Morishige K, et al., Supplement Data

Methods

Animal Protocol

All experimental protocols were approved by the Harvard Medical Area Standing Committee on Animals. Aortic atherosclerosis was induced in nine male New Zealand White rabbits (Millbrook Farms, Amherst, MA) through high-cholesterol diet (purified rabbit chow supplemented with 0.3% cholesterol and 4.7% coconut oil, Research Diets, Inc.) and mechanical injury as previously described.\(^1\)\(^,\)\(^2\) One week after initiating an atherogenic diet, rabbits underwent balloon injury (4F embolectomy catheter, Applied Medical) of the abdominal aorta under anesthesia with intramuscular ketamine, 35 mg/kg, and xylazine, 7 mg/kg. In three rabbits, an atherogenic diet was halted at three months and rosuvastatin (1 mg/kg/day, AstraZeneca, London, UK) was administered mixed in a regular diet, whereas the six other rabbits continued the atherogenic diet. Rosuvastatin was chosen as a representative of the class of hydrophilic statins, which limits the effects to only cholesterol lowering, whereas lipophilic statins may also exert lipid-independent direct effects on macrophages. Animals were euthanized after the final MRI sessions for the ex vivo MRI and histological examinations.

Superparamagnetic nanoparticles

MION-47 was produced at the Center for Molecular Imaging Research, Massachusetts General Hospital. The details of the compound have been described elsewhere.\(^3\)\(^,\)\(^4\) Briefly, MION-47 was synthesized by adding ammonium hydroxide to a mixture of T-10 dextran and ferrous and ferric salts chilled to 5-10°C, followed by heating to 60-80°C. The resulting nanoparticle has a \(\sim\)5-nm diameter core of superparamagnetic iron oxide, coated by a \(\sim\)10-nm-thick dextran layer. The particle size as determined by unimodal analysis was approximately 27 nm in diameter. As a consequence of the dextran coating, the nanoparticles are monodisperse and have a relatively
long blood half-life, which facilitates their accumulation in macrophages of atherosclerotic plaques. The R1 relaxivity was 28.8 (mM.sec)-1 and R2 relaxivity was 49.3 (mM.sec)-1 in aqueous solution at 37°C and 0.47 T. Plasma concentrations of the compound were determined using quantitative colorimetric iron assay kit (Bioassay Systems). Further physical, biological, and relaxivity characteristics of MION have been previously described.\textsuperscript{1, 2}

A previous study rigorously evaluated toxicity of a similar class of nanoparticles.\textsuperscript{3} In this study, up to 168 mg Fe/kg AMI-25 was infused in rats and dogs. Peak concentrations of Fe were found in the liver after 2 hr and in the spleen after 4 hr. Fe slowly cleared from the liver (half-life, 3 days) and spleen (half-life, 4 days) and was incorporated into hemoglobin of erythrocytes in a time-dependent fashion. Histologic and serologic studies detected no acute or subacute toxic effects. In addition, MION-47 has similar size and biological properties as ferumoxtran-10, which complies with the Food and Drug Administration guidelines.\textsuperscript{7}

MION-47 was infused into the rabbit via an ear vein (10 mg Fe/kg, approximately 40 mg Fe/rabbit). This dose was chosen based on a preliminary in vivo MRI study comparing the effects of 2.6 (a clinical dose), 10, and 30 mg Fe/kg on changes in T2 signal intensity (T2-SI). While 2.6 mg Fe/kg produced no significant T2 effect on the aorta, 30 mg Fe/kg brought about overwhelming T2-SI reduction effects in various tissues surrounding the aorta, e.g., back muscles. Seventy-two hours after injections of 10 mg Fe/kg MION-47, the T2-SI loss in back muscles had already returned to the original level, while reliably producing T2-SI reduction of the atherosclerotic aorta.

**MRI procedure**

Rabbits were sedated with ketamine (35 mg/kg)/xylazine (7 mg/kg) and imaged supine in a 3.0 Tesla MRI system using a four-channel phased array knee coil (Excite HD, GE Medical Systems, St Giles, UK). Twenty-eight axial slices on T2-weighted spin-echo sequence [echo time (TE) 34 ms, repetition time (TR) 1800 ms] and 20 axial slices on T1-weighted fast spin...
echo (TE 13 ms, TR 800 ms) of the abdominal aorta were obtained. Other general conditions included number of excitation (NEX)=6, field of view (FOV) 6×6 cm, slice/gap = 2.0/1.0 mm, data matrix 256×192, and in-plane resolution 234×312 μm. T2 spin echo was employed over the more conventional choice of T2 fast spin echo because less blurring of the vessel wall has been observed in former pilot trials utilizing this method. More sophisticated pulse sequences, such as double inversion-recovery black-blood imaging, which nullifies signal from fat and blood, were not employed due to limited image quality for small fields of view and long acquisition time per slice. Instead, superior and inferior spatial saturation slabs minimized signal from blood in conjunction with spectral fat suppression, which negated signal from peri-adventitial fat. Blurring of the vessel wall increased with electrocardiographic (ECG) gating as a result of the necessary extended imaging time in the presence of respiratory motion, so it was not used in the procedure. Acquisition times with combined ECG and respiratory gating were prohibitively long, and bulk motion artifact occurred as anesthetic dose wore off. With the limited vessel motion of the abdominal aorta, keeping acquisition time as low as possible minimized blurring. More time-efficient gradient echo sequences yielded images much lower in signal-to-noise ratio compared with spin echo images. Efficiency suffered due to the time necessary to create useful images.

Pre- and post-images were paired at the same levels according to distance from the right or left renal artery. Ex vivo MRI was also performed on excised 4% paraformaldehyde-fixed aortas in 0.9% NaCl solution.

**MR image analysis**

The aortic wall area and SI were obtained from MR images and correlated with wall area from histological assays. MR images were transferred from the MRI scanner to a DICOM image server for quantitative analysis. An observer unaware of the experimental protocol manually
traced the aortic lumen and outer wall on T2SE before MION-47 injection, using ImageJ software (National Institutes of Health). The aortic wall area and the average SI of each T2SE image were calculated by the software. In order to account for spatial SI variations of the MRI coil as well as variation across imaging sessions, the SI of the aortic wall was normalized by the average SI of a region of interest in back muscle for each slice.

The ratio of the aortic wall SI and the back muscle SI served as a metric in the quantitative analyses. In order to appreciate the T2 SI variation along the length of the vessel, the axial T2SE slices were reformatted into a coronal view of the aorta. Rigid registration between pre- and post-images was performed prior to image subtraction. The subtraction image was pseudo-colored and superimposed on the pre-image to demonstrate the magnitude of T2–SI reduction.

**Histological assays**
Rabbits were euthanized (IV sodium pentobarbital, 150 mg/kg) and heparinized (IV 1000 U/kg) immediately following the final MRI session. The abdominal aorta was marked at 3-mm intervals from the bifurcations of the renal arteries, which were used to establish correspondence with the MRI slice positions, and perfusion fixed with 4% paraformaldehyde. Each excised aortic ring was marked for orientation and frozen in O.C.T. compound (Sakura Finetek, Torrance, CA) for histological assays. A monoclonal anti-rabbit macrophage antibody (RAM11, Dako North America Inc, Carperiera, CA) and the ABC method (Vector Laboratories Inc, Burlingame, CA) were used to localize plaque macrophages. Accumulation of MION-47 in the atherosclerotic lesions was examined by Perl’s Prussian Blue stain enhanced with 3,3’-diaminobenzidine. Quantitative analyses employed ImagePro Plus v5.1 software (Media Cybernetics Inc., Bethesda, MD) to measure vessel wall areas of histological specimens as well as to evaluate the immunopositive area for RAM11 (macrophages).
In vitro experiments

To assess uptake of MION-47 in macrophages, human monocytes isolated from peripheral blood of healthy donors by density gradient centrifugation and adherence were cultured in RPMI 1640 medium containing 5% human serum for 14 days to facilitate differentiation into macrophages. Monocytes (day 1) or macrophages (day 14) were then incubated in medium containing MION-47 in a range of concentrations (0, 30, 100, and 300 mg/ml). The concentrations were chosen based on the in vivo kinetics data after bolus injection of the compound (10 mg Fe/kg) in vivo. After 72 h, cells were lysed and assayed for iron content by quantitative colorimetry (Bioassay Systems, Hayward, CA).

Statistical Analysis

All results are expressed as the mean ± SD. Throughout the text and figures, n represents the number of samples examined as indicated in the method. The number varies across tests since issues during histopathology processing results in a varied number of useful slides. Pre-contrast and post-contrast SI, as well as macrophage content and T2-SI were analyzed with a mixed-effects ANOVA model. The between-animal variability is low compared to within-animal variability. The animal was taken as the random effect and the group (control vs. rosuvastatin) as a fixed effect, and testing was performed for significance of the group effect. P-values of < 0.05 were considered to be statistically significant. Each graph of the regression analysis shows the values of correlation coefficient, r, measuring the strength of a linear relationship between the two variables.

References


