Renal Inflammation: Targeted Iron Oxide Nanoparticles for Molecular MR Imaging in Mice¹

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Purpose:

To determine the feasibility of T2-weighted magnetic resonance (MR) imaging in the noninvasive quantification of renal inflammation by using superparamagnetic iron oxide (SPIO) nanoparticles targeted to tissue-bound C3 activation fragments in a mouse model of lupus nephritis.

Materials and Methods:

All animal procedures were approved by the University of Colorado-Denver animal care and use committee. SPIO nanoparticles were encapsulated by using amine-functionalized phospholipids. A recombinant protein containing the C3d-binding region of complement receptor type 2 (CR2) was then conjugated to the surface of the SPIO nanoparticle. Five MRL/lpr mice (a model of lupus nephritis) and six C57BL/6 wild-type mice were assessed with T2-weighted MR imaging at baseline and after SPIO injection. The same five MRL/lpr mice and three C57BL/6 mice also underwent MR imaging after injection of CR2-targeted SPIO. A series of T2-weighted pulses with 16 echo times was used to enable precise T2 mapping and calculation of T2 relaxation times in the cortex and outer and inner medulla of the kidneys, as well as in the spleen, muscle, and fat. The effects of treatment and animal genotype on T2 relaxation times were analyzed with repeated-measures analysis of variance.

Results:

At baseline, the T2-weighted signal intensity in the kidneys of MRL/lpr mice was higher than that in the kidneys of wild-type mice. Injection of untargeted SPIO did not alter the T2-weighted signal in the kidneys in either strain of mice. Injection of CR2-targeted SPIO in MRL/lpr mice, however, caused a significant accumulation of targeted iron oxide with a subsequent decrease in T2 relaxation times in the cortex and outer and inner medulla of the kidneys. No changes in T2 relaxation time were observed in the wild-type mice after injection of targeted SPIO.

Conclusion:

Injection of CR2-conjugated SPIO caused a significant reduction in T2-weighted MR imaging signal and T2 relaxation time in nephritic kidneys.

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In recent years, there has been increasing interest in the use of iron oxide nanoparticles in molecular imaging (noninvasive depiction of specific target molecules on cell surfaces). Conjugation of superparamagnetic iron oxide (SPIO) nanoparticles to targeting proteins enables the detection of specific molecules at the cellular level (1). For example, magnetic resonance (MR) imaging with SPIO-based detection agents can enable imaging of cell migration (2) and apoptosis (3) and detection of small foci of cancer (4,5). Such an approach is well suited for use in the dynamic noninvasive imaging of macromolecules or molecular events, but it requires targeting ligands that specifically bind to the molecule of interest.

Macromolecular contrast agents, including SPIO nanoparticles (60–150-nm diameter) and ultrasmall particles of iron oxide (5–40-nm diameter), have been used to detect functional and anatomic renal abnormalities (6,7). This approach has been used to successfully detect inflammation in models of antibody-mediated and ischemic renal disease (7–9), as well as inflammation at other tissue sites (10). When injected into the blood stream, SPIO nanoparticles or ultrasmall particles of iron oxide are taken up by macrophages and

accumulate in inflamed tissues. Their iron moiety negatively enhances signal attenuation on T2-weighted images, and their relative concentrations can be assessed by the presence of decreased T2 signal intensity or, more precisely, by the presence of decreased spin-spin T2 relaxation time. The decreased T2 relaxation time can be used to detect inflammation.

Systemic lupus erythematosus is a complex autoimmune disease. Up to 80% of patients with lupus develop renal abnormalities, but the renal prognosis varies greatly within this population (11). One of the great challenges of caring for patients with lupus nephritis is accurately assessing the activity of the renal disease and tailoring pharmacologic therapy to achieve remission while minimizing toxicity. Percutaneous renal biopsy is the reference standard with which to assess disease activity, and glomerular deposition of C3 activation fragments (C3b, iC3b, C3d) is a hallmark of active renal disease. During renal biopsy, however, only a small portion of the kidney is sampled. Furthermore, major complications may occur in more than 6% of biopsies (12,13). A noninvasive method with which to monitor disease activity in patients with

lupus nephritis would, therefore, be of great clinical utility.

The complement system is comprised of more than 30 proteins, and it is an important part of the innate immune system. Complement activation helps to eliminate invasive pathogens, but it also causes injury to host tissues in several autoimmune diseases. During complement activation, fragments of the protein C3 are covalently bound to tissue surfaces, and tissue-bound C3 fragments are characteristic of several types of renal disease. Complement receptor type 2 (CR2) (CD21) is a 140-kD transmembrane protein that binds the C3b, iC3b, and C3d cleavage fragments of C3. CR2 is comprised of multiple tandem short consensus repeats. Short consensus repeats are a conserved domain found in many complement proteins. Binding is mediated by short consensus repeats 1–2 of the protein (14). Consequently, the short consensus repeat 1–2 region is capable of enabling discrimination of cleaved (activated) forms of C3 from intact circulating C3. The purpose of the current study was to determine the feasibility of using T2-weighted MR imaging in the noninvasive quantification

Advances in Knowledge

- Superparamagnetic iron oxide (SPIO) nanoparticles conjugated with recombinant complement receptor type 2 (CR2) specifically bind to renal C3 deposits and enable noninvasive visualization of renal inflammation in a murine model of lupuslike glomerulonephritis.
- T2 mapping and precise calculation of T2 relaxation times and R2 relaxivity can be used to quantitatively assess the severity of renal inflammation in various kidney compartments in vivo.
- No toxicities were observed with the CR2-conjugated SPIO in mice, and the elimination half-life of targeted nanoparticles was reasonably short (48–96 hours).

Implications for Patient Care

- T2-weighted MR imaging with the CR2-conjugated SPIO nanoparticles may provide a quantitative noninvasive method with which to monitor disease activity in patients with autoimmune renal diseases, such as lupus nephritis.
- A method with which to measure complement activation throughout both kidneys might enable a more accurate assessment of disease activity than that yielded by currently available clinical tests.
- Noninvasive detection of intrarenal complement activation may reduce the need for repeated renal biopsies in patients with autoimmune diseases of the kidneys.

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Abbreviations:

CHO = Chinese hamster ovary
CR2 = complement receptor type 2
SPIO = superparamagnetic iron oxide

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See Materials and Methods for pertinent disclosures.

See also Science to Practice in this issue.

of renal inflammation by using SPIO nanoparticles targeted to tissue-bound C3 activation fragments in a mouse model of lupus nephritis. We hypothesized that changes in the T2 relaxation time after injection with the CR2-conjugated particles would enable quantitative assessment of the abundance of C3 deposition within the kidneys. This could then be used to detect renal inflammation due to immune complex deposition and complement activation.

Materials and Methods

One author (J.M.T.) is a stockholder in and consults for Taligen Therapeutics (Cambridge, Mass). Another author (V.M.H.) is a stockholder in and chief scientific officer of Taligen Therapeutics. All other authors had control of data that may have presented a conflict of interest. Animal care before and during the experimental procedures was conducted in accordance with the policies of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All animal procedures were approved by the University of Colorado–Denver animal care and use committee. All animals were obtained from Jackson Laboratories (Bar Harbor, Me) and housed under standard laboratory conditions (a 12-hour light-dark cycle, standard food and water ad libitum). The study design is shown in Figure 1a.

Generation of Targeted SPIO

SPIO nanoparticles were generated (B.A.L., who had recently received his doctoral degree; C.R.S., who had 10 years of experience in mechanical engineering and nanoscale materials synthesis) and functionalization for conjugation to proteins was confirmed, as described previously (15,16). The synthesis description is provided in Appendix E1 (online) and Figure E1 (online). The mean particle diameter was $9.7 \text{ nm} \pm 2.0$ (standard deviation) (1151 particles measured with ImageJ particle analysis software [National Institutes of Health, Bethesda, Md]).

A recombinant protein containing the first two short consensus repeats

of CR2 linked to the Fc portion of a mouse immunoglobulin G1 was generated (V.M.H., 25 years of experience with complement regulators and receptors), as described previously (17). One milligram of the recombinant protein ($6.7 \text{ } \mu\text{mol}$) was mixed (B.R., 7 years of experience with molecular biology) with 10 mg (1.5 nmol) of SPIO and 2 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce Biotechnology, Rockford, Ill); we added 0.2 mg of *N*-hydroxysulfosuccinimide (Sulfo-NHS; Pierce Biotechnology) and left the mixture at room temperature for 2 hours while vortexing every 15 minutes. The mixture was then washed and resuspended in phosphate-buffered saline.

Flow Cytometry

To confirm conjugation of CR2-Fc to the surface of the SPIO nanoparticle, $10 \text{ } \mu\text{g}$ of conjugated or unconjugated particles were incubated with $1 \text{ } \mu\text{g}$ of Cy3-labeled antimouse immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, Pa) or with biotinylated mouse anti-CR2 [mAb 171] (18) for 40 minutes on ice (B.R.). The biotinylated antibody was then detected with fluorescein isothiocyanate-labeled streptavidin. Surface staining was detected with flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ) and analyzed with software (CellQuest Pro; BD Biosciences). To test whether the conjugated particles bound to C3 opsonized cells, CHO cells (American Type Culture Collection, Manassas, Va) were incubated with 10% normal mouse serum for 1 hour at room temperature. Surface deposition of C3 was confirmed with fluorescein isothiocyanate-conjugated goat antimouse C3 antibody staining (MP Biomedicals, Akron, Ohio) and detected with flow cytometry (B.R., 2 years of experience with flow cytometry). The binding of CR2-Fc-conjugated SPIO CHO cells was performed by incubating opsonized or unmanipulated CHO cells with conjugated SPIO for 40 minutes at 4°C . Cy3-conjugated antimouse immunoglobulin G was used to detect the conjugated SPIO, and forward and side scatter were used to identify the CHO cells. Binding of SPIO to CHO cells was then

identified by examining Cy3 staining in the CHO cell population.

Animals

Sixteen-week-old MRL/lpr mice ($n = 5$) and C57BL/6 wild-type mice ($n = 6$) were assessed with MR imaging. All imaging and analysis was performed by an MR physicist (N.J.S., 12 years of pre-clinical imaging experience) with the assistance of a radiology technologist (K.M.H., 11 years of experience), both of whom were blinded to the animal group assignment. First, baseline images were obtained, and untargeted SPIO was injected (followed by acquisition of a series of T2-weighted images [Fig 1a]). After complete washout of unconjugated nanoparticles (2 weeks), baseline images were again obtained, and the T2 experiments were repeated with CR2-Fc-conjugated SPIO. (Five MRL/lpr mice and three C57BL/6 mice were used for this experiment.) The mice were injected with 0.4 mg ($10\text{--}16 \text{ mg}$ per kilogram of body weight) of CR2-Fc-conjugated or nontargeted SPIO via the tail vein in a volume of less than $200 \text{ } \mu\text{L}$.

MR imaging protocols.—At each time point, animals were weighed, anesthetized with isoflurane, and inserted into a 4.7-T MR imager (PharmaScan; Bruker BioSpin, Billerica, Mass). Proton-density-weighted MR imaging (for anatomic assessment) and T2-weighted MR imaging (for molecular assessment based on T2 values) were performed at baseline (prior to SPIO injection) and 4, 24, 48, and 72 hours after SPIO injection. A volume coil (38-mm diameter) tuned to the ^1H frequency of 200 MHz was used for radiofrequency transmission and reception. First, fast spin-echo rapid acquisition with relaxation enhancement tripilot imaging was performed. Thereafter, high-spatial-resolution rapid acquisition with relaxation enhancement proton-density-weighted MR images were acquired with the following parameters: repetition time msec/echo time msec, 3000/31.9; field of view, 4.00 cm ; section thickness, 1.0 mm ; intersection distance, 1.0 mm (no intersection gap allowed); axial orientation; number of sections, 16; number of signals

Figure 1

a.

Figure 1: (a) Flowchart of the study design. CHO = Chinese hamster ovary. (b) Flow cytometry profiles obtained with a monoclonal antibody to the C3d binding region of CR2. We confirmed successful conjugation of CR2-Fc to the surface of SPIO nanoparticles. No staining of unconjugated particles was seen. (c) Flow cytometry profiles show that incubation of CHO cells with 10% mouse serum opsonized the cells with C3, whereas no C3 was detected on unmanipulated cells. (d) Flow cytometry profiles show CR2-targeted SPIO bound to opsonized CHO cells, whereas binding to untargeted particles was similar to isotype staining of CHO cells.

acquired, two; matrix, 256×256 ; total acquisition time, 4 minutes 16 seconds. Subsequently, a series of multisection multiecho T2-weighted pulses with 16 echo times was used for precise T2 mapping and calculation of T2 relaxation times. The imaging parameters were as follows: 2650/10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160; field of view, 4.00 cm; section thickness, 1.50 mm; intersection distance, 1.80 mm; axial orientation; number of sections, 16; number of signals acquired, two; matrix, 128×256 ; total acquisition time, 11 minutes. All imaging protocols were developed by the director of the animal imaging core (N.J.S.).

MR imaging data analysis.—All images were processed with ParaVision software (version PV3.0.2; Bruker Biospin). For the series of T2-weighted images acquired with 16 echo times, a low-volume region of interest was placed

over the muscle, fat, spleen, left and right kidney cortex, left and right outer medulla, and left and right inner medulla on a corresponding section for each set of the echo time series. Three independent regions of interest (circles ranging from 0.5 to 0.9 mm²) were placed in the organ of interest, and the average T2 value was reported. All analysis was performed by the MR physicist with the assistance of the radiology technologist, both of whom were blinded to the animal group assignment. For the series of 16 echo times in our MR protocol, the

t2vtr-fitting function (Bruker Biospin), which was based on the following equations, was used to calculate T2 relaxation time (TR) as a function of signal intensity (S) and echo time (TE) of each image:

$$S = M_0(1 - e^{-TR/T1})e^{-TE/T2} \quad (1)$$

and

$$S = C_2 e^{-TE/T2}, \quad (2)$$

where $C_2 = M_0(1 - e^{-TR/T1})$ is a constant that gets fitted, M_0 represents magnetization, T1 is spin-lattice relaxation time, T2 is spin-spin relaxation time, and C_2

is a fitting constant. All calculated T2 times are given in milliseconds.

Immunofluorescence microscopy and histology.—For immunofluorescence on C3 expression, sagittal sections of the kidneys were snap frozen in an optimum-cutting-temperature compound (Sakura Finetek, Torrance, Calif). The 4- μ m-thick tissue slices were cut, fixed with acetone, and stained with fluorescein isothiocyanate-conjugated antimouse C3. Tissue slices were counterstained with hematoxylin (Vector Laboratories, Burlingame, Calif). Staining was evaluated with a fluorescent microscope (T2000; Nikon, Melville, NY).

For light microscopy, sagittal kidney slices were fixed in formalin, embedded in paraffin, and 4- μ m-thick slices were cut and stained with periodic acid-Schiff. Twenty glomeruli from each slice were examined. At the end of the study, kidneys were stained for iron with Perls Prussian blue, and 4- μ m-thick slices were cut. At least 10 glomeruli and 10 high-powered fields in the tubulointerstitium were examined in each kidney slice. Two authors (J.M.T., B.R.; 8 and 2 years of experience, respectively, with tissue processing and histologic analysis) performed all immunocytochemistry and histology experiments.

Statistical Analysis

Repeated-measures analysis of variance was used to assess the effects of treatment, group (lupus or control), and time, as well as their two-way interactions on T2 values as measured in the renal cortex, outer and inner medulla, spleen, muscle, and fat. Animal weight was also assessed. The animal was entered as a random variable in the model, and all other terms were fixed. The covariance structure over time was modeled as first-order autoregressive. In cases in which the group by treatment interactions was significant, simple effects modeling—applying the same model without group and all group interactions—was used to explore the effect of treatment within lupus and control animals separately. A *T* test was used for direct comparison of baseline characteristics between the two groups. All numerical data for T2 values are

presented as means $\pm$ standard deviations from the replicate experiments.

Results

CR2-conjugated Iron Oxide Nanoparticles Bind to Cell-bound C3

Successful conjugation of CR2-Fc to the surface of SPIO nanoparticles was confirmed at flow cytometry (Fig 1b). CHO cells were incubated with normal mouse serum to opsonize them with C3 activation fragments (Fig 1c). CR2-conjugated particles bound to the CHO cells, but unconjugated particles did not (Fig 1d). Furthermore, conjugated SPIO did not bind to unopsonized CHO cells. These experiments revealed that surface CR2-Fc can mediate specific binding of targeted SPIO nanoparticles to C3 fragments on the cell surface.

Immune-complex Deposition and Complement Activation in the Glomeruli of MRL/lpr Mice

The MRL/lpr strain of mice spontaneously develops lupuslike renal disease characterized by glomerular immune-complex deposition and complement activation (19). At histologic examination of the kidneys of 16-week-old mice, we confirmed that proliferative changes were present in the glomeruli of MRL/lpr mice; however, these changes were not present in wild-type mice (Fig 2a, 2b). At 18–20 weeks of age, glomerulosclerosis (Fig 2c) and tubulointerstitial fibrosis were prominent. At immunofluorescence microscopy, we confirmed the presence of C3 in the glomeruli of 16-week-old MRL/lpr mice (Fig 2d). In the MRL/lpr mice, C3 was also deposited along the tubular basement membrane in the outer (Fig 2e) and inner (Fig 2f) medulla. Small C3 deposits were present along the Bowman capsule and tubular basement membrane of unmanipulated wild-type mice (not shown).

Baseline T2 Values in Kidneys Are Higher in MRL/lpr Mice than in Wild-Type Mice

The mean body weight of MRL/lpr mice (35.1 g $\pm$ 2.3) was greater than that of wild-type mice (27.6 g $\pm$ 2.9, *P* =

.006) (Table). Rapid acquisition with relaxation enhancement proton-density-weighted MR imaging in the MRL/lpr mice revealed multiple areas of lymphadenopathy and hyperplasia of the lymphoid organs (Fig 3a, 3b). Multisection multiecho T2-weighted MR imaging revealed similar T2 relaxation times in the muscle, fat, and spleen in both groups (Table). T2-weighted signal intensity and corresponding T2 relaxation times in the renal cortex of MRL/lpr mice were higher than those in the renal cortex of wild-type mice (Table; Fig 3c, 3d), most likely because of higher water content due to tissue inflammation.

Untargeted SPIO Does Not Alter T2-weighted Signal in Kidneys of MRL/lpr Mice

T2 values in the kidneys of MRL/lpr mice did not significantly decrease after injection of unconjugated SPIO (Table, Fig 4). In wild-type mice, a small decrease in the signal in the outer medulla was observed 48 hours after injection with unconjugated SPIO (Table). No significant decrease in the T2-weighted signal intensity of muscle or fat was observed in MRL/lpr or wild-type animals. No changes in body weight were observed after injection of unconjugated SPIO at any time (Table), nor were any toxicities observed.

CR2-conjugated SPIO Reduces T2 Relaxation Time in Kidneys of MRL/lpr Mice

Injection of CR2-conjugated SPIO in wild-type mice did not cause a significant reduction in T2-weighted signal in any region of the kidneys (Table). Signal in the spleen, muscle, and fat of wild-type mice also did not change after injection with CR2-conjugated SPIO.

In marked contrast, injection of CR2-targeted SPIO into MRL/lpr mice caused a significant decrease in T2-weighted signal in the inner medulla (Table, Fig 4). A trend toward reduction of T2-weighted signal in the cortex was observed from 4 to 48 hours after injection, and signal in the outer and inner medulla was reduced from 4 to 72 hours after injection. No significant reduction in signal was seen in the

Figure 2

Figure 2: Renal disease in MRL/lpr mice. (a–c) Histologic images show (a) the normal glomerulus (arrowhead) in a wild-type mouse, (b) proliferative changes in a 16-week-old MRL/lpr mouse, and (c) glomerulosclerosis in an 18-week-old MRL/lpr mouse. (Periodic acid-Schiff stain; original magnification, $\times 400$.) (d–f) Immunofluorescence microscopy for C3 was performed in 16-week-old MRL/lpr mice. Photomicrographs show deposition of C3 in (d) glomeruli (arrowhead), (e) tubules of the outer medulla, and (f) the inner medulla. (C3 antibody stain; original magnification, $\times 400$.)

T2 Relaxation Time in Various Organs and Tissues at Baseline and 4, 24, 48, and 72 Hours after Injection of CR2-targeted Iron Oxide Nanoparticles

Organ, Tissue, or Weight	Baseline		4 Hours after Injection		24 Hours after Injection		48 Hours after Injection		72 Hours after Injection	
	MRL/lpr Mice	Wild-Type Mice	MRL/lpr Mice	Wild-Type Mice	MRL/lpr Mice	Wild-Type Mice	MRL/lpr Mice	Wild-Type Mice	MRL/lpr Mice	Wild-Type Mice
Cortex	52.6 $\pm$ 4.8	47.4 $\pm$ 2.6	47.1 $\pm$ 3.6	47.6 $\pm$ 4.8	46.0 $\pm$ 3.3	49.2 $\pm$ 4.1	47.5 $\pm$ 3.5	50.7 $\pm$ 3.7	49.8 $\pm$ 5.3	50.0 $\pm$ 3.4
Outer medulla	68.8 $\pm$ 8.4	61.3 $\pm$ 4.9	57.4 $\pm$ 3.4	63.3 $\pm$ 5.8	55.5 $\pm$ 3.4	68.9 $\pm$ 10.0	54.9 $\pm$ 3.2	64.2 $\pm$ 4.9	56.4 $\pm$ 6.5	62.5 $\pm$ 6.3
Inner medulla*	93.3 $\pm$ 10.3	89.7 $\pm$ 3.0	77.7 $\pm$ 8.3	91.1 $\pm$ 8.2	66.3 $\pm$ 6.4	92.7 $\pm$ 7.4	67.6 $\pm$ 7.1	91.9 $\pm$ 6.0	77.6 $\pm$ 9.6	92.2 $\pm$ 6.3
Spleen	41.8 $\pm$ 1.0	38.8 $\pm$ 4.0	41.5 $\pm$ 5.4	40.2 $\pm$ 7.3	43.2 $\pm$ 4.1	38.6 $\pm$ 4.2	44.5 $\pm$ 2.9	34.5 $\pm$ 4.8	44.4 $\pm$ 6.5	38.2 $\pm$ 7.1
Muscle	33.1 $\pm$ 0.8	32.8 $\pm$ 2.6	31.0 $\pm$ 3.1	37.0 $\pm$ 1.4	32.7 $\pm$ 3.1	33.0 $\pm$ 0.3	35.5 $\pm$ 2.8	33.0 $\pm$ 1.3	35.4 $\pm$ 1.3	33.0 $\pm$ 0.4
Fat	103.0 $\pm$ 5.1	100.4 $\pm$ 7.8	102.0 $\pm$ 3.0	100.0 $\pm$ 4.1	99.4 $\pm$ 6.4	100.2 $\pm$ 2.8	102.3 $\pm$ 3.1	97.8 $\pm$ 3.3	101.7 $\pm$ 3.5	98.7 $\pm$ 3.9
Body weight (g)†	35.1 $\pm$ 2.3	27.6 $\pm$ 2.9	35.1 $\pm$ 6.3	27.6 $\pm$ 3.1	34.4 $\pm$ 2.8	25.5 $\pm$ 2.3	34.8 $\pm$ 2.2	26.4 $\pm$ 2.2	34.6 $\pm$ 2.4	26.8 $\pm$ 2.6

Note.—Data are means $\pm$ standard deviations. For each data point, there were five repetitions for MRL/lpr mice and three repetitions for wild-type mice for examination of the spleen, muscle, and fat. For evaluation of the kidneys, the number of repetitions was 10 and six, respectively, since the left and right kidneys of each animal were evaluated.

* $P = .0077$ for the effects of treatment on T2 relaxation time in the inner medulla.

† $P = .006$ for baseline MRL/lpr mice versus baseline wild-type mice.

Figure 3

Figure 3: Abdominal MR images in MRL/lpr and wild-type mice. Arrowhead = left kidney. (a, b) Proton-density-weighted rapid acquisition with relaxation enhancement MR images in (a) a control mouse and (b) an MRL/lpr mouse show extrarenal abnormalities in the MRL/lpr mouse, including significant lymphadenopathy (small arrow). (c, d) Baseline T2-weighted multisecution multiecho images in (c) a control mouse and (d) an MRL/lpr mouse show higher T2-weighted signal intensity in the kidney of the MRL/lpr mouse than in the kidney of the wild-type control mouse (Table). Substantial lymphadenopathy can be seen in the MRL/lpr mouse (small arrow). T2 times in other organs were similar between the two groups (Table).

muscle, fat, or spleen of MRL/lpr mice (Table). Although the baseline T2 relaxation times in the cortex were higher in MRL/lpr mice than in wild-type control mice (Table), injection of CR2-targeted SPIO reduced the T2 relaxation times in the kidneys of MRL/lpr mice below the T2 relaxation times in the kidneys of control mice. Negative enhancement of T2 signal in the MRL/lpr kidneys was clearly visible on the T2 MR images with increasing echo times (Fig 5).

Location of Iron in the Kidneys of Mice Injected with CR2-targeted Nanoparticles

Iron was still detectable 72 hours after injection in some glomeruli and in tubules of MRL/lpr mice injected with the CR2-targeted SPIO; however, it was not seen in the kidneys of control mice, as

indicated by Perls Prussian blue stain (Fig 6).

Discussion

We developed specific CR2-conjugated SPIO nanoparticles to target the C3 inflammation protein in order to noninvasively depict renal inflammation with T2-weighted MR imaging in a mouse model. We used a recombinant form of CR2 to target SPIO nanoparticles to sites of complement activation. In vitro assays enabled us to confirm that CR2 linked to the surface of SPIO nanoparticles mediated binding to cells that were opsonized with C3 activation fragments; however, the particles did not bind to unopsonized cells. We used the

MRL/lpr model of lupus nephritis to show that injection of CR2-conjugated SPIO in mice caused a significant reduction in T2-weighted MR imaging signal and T2 relaxation times in nephritic kidneys. Injection of targeted nanoparticles into healthy control mice did not change these quantitative imaging endpoints. Furthermore, injection of untargeted SPIO particles did not affect the T2-weighted signal in the kidneys of MRL/lpr mice. Thus, by conjugating the SPIO nanoparticles with recombinant CR2 protein we have developed a contrast agent that specifically targets the site of complement activation. This method can be used to noninvasively detect active inflammation in immune-complex glomerulonephritis.

Complement activation is central to the pathogenesis of many inflammatory and autoimmune diseases (20). It is also a robust marker of tissue inflammation since activation of the complement system leads to the rapid covalent linkage of multiple C3b, iC3b, and C3d molecules to cell surfaces. Classical pathway activation by immunoglobulin, for example, can deposit more than 240 molecules of C3b to the surface for every molecule of C1q engaged by the immunoglobulin (21). Immunostaining for tissue C3 fragments is routinely performed on kidney biopsy samples when patients are suspected of having an autoimmune disease and when the presence of glomerular C3 deposits indicates active glomerulonephritis. The method described in our study could yield similar information noninvasively. It could also lead to the development of a more global picture of complement activation throughout both kidneys than that which is currently obtained from a single tissue sample.

SPIO nanoparticles are regarded as safe, and we did not observe any toxicities in mice after they received targeted SPIO. Nontargeted SPIO nanoparticles and ultrasmall particles of iron oxide have been used in patients with several types of disease, including renal disease, mostly to detect macrophage accumulation (22,23). Thus, this is a clinically feasible method with which to detect renal inflammation.

Figure 4

Figure 4: Relative changes in T2 values after injection with CR2-targeted and untargeted SPIO in MRL/lpr mice. **(a)** T2-weighted signal intensity was measured at various times in the cortex, inner and outer medulla, and muscle (as an unchanged comparison) of MRL/lpr mice. After injection with untargeted SPIO, no changes in T2 signal intensity were seen in the cortex, outer or inner medulla, or muscle. In contrast, after injection of CR2-targeted SPIO, significant decreases in T2 relaxation time were seen in the cortex and the outer and inner medulla. Changes were observed as soon as 4 hours after injection and persisted up to 72 hours after injection. **(b, c)** T2 signal intensity graphs are shown for the outer medullas of kidneys from **(b)** control and **(c)** MRL/lpr mice after injection with untargeted and CR2-targeted SPIO. Data are means $\pm$ standard deviations. Five animals were examined, and the signal intensities in both kidneys were measured. $P = .0077$ for the effect of treatment on T2 signal intensity in the inner medulla. BL = baseline, TE = echo time.

We initially anticipated that the CR2-targeted SPIO nanoparticles would have a stronger effect in the cortex since the glomeruli are limited to this renal compartment. In fact, our results showed a greater change in T2 relaxation times in the medulla. Immunofluorescence microscopy showed that the 16-week-old MRL/lpr mice have C3 deposits throughout the tubulointerstitium. The tubules occupy a greater volume within the kidney than do the glomeruli. Thus, although lupus nephritis is primarily regarded as a glomerular disease, tubular C3 may provide a more abundant target for the CR2-targeted nanoparticles. Thus, it is possible that this method will reveal spatiotemporal patterns of inflammation that are different from those

revealed by histologic analysis. We also had anticipated that nontargeted SPIO might be taken up by the inflamed kidneys and might cause a change in the T2 relaxation times, but we did not see this. It is possible that the substantial extrarenal disease (lymphadenopathy) provided enough nonspecific uptake of SPIO that too few particles accumulated in the kidneys to be detected.

This method, as performed in our study, had several limitations. The dose of SPIO used in our study was higher than that used clinically. Furthermore, the changes in T2 relaxation times seen after injection with the targeted SPIO were modest. Future improvements in nanoparticle design, as well as introduction of T2*- and/or ultrafast T2-based

protocols (currently in clinical development) may lead to improvements in the sensitivity of this method. In addition, introduction of T1-relaxing nanoprobe based on gadolinium ions instead of on iron oxide may further increase the clinical relevance of our findings. If not, the diagnostic power of this method may be limited. Even with the modest contrast effect, however, the method could still be useful in following up individual patients over time or in assessing the response to various therapies in groups of patients. Another limitation was that glomerular C3 deposition is not specific to lupus nephritis; it is seen in other types of renal disease. The definitive diagnosis of a given disease could therefore require the acquisition of other

Figure 5

Figure 5: T2-weighted multislice multiecho MR images (2650/10, 30, 50, 70) of (a) a wild-type mouse and (b) an MRL/lpr mouse 48 hours after injection of CR2-conjugated SPIOs. A decrease in T2 signal intensity is visible in all regions of the kidneys in the MRL/lpr mouse, particularly when an echo time (TE) of 70 msec was used. Arrowhead = left kidney.

Figure 6

Figure 6: Histologic analysis was performed to detect iron in the glomeruli and tubules of MRL/lpr mice injected with CR2-targeted SPIO. MRL/lpr and wild-type mice were sacrificed at the completion of the study. Kidney slices were stained with Perls Prussian blue to enable us to detect iron deposits in the tissue. (Original magnification, $\times 400$.) (a, b) In the MRL/lpr mice, iron was detected in the (a) glomeruli and (b) tubules. (c, d) In wild-type mice, iron was not seen in any (c) glomeruli or (d) tubules.

clinical and histologic data. It is possible, though, that this method could be useful in monitoring renal inflammation in a wide variety of diseases given the fact that C3 deposition is characteristic of several types of autoimmune and inflammatory kidney diseases.

In summary, we have developed a quantitative imaging technique with which to noninvasively detect intrarenal complement activation with an MR imaging contrast agent that is targeted to sites of C3 deposition. This technique uses a recombinant fragment of the complement receptor CR2 to target SPIO to tissue-bound C3b, iC3b, or C3d, and the particles are then detected by their negative enhancement on T2-weighted MR images. Injection of this agent led to a reduction in the T2-weighted signal intensity and, consequently, decreased T2 relaxation times in the kidneys of mice with active immune-complex glomerulonephritis; however, this did not affect the signal intensity in healthy control mice. No toxicities were observed in mice that received the SPIO imaging probe.

Practical applications: Such a technique may be useful as a noninvasive method with which to monitor the activity of renal disease in patients with lupus nephritis without performing serial renal biopsies. This technique may offer several potential benefits when monitoring lupus nephritis. Because of the difficulties in monitoring disease activity in patients with lupus nephritis, repeat renal biopsies are frequently necessary to assess the response to therapy or to diagnose disease relapse (24). The assessment of disease activity within the kidney with our technique may be more comprehensive than that afforded by biopsy and may enable one to avoid sampling error. MR images obtained after injection with CR2-conjugated SPIO can depict complement activation throughout both kidneys and enable one to distinguish renal C3 activation from that occurring at extra-renal locations. Longitudinal assessment with a technique such as this may offer important insight into the rate at which active disease responds to therapy and resolves.

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