Briefly, a single bolus of CF at 3.3mg/100 g was given, and the animal was sacrificed and kidneys were perfused and imaged with MRI as described. When a single bolus failed to give contrast by MRI, we measured the blood half-life of CF to determine the optimal time to deliver another bolus of CF, assuming that circulating CF would label the GBM continuously until it had been removed from circulation. Blood half-life was measured by removing samples of blood in 200 μ l volumes from a femoral arterial catheter at 15 minute intervals after CF administration for 1.5 hr. The blood was placed in 25 μ l of heparin, and iron content was measured by *atomic absorption* (West Coast Analytical Service, Inc., Santa Fe Springs, CA). Based on these results (data not shown), the blood half-life for CF was approximately 45 minutes. Second and third doses were chosen to be twice the half-life. By the second dose, MRI contrast in the glomeruli was observed, and by the third dose the contrast was clear, as presented here. Similarly, when we were first unable to readily detect CF by MRI after three systemic injections *in vivo*, presumably due to intrinsic shortening of the transverse relaxation times from circulating blood, we administered a fourth and then a fifth injection until the changes became apparent in MRI. We have not to date studied whether this dose could be administered as a single bolus at higher CF concentration.

Both kidneys and the liver were visually clear of blood after perfusion. The kidneys and were removed, dissected from adipose tissue, and placed in 2% gluteraldehyde in 0.1 M cacodylate buffer.

Kidneys were imaged in gluteraldehyde in a 6 ml syringe. The syringe was inserted into a 25 mm ID Bruker volume coil and imaged in a Bruker 11.7T/31 cm Magnex scanner (Bruker Biospin MRI) with a Bruker Avance console controlled by Paravision 4.0 software. After localization, kidneys were imaged using a gradient-echo (FLASH) pulse sequence, with a 30° flip-angle and TE/TR = 20/800 ms. The resolution was 100 $\times$ 100 $\times$ 500 microns, with a 256 $\times$ 256 matrix. The scan time was 3 minutes. For high-resolution gradient-echo images, a 30° flip-angle was used, and TE/TR = 12/30 ms. The resolution was 50 $\times$ 50 $\times$ 50 microns, with a 320 $\times$ 512 $\times$ 512 3D matrix, and scan time was 12 h 48 minutes.

Disease Model

Eight Male Sprague-Dawley rats, weighing 150-170 g, were given an intraperitoneal injection (100 mg/kg body weight) of puromycin aminonucleoside (PAN) (Biomol Research laboratories, PA) and additional doses (20 mg/kg) at weeks 2, 4, 6, 8, 10, and 12 later. Proteinuria and blood chemistry were examined at day 7 after first PAN injection. After confirmation of nephritic syndrome based on intensive proteinuria by a urine test Chemostrip (Roche, IN), and hypoalbuminemia and hyperlipidemia by an autoanalyzer (Hitachi 917, Boehringer Mannheim, Indianapolis, IN), FSGS rats and normal controls were given two injections of CF or NF at 2 weeks after the first PAN injection in an early phase study, and 13 weeks after multiple injections in a phase study. Animals presented intensive proteinuria (4+), hypoalbuminemia ($<3.0\text{g/dl}$), and hyperlipidemia 7 days after PAN injection. The ratio of kidney weight to bodyweight was significantly increased in PAN-treated rats compared to normal controls 13 days after PAN injection ($0.9 \pm 0.04\%$ vs. $0.6 \pm 0.004\%$, $p<0.01$).

Rats were anesthetized as described above and given three injections of $3.3\text{mg}/100\text{g}$ of either native horse spleen ferritin (NF, Sigma, St. Louis), as described above, or cationized horse spleen ferritin, in physiological saline.

Histology and immunofluorescence

Following MRI, the animals were transcardially perfused with PBS followed with 10% formalin. In the disease model, the animals were perfused locally through the renal artery. The kidneys were removed stored in the same 10% formalin solution for at least 5 hrs and placed in a 30% sucrose solution for 2-3 days. Tissues were processed for frozen sections (Histoserv, Inc, Germantown, MD). Serial $16\text{ }\mu\text{m}$ sagittal sections were cut. Histology sections were rinsed 2x with Dulbecco's Phosphate-Buffered Saline (DPBS). Anti-horse spleen ferritin (Sigma-Aldrich, St. Louis, MO) was applied to sections in DPSS with 1% BSA for 1 hr at 25°C . The sections were washed 3x with NPM or DPSS. The secondary antibody, Alexa Fluor 594 anti-rabbit IgG (Invitrogen, Carlsbad, CA) and/or wheat-germ agglutinin (WGA)-conjugated Alexafluor 488 were applied in the same manner as the primary antibody. 4',6-diamidino-2-phenylindole (DAPI) was used to counterstain the cell nuclei by applying a $1\mu\text{g/mL}$ DAPI solution for 10 min. The slides were then rinsed with the 1% BSA solution three times. Fluorescence images were acquired on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc., Germany) or an Olympus IX71 microscope (Olympus America Inc., Center Valley, PA). To verify that the changes in MRI were a result of CF uptake in kidney glomeruli, we performed immunofluorescence to detect CF, and counterstained with wheat germ agglutinin (WGA), which is known to accumulate in the GBM [43]. Three-dimensional co-localization of CF and WGA was done using Velocity (Velocity Software Inc., Mountain View, CA).

The harvested half of right kidney from each animal was immediately fixed in 10% neutral buffered formalin solution. The kidney tissue block was dehydrated through a graded alcohol series, embedded in paraffin, and cut into $4\text{-}\mu\text{m}$ sections and then stained with periodic acid Schiff (PAS) reagent.

Electron microscopy

Small specimens of approximately 1 mm were dissected from the fixed kidneys and placed in 1 N cacodylate buffer with 2% glutaraldehyde and stored at 4°C . Selected areas were trimmed, dehydrated with a graded series of ethanol mixtures, infiltrated with graded mixtures of ethanol and epoxy resin (SPI Supplies; West Chester, PA), and embedded in epoxy resin. Polymerization of the resin was carried out at 60°C for at least 48 hours. Images were taken with a Jeol (Peabody, MA) JEM-1200CX electron microscope, and

images were collected with a CCD digital camera system (XR-100 from AMT, Danvers, MA).

In vivo magnetic resonance imaging

For MRI, rats were prepared as described above, but were imaged rather than sacrificed at the final time-point of the inoculation in the same MRI scanner system as the *in vitro* scans. The rats were anesthetized and orally intubated with a 14-gauge, 2 in. intravenous-type catheter (Abbocath Labs). The rats were placed under ventilation with the isofluorane/air mixture, both before and during the MRI session. Animals were secured supine and to the side in an MRI cradle, and a 30 mm diameter home-built oval surface coil was placed over the left kidney.

A micro-capnometer was used to maintain end-tidal CO₂ and respiratory waveforms within normal range. A thermistor type rectal probe was placed under the animal, and external temperature was maintained at 37°C throughout the experiment by a feedback controlled warm water circulating heating pad. The ventilator was used to gate the MRI pulse sequence based on the respiratory cycle to avoid motion artifacts. Multi-slice gradient-echo images were acquired with a 30° flip-angle and TE/TR = 6/30 ms. The resolution was 100 × 100 × 500 microns, with a 256 × 256 matrix. Four averages were acquired.

Data processing and analysis

Image reconstruction was done in Paravision (Bruker, Billerica, MA.), and MR images were analyzed using Bruker Paravision software, as well as ImageJ (National Institutes of Health, Bethesda, MD). Because receiver gains on the MRI system were adjusted between imaging sessions, we adjusted image contrast and brightness to highlight contrast between the kidney and surrounding tissue, but all observed structures and changes were readily seen without manipulation. All mathematical analysis on the MRI data was done on unprocessed images using either ImageJ or Matlab (The Mathworks, Inc). To determine inter-glomerular spacing, the fast Fourier transform (FFT) was computed using 32 pixel intensity values along a profile line through the kidney cortex. Prior to the FFT, linear trends in the data were removed by subtracting the slope obtained by linear regression. The power spectrum was obtained by taking the square of the Fourier transform data. Power at each frequency was computed as a percentage of the total frequency spectrum. The average power of a several profile lines was computed to determine the repeatability of the power spectrum measurement.

Results

Detection of Basement Membrane with MRI

To label kidney GBM via the blood stream, rats were given two or three intra-venous injections of CF or NF. Rats were either imaged *in vivo* or sacrificed. After sacrifice, the kidneys were removed and imaged in fixative. Typical *ex vivo* GRE-MRI images of the kidney are shown in Figure 1. The top images (a,d, and g) are axial images, while images b,e, and h are sagittal images from the 3D dataset. The bottom images (c,f and i) show magnifications of the kidney cortex. The images show distinct spots of decreased signal intensity in the kidney cortex in CF-injected rats(g-i), compared to the NF-injected controls (d-f). The dark spots were approximately two pixels across, and were observed in all CF-injected rats, but not in normal or NF-injected rats. The location, size, and density of the dark spots are consistent with uniform and specific labeling of glomeruli in the cortex with CF. Additionally, CF accumulation was visible in the renal pelvis, consistent with the urinary excretion of CF after filtration.

Figures 1(j-m) show the signal intensity profiles of a line through the cortex and the average spatial power spectrum of $n = 8$ line profiles for the NF- and CF- inoculated rats. As seen by the profile, there was little variation in the signal intensity for NF-inoculated rats, but the intensity varied by 60% about the mean for CF-inoculated rats. This variation was observed in the spatial power spectrum, corresponding to a peak at approximately 2.25/mm, or a dark spot approximately every 440 μm .

Figure 2 shows typical fluorescent images of the kidney, with red stain for ferritin detected with Alexafluor 586, and blue showing cell nuclei stained with DAPI. Figures 2a and 2d show cortical fluorescence in NF- (a) and CF-injected (d) rats. The figures show the spotted accumulation of CF in glomeruli in cortex, consistent with the spots observed in MRI. These glomerular spots were not present in the kidneys of NF-injected rats. Figures 2b and 2e show 40x confocal images of single glomeruli in the NF- and CF- injected rats, respectively. There was little red fluorescence in NF-inoculated rat glomeruli. The uptake of CF, by contrast, was localized to an amorphous ribbon surrounding the capillaries, corresponding to the location of the GBM. The CF localization to the GBM was verified by fluorescent co-localization with WGA, as shown in the 3D reconstruction in Figure 2g-h at 40x magnification. Because the WGA was applied after sectioning, it labeled proteoglycans in the entire kidney, whereas the CF was limited to the GBM. However, the two labels were co-localized to the ribbon of the GBM. Glomeruli were also identified with electron microscopy (Figs 1c, f, and i). In the kidneys of rats inoculated with NF, no accumulation was seen in the GBM (Fig 2c). However, CF accumulated in the GBM, as well as in the filtration slits between podocytes (Fig. 2f). This is consistent with previous observations of uptake of CF, but not NF, in the GBM using TEM [29]. In some cases, as shown in Fig. 2i, CF accumulated in vacuoles inside podocytes and endothelial cells. Vacuoles were approximately 900 nm in diameter, and were often partially filled with CF. No similar accumulation was found in kidneys of NF-injected rats.

The accumulation of CF in the kidney cortex was detected with MRI *in vivo* after five intravenous injections, as shown in Figure 3. Single kidney slices in a $100 \times 100 \times 500 \mu\text{m}$ resolution image are shown for a NF-injected rat and a CF-injected rat (Figures 3a and 3d, respectively). Accumulation of CF in the kidney cortex was visible as punctuate dark spots, consistent with the observations with *ex vivo* MRI and TEM. Some accumulation of CF is also visible in the medulla and pelvis. There was good correspondence between *in vivo* and *ex vivo* MRI after CF accumulation. The signal intensity, normalized to the mean, is shown in Figs. 3b and 3e, and the average spatial power spectrum of this signal intensity for $n = 9$ lines is shown in Fig 3c and 3f. There was an oscillation in the signal intensity along the profile, which was not detected on average in the NF controls. The peak associated with the line profile oscillation was 1.25/mm (1 spot per 800 μm), or approximately half of the frequency determined at high-resolution *ex-vivo*. This is consistent with partial-volume effects between glomeruli with the lower *in vivo* imaging resolution.

Detection of Basement Membrane Disruption

Figure 4 shows typical *ex vivo* (50 μm isotropic) and *in vivo* ($100 \times 100 \times 500 \mu\text{m}$) GRE-MRI images of the normal and PAN-inoculated (FSGS) rats. CF-inoculated normal kidneys had hypointense spots in cortex (Fig 4a). With increasing time after the start of the PAN regimen (4b-c), the spots became surrounded by areas of hypointensity, such that the animals at late-stage FSGS showed no contrast between the glomeruli and surrounding cortex. This effect was seen *in vivo* in early FSGS (Fig 4e), where the cortical signal was darkened over background compared to NF-inoculated animals with comparable proteinuria (Fig. 4d). Figures 4f and 4g show that typical line profiles from images of cortex in CF-inoculated early FSGS rats varied by 50% from the mean, compared to a variation of 15% in NF-inoculated FSGS rats. The average spatial power spectra ($n = 9$ profile plots, Figs 4h and

4i) showed a peak at a frequency of 1.25/mm in the CF-inoculated rats and no deviation in the average spectrum of NF-inoculated rats. The spectrum of CF-inoculated FSGS rats had a standard deviation exceeding the amplitude of the peak in the spectrum. On average, the major impact of early-stage FSGS was increased CF accumulation rather than spatial redistribution. This is consistent with sclerosis being located in only a fraction of the cortex, which is characteristic of FSGS. These images are consistent with the progression from early- to late-stage sclerotic glomerular damage in FSGS, with loss of glomerular integrity, permeation of large proteins through the glomerulus, and uptake of filtered proteins by the proximal tubule, which starts as a focal process only in parts of the kidney.

As shown in Figure 5, the *in vivo* renal cortical signal intensity, normalized to the intensity of the liver, was significantly ($p < 0.05$) lower in early FSGS compared to normal controls - for both CF and NF, and the width of the distribution was noticeably larger in early FSGS animals. The signal was also lower in CF-inoculated normal animals compared to NF-inoculated normal controls, consistent with the presence of general proteinuria. The mean value of the signal intensity was significantly lower in CF-inoculated FSGS animals than in CF-inoculated controls, and the width of this distribution was larger in FSGS animals, consistent with the breakdown of the GBM.

The effects of FSGS on the accumulation of CF was also detected using anti-CF immunofluorescent staining using anti-ferritin IgG, and compared to changes detected by periodic acid-Schiff staining (Figure 6). In PAS staining compared to normal glomeruli (6a), early sclerosis was detected in a few glomeruli at day 13 (6b) and typical focal sclerosis was much more readily found in more glomeruli 13 weeks after PAN injection (6c). Morphological alterations include a reduced magenta PAS stain indicating GMB injury, cell death (reduction in labeled nuclei), and podocyte foot process fusion. The main difference between glomeruli in early- and late-stage FSGS was the number of severely sclerotic glomeruli detected with immunofluorescence. The glomerular accumulation of CF was visible as bright red spots in the fluorescent images in healthy animals (Fig 6d). By comparison, CF (Fig 6e) was diffusely distributed in the cortex in PAN- inoculated rats at early-stage FSGS, consistent with the data from MRI and EM. There was no NF accumulation in cortex in healthy animals, but light accumulation of NF in cortex in PAN-inoculated rats (data not shown), consistent with glomerular leakage and proteinuria. In confocal fluorescence images there was altered CF accumulation in approximately 5% of glomeruli in PAN-inoculated rats 2 weeks into the PAN regimen, and in approximately 10% of glomeruli 12 weeks into the regimen. This altered distribution is demonstrated in glomeruli in normal healthy kidney (Fig 6d,g), early FSGS (Fig 6e,h), and late FSGS (Fig 6f,i).

The accumulation of CF in early stage FSGS was similar to that observed after 12 weeks, with light accumulation of CF in the GBM and some accumulation in the surrounding proximal tubule. The difference in background intensity in fluorescence in late stage was attributed to the cellular internalization of CF in late-stage, as shown in Fig 6j-k. Podocyte foot processes were fused, and endothelia were detached from the GBM. In early FSGS, there was reduced binding of CF to the GBM, and an increase in the number of vesicles accumulating in the podocytes in some of the glomeruli observed. By 13 weeks, there was almost no accumulation of CF in the GBM, consistent with GBM disruption. Additionally, we observed CF in vesicles in the podocytes and in the urinary space (Fig 6j) and diffusely distributed in the podocyte cell body (Fig 6k). This indicates a redistribution of CF from the GBM to the cell body after GBM breakdown, which is consistent with diffuse accumulation of CF in cortex in the MRI data.

Discussion

The basement membrane is structurally and functionally integrated with cells and vasculature to form the tissue of every organ of the body. Cellular differentiation, communication, and proliferation are tightly linked to the function of the extracellular matrix, and the basement membrane forms an interface between tissues. In this work, a noninvasive imaging technique has been demonstrated for detecting and evaluating the integrity and function of the basement membrane using MRI. Using superparamagnetic, charged iron oxide nanoparticles that accumulate due to the high density of negative charges on proteoglycans in the kidney GBM, molecular structural changes associated with GBM breakdown were detected.

While anionic agents have previously been utilized to detect cartilage destruction [33], to the best of our knowledge this is the first demonstration of targeting of systemically injected contrast agents to the basement membrane. *In vitro* and *in vivo* images showed the uptake of CF in the kidney cortex, and high resolution MRI allows the identification of spots of CF accumulation by the decrease in signal intensity. The accumulation of CF in the glomeruli was confirmed by TEM of fixed kidneys, and large aggregates of CF were present in the basement membrane and filtration slits. There was no such accumulation in NF-injected controls. Fluorescence microscopy confirmed the accumulation of CF in every kidney glomerulus, and specifically in the GBM. These results suggest that MRI with CF as contrast might early detect the loss of proteoglycans from the GBM in glomerular disease earlier than detection by biopsy. The other possible renal applications of this method are thus to measure the function of single glomeruli *in vivo* or study the mechanism of glomerular diseases related to podocyte damage.

Cationized ferritin was used in this work because of its size and ease of uptake into the GBM. However, the MR relaxivity of ferritin is low ($1\text{--}10\text{ mM}^{-1}\text{ sec}^{-1}$, depending on field strength). It is practical to cationize other MR-visible nanoparticles. For instance, ferritin can be completely filled with iron, forming “magnetoferritin”, which would allow it to be detected in lower concentrations [34,35]. Alternatively, CF can be filled with gadolinium or clinically approved gadolinium chelates to form very potent MRI contrast agents, which should target the GBM [36]. This leads to the possibility for clinical application of cationized nanoparticles to visualize single glomeruli in humans.

The primary mechanism for cationic particle accumulation in the GBM is likely electrostatic. However, after attachment to the GBM and subsequent passage through the filtration slits, CF was in some instances taken up by podocytes. The accumulation of CF in podocytes may persist for some time, or may lead to excretion of CF. This work provides some evidence of CF excretion, because there is accumulation of CF in the renal pelvis.

There is *in vitro* evidence that CF binds to proteoglycan sites in organs such as heart, eye, and muscle tissue [5,25,28,37-40]. Other targets of cationic nanoparticles may exist outside of kidney, and other cationic probes have been developed to and overcome the barriers to delivery [41,42]. Cationization and subsequent uptake at specific sites therefore represents a simple modification of MR contrast agents that may be useful to study specific diseases.

Further work will be needed to assess the toxicity of cationic nanoparticles used as contrast agents, either systemically injected or otherwise. In this study, the total dose of iron delivered per bolus was approximately 5 mg/kg. This is about 10-fold the total daily dose of iron in a typical chronic transfusion in patients with severe anemia syndromes, and these patients are known become iron-overloaded after one year of therapy [44]. The use of magnetoferritin may reduce the required dose of iron, because relaxivity will increase an order of magnitude, while iron concentration will only double. Another concern arises if

apoferritin is loaded with gadolinium, given the current awareness of nephrogenic systemic fibrosis in patients with Gd chelates as contrast agents [45-46]. In normal patients, 0.1 - 0.3 mM/kg of Gd has been safely administered [46]. Since the T_1 relaxivity of Gd-filled ferritin is an order of magnitude higher than that of CF presented here, the detectable dose could be reduced. Indeed, Gd-loaded ferritin has been detected in a tumor *in vivo* with a bolus of 0.01 mM/kg Gd [36]. CF in its present or modified form may thus be a safe route of contrast delivery, but each specific modification of ferritin will have to be tested for toxicity.

In conclusion, cationic nanoparticles can be used as highly specific contrast agents to noninvasively detect basement membrane structure throughout the body. The structure of the basement membrane is important for the integrity of tissue throughout the body, and the *in vivo* determination of its structure should be useful for the early detection of tissue development and disease. The use of cationic nanoparticles as contrast agents opens the possibility of noninvasive imaging of the basement membrane in animal models and humans.

Acknowledgments

The authors gratefully acknowledge the assistance of S. Cheng, R. Azzam, and V. Crocker at the NINDS EM facility, and of M. Kiganda in the Laboratory of Functional and Molecular Imaging. R. Balaban of the NHLBI is thanked for stimulating discussion. We especially appreciate the assistance and expertise of C. Smith at the NINDS Light Imaging Facility. This research was supported in part by the Intramural Research Programs of the NIH/NINDS and NIDDK.

References

1. Hallmann R, Horn N, Selg M, Wendler O, Pausch F, Sorokin LM. Expression and function of laminins in the embryonic and mature vasculature. *Physiol Rev.* 2005; 85(3):979–1000. [PubMed: 15987800]
2. Sherwood DR. Cell invasion through basement membranes: an anchor of understanding. *Trends Cell Biol.* 2006; 16(5):250–256. [PubMed: 16580836]
3. Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell.* 2006; 127(4):679–695. [PubMed: 17110329]
4. Schindler M, Nur-E-Kamal A, Ahmed I, Kamal J, Liu HY, Amor N, Ponery AS, Crockett DP, Grafe TH, Chung HY, Weik T, Jones E, Meiners S. Living in three dimensions: 3D nanostructured environments for cell culture and regenerative medicine. *Cell Biochem Biophys.* 2006; 45(2):215–227. [PubMed: 16757822]
5. Olsson R, Carlsson PO. The pancreatic islet endothelial cell: emerging roles in islet function and disease. *Int J Biochem Cell Biol.* 2006; 38(4):492–497. [PubMed: 16162421]
6. Deen WM, Lazzara MJ, Myers BD. Structural determinants of glomerular permeability. *Am J Physiol Renal Physiol.* 2001; 281(4):579–596.
7. Chodobska A, Szmydynger-Chodobska J. Choroid plexus: target for polypeptides and site of their synthesis. *Microsc Res Tech.* 2001; 52(1):65–82. [PubMed: 11135450]
8. Geninatti Chrich S, Barge A, Battistini E, Cabella C, Coluccia S, Longo D, Mainero V, Tarone G, Aime S. Magnetic resonance imaging visualization of targeted cells by the internalization of supramolecular adducts formed between avidin and biotinylated Gd³⁺ chelates. *J Biol Inorg Chem.* 2005; 10(1):78–86. [PubMed: 15616832]
9. Lowe MP. Activated MR contrast agents. *Curr Pharm Biotechnol.* 2004; 5(6):519–528. [PubMed: 15579041]
10. Josephson L, Kircher MF, Mahmood U, Tang Y, Weissleder R. Near-infrared fluorescent nanoparticles as combined MR/optical imaging probes. *Bioconj Chem.* 2002; 13(3):554–560.
11. Zhang S, Merritt M, Woessner DE, Lenkinski RE, Sherry AD. PARACEST agents: modulating MRI contrast via water proton exchange. *Acc Chem Res.* 2003; 36(10):783–790. [PubMed: 14567712]

12. Caruthers SD, Winter PM, Wickline SA, Lanza GM. Targeted magnetic resonance imaging contrast agents. *Methods Mol Med.* 2006; 124:7–400.
13. Farquar MG. The glomerular basement membrane: not gone, just forgotten. *J Clin Invest.* 2006; 116:2090–2093. [PubMed: 16886057]
14. Tryggvason K, Patrakka J, Wartiovaara J. Hereditary proteinuria syndromes and mechanisms of proteinuria. *N Engl J Med.* 2006; 354(13):1387–401. [PubMed: 16571882]
15. Russo LM, Sandoval RM, Mckee M, Osicka TM, Collins AB, Brown D, Molitoris BA, Comper WD. The normal kidney filters nephrotic levels of albumin retrieved by proximal tubule cells: retrieval is disrupted in nephrotic states. *Kidney Int.* 2007; 71(6):504–513. [PubMed: 17228368]
16. Kobayashi H, Jo SK, Kawamoto S, Yasuda H, Hu X, Knopp MV, Brechbiel MW, Choyke PL, Star RA. Polyamine dendrimer-based MRI contrast agents for functional kidney imaging to diagnose acute renal failure. *J Magn Reson Imaging.* 2004; 20(3):512–518. [PubMed: 15332261]
17. Baumann D, Rudin M. Quantitative assessment of rat kidney function by measuring the clearance of the contrast agent Gd(DOTA) using dynamic MRI. *Magn Reson Imaging.* 2000; 18:587–595. [PubMed: 10913720]
18. Annet L, Hermoye L, Peeters F, Jamar F, Dehoux JP, Van Beers BE. Glomerular filtration rate: assessment with dynamic contrast-enhanced MRI and a cortical compartment model in the rabbit kidney. *J Magn Reson Imaging.* 2004; 20:843–849. [PubMed: 15503326]
19. Bae KT, Heiken JP, Brink JA. Aortic and hepatic contrast medium enhancement at CT. Part I. Prediction with a computer model. *Radiology.* 2004; 207:843–849.
20. Buckley DL, Shurraf AE, Cheung CM, Jones AP, Mamtara H, Kalra PA. Measurement of single kidney function using dynamic contrast-enhanced MRI: comparison of two models in human subjects. *J Magn Reson Imaging.* 2006; 24:1117–1123. [PubMed: 16941606]
21. Alexander CS, Swingle KF, Nagasawa HT. Teratogenic effect of puromycin aminonucleoside on rat kidney. *Nephron.* 1966; 3(6):344–351. [PubMed: 6006204]
22. Ryan GB, Karkovsky MJ. An ultrastructural study of the mechanisms of proteinuria in aminonucleoside nephrosis. *Kidney Int.* 1975; 8:219–232. [PubMed: 1104966]
23. Abrahamson DR, Perry EW. Distribution of intravenously injected cationized ferritin within the developing glomerular basement membranes of newborn rat kidneys. *Anat Rec.* 1986; 216(4): 534–543. [PubMed: 3800001]
24. Alcorn D, Ryan GB. Distribution of anionic groups in the glomerular capillary wall in rat nephrotoxic nephritis and aminonucleoside nephrosis. *Pathology.* 1981; 13(1):37–50. [PubMed: 6164035]
25. Simionescu M, Simionescu N, Palade GE. Preferential distribution of anionic sites on the basement membrane and the abluminal aspect of the endothelium in fenestrated capillaries. *J Cell Biol.* 1982; 95(2 pt 1):425–434. [PubMed: 6815207]
26. Simionescu N, Simionescu M, Palade GE. Differentiated microdomains on the luminal surface of the capillary endothelium. I. Preferential distribution of anionic sites. *J Cell Biol.* 1981; 90(3):605–613. [PubMed: 7287817]
27. Bertolatus JA, Hunsicker LG. Polycation binding to glomerular basement membrane. Effect of biochemical modification. *Lab Invest.* 1987; 56(2):170–179. [PubMed: 3807316]
28. Vorbodt AW, Lossinsky AS, Dobrogowska DH, Wisniewski HM. Distribution of anionic sites and glycoconjugates on the endothelial surfaces of the developing blood-brain barrier. *Brain Res.* 1986; 394(1):69–79. [PubMed: 3756533]
29. Kanwar YS, Farquhar MG. Anionic sites in the glomerular basement membrane. In vivo and in vitro localization to the laminae rarae by cationic probes. *J Cell Biol.* 1979; 81(1):137–153. [PubMed: 90048]
30. Schmidley JW, Wissig SL. Anionic sites on the luminal surface of fenestrated and continuous capillaries of the CNS. *Brain Res.* 1986; 363:265–271. [PubMed: 3942898]
31. Brooks RA, Goldfarb RB, Bulte JWM, Aisen P. Relaxometry and magnetometry of ferritin. *Magn Reson Med.* 1998; 40:227–235. [PubMed: 9702704]
32. Danon D, Goldstein L, Marikovsky Y, Skutelsky E. Use of cationized ferritin as a label of negative charges on cell surfaces. *J Ultrastruct Res.* 1972; 38(5):500–510. [PubMed: 4111070]

33. Lesperance LM, Gray ML, Burstein D. Determination of fixed charge density in cartilage using nuclear magnetic resonance. *J Orthop Res*. 1992; 11(4):1–13. [PubMed: 1309384]
34. Bulte JW, Douglas T, Mann S, Frankel RB, Moskowitz BM, Brooks RA, Baumgamer CD, Vymazal J, Strub MP, Frank JA. Magnetoferritin: characterization of a novel superparamagnetic MR contrast agent. *J Magn Reson Imaging*. 1994; 4(3):497–505. [PubMed: 7802866]
35. Meldrum FC, Heywood BR, Mann S. Magnetoferritin: in vitro synthesis of a novel magnetic protein. *Science*. 1992; 257(5069):522–523. [PubMed: 1636086]
36. Geninatti Chric S, Bussolati B, Tei L, Grange C, Esposito G, Lanzardo S, Camussi G, Aime S. Magnetic resonance visualization of tumor angiogenesis by targeting neural cell adhesion molecules with the highly sensitive gadolinium-loaded apoferritin probe. *Cancer Res*. 2006; 66(18):9196–9201. [PubMed: 16982763]
37. Mills AN, Haworth SG. Greater permeability of the neonatal lung. Postnatal changes in surface charge and biochemistry of porcine pulmonary capillary endothelium. *J Thor Cardio Surg*. 1991; 101:909–916. [PubMed: 2023449]
38. Thethi K, Duszyk M. Decreased cell surface charge in cystic fibrosis epithelia. *Cell Biochemistry and function*. 1998; 15(1):35–38. [PubMed: 9075335]
39. Uzgiris EE, Lockwood SH, Kaplan JH. Oscillation of cell surface charge of human peripheral blood lymphocytes after stimulation with concanavalin A. *J Immun*. 1982; 128(5):1975–1978. [PubMed: 7199546]
40. Matsuoka T, Aiyama S, Kikuchi KI, Koike K. Uptake of cationized ferritin by the epithelium of the main excretory duct of the rat submandibular gland. *Anat Rec*. 258(1):10–113. 1000.
41. Masotti A, Magniola A, Sabatino G, Maira G, Denaro L, Conti F, Ortaggi G, Capuani G. Intracerebral diffusion of paramagnetic cationic liposomes containing GdDTPA₂-followed by MRI spectroscopy: assessment of pattern of diffusion and time steadiness of a non-viral vector model. *Int J Immunopathol Pharmacol*. 2006; 19(2):379–390. [PubMed: 16831304]
42. Shieh DB, Cheng FY, Su CH, Yeh CS, Wu MT, Wu YN, Tsai CY, Wu CL, Chen DH, Chou CH. Aqueous dispersions of magnetite nanoparticles with NH₃⁺ surfaces for magnetic manipulations of biomolecules and MRI contrast agents. *Biomaterials*. 2005; 26(34):1783–1791.
43. Ramos-Vara JA, Miller MA, Ojeda JL, Reid R, Craft D, Watson GL. Glomerulocystic kidney disease in a Belgian Malinois dog: an ultrastructural, immunohistochemical, and lectin-binding study. *Ultrastruct Pathol*. 2004; 28(1):33–42. [PubMed: 14967597]
44. Wood JC. Diagnosis and management of transfusion iron overload: The role of imaging. *Am J Hematol*. 2007; 82:1132–1135. [PubMed: 17963249]
45. Thakral C, Alhariri J, Abraham JL. Long-term retention of gadolinium in tissues from nephrogenic systemic fibrosis patient after multiple gadolinium-enhanced MRI scans: case report and implications. *Contrast Media Mol. Imaging*. 2007; 2:199–205. [PubMed: 17712863]
46. Ersoy H, Rybicki FJ. Biochemical safety profiles of gadolinium-based extracellular contrast agents and nephrogenic systemic fibrosis. *J Magn Reson Imag*. 2007; 26:1190–1197.

Figure 1.

Ex vivo study of gradient-recalled echo magnetic resonance images using cationic ferritin as a contrast agent. Images are (a-c) No ferritin injection, (d-f) native ferritin (NF), and (g-i) cationized ferritin (CF). c, f, and i show a magnification of the kidney cortex. No spots were detected in the kidney without ferritin or with NF injection. Spots were seen in the cortex after CF administration. The graphs show the signal intensity profiles of a line through the cortex (j and k) and averaged spatial power spectrum of intensity profiles (l and m) in NF (j and l) and CF (k and m)-inoculated rats. Error bars show mean $\pm$ 1SD between power spectra of $n = 8$ randomly chosen profile plots.

Figure 2.

Immunofluorescence and transmission electron microscopy showing the accumulation of systemically injected cationic iron oxide nanoparticles (ferritin, CF) in glomerular basement membrane of rat kidneys *in vivo*, and little accumulation of native iron oxide nanoparticles (NF). Ferritin was detected with anti-ferritin IgG and a secondary IgG conjugated to Alexa594 (shown in red), and wheat germ agglutinin (WGA) was detected by conjugation to Alexa488 (shown in green). DAPI was used to label cell nuclei (shown in blue). (a,d) 12 x fluorescent image of kidney cortex after injection of NF(a) and CF (d) and perfusion, showing CF was localized to glomeruli in cortex that are not heavily labeled in (a). (b,e) 40x confocal images of NF- and CF- labeled glomeruli, respectively, showing the

accumulation of CF in the basement membrane presenting as an amorphous ribbon, but little accumulation of NF. (c,f) Transmission electron microscopy of normal rat kidney glomeruli after three systemic injection of native horse-spleen ferritin (NF,c) and cationized horse spleen ferritin (CF, f). Scale bars are 100 nm. The images show endothelial cells (E) neighboring the basement membrane (BM) and the capillary lumen (CL). Podocytes (P) are shown adjacent to the BM, with filtration slits between the podocyte foot processes. Electron-dense CF is accumulated in the BM, and in some cases in the filtration slits. Some of the CF clusters are marked by arrows. No accumulation of NF is seen in the GBM. (g-h) Trichromic staining with additional WGA further exhibited the localization of CF in GBM at 40x confocal magnification. (i) In some cases CF was detected in aggregates inside endothelial cells and podocytes with TEM (scale 500 nm).

Figure 3.

In vivo detection of CF after systemic administration using respiratory-gated MRI. (a) Typical GRE image of a rat kidney after five NF injections, showing darkening of the MRI signal due to blood vessels in the cortex and medulla. (d) Typical GRE image of a rat kidney after five injections of CF, showing darkening of glomeruli in the cortex due to accumulation of CF. (b-c) and (e-f) show line profiles of signal intensity in the cortex, as well as the power spectrum of the line profile, for NF, and CF- injected rats, respectively. As seen in the power spectra, a peak is present at 1.25/mm in the kidney of the CF-injected rats that is not present in the kidney of the NF-injected rats. Error bars show mean $\pm$ 1SD between $n = 9$ randomly chosen profile plots.

Figure 4.

Detection of glomerular injury in puromycin-induced FSGS by gradient-recalled echo magnetic resonance images with cationized ferritin (CF). The accumulation of CF showed a clear spotted distribution in glomeruli from normal kidney (a), the spots were surrounded by areas of signal hypointensity but still visible in cortex of kidney from early FSGS (b) at 13 days after PAN injection, and then cortical signal was darkened without visible spots in kidneys of late FSGS (c) 13 weeks after PAN administration in an *ex vivo* study. A similar image was seen in kidney from early FSGS rat after CF injection (e) compared with NF administration (d) *in vivo*. Graph (f-i) showed a single line profile and averaged spatial power spectrum of the cortex image between NF (f and g) and CF (h and i) injection in early FSGS rats. Error bars show mean $\pm$ 1SD between power spectra of $n = 9$ randomly chosen profile plots.

Figure 5.

Distribution of signal intensity in gradient-recalled echo magnetic resonance images of kidneys from normal and early FSGS rats *in vivo* after administration of native ferritin (NF) or cationic ferritin (CF). Signal intensity in the kidney was normalized to the value in an ROI of the liver. Signal intensity after CF administration was approximately half of the value after NF-administration. The signal intensity was significantly decreased in early FSGS rats compared to normal animals in both CF and NF (* $p < 0.05$).

Figure 6.

The microscopic detection of glomerular structure injury in puromycin-induced FSGS rats after cationized ferritin (CF) injection. PAS staining (a-c) indicated the expansion of mesangial matrix (arrow 1) and protein cast (arrow 2) in glomerular from early FSGS rats (50 x magnification) (b); segmental sclerosis (arrow 3) in late FSGS (c) compared to normal glomeruli (a). Immunofluorescent staining of ferritin (d-f) showed a focal accumulation of CF in glomeruli in the normal kidney (d), progressive nonspecific accumulation of CF outside of the glomeruli in early FSGS rats (e), and few glomeruli with distinctly labeled fluorescence by late-stage (f, 10x magnification). Double staining (g-i) with DAPI (blue) and ferritin (red) showed the ribbon pattern of glomerular basement membrane (GBM) (g),

breakdown of GBM (h) in early FSGS and lumpy bumpy GBM (i) in late FSGS (30x confocal). Transmission electron micrographs (j and k) of glomeruli in FSGS animals, showed (j) localization of CF to podocyte vacuoles and (k) diffuse accumulation of CF in the podocyte cytosol with little CF present in the basement membrane.