High-Resolution Magnetic Resonance Imaging Enhanced With Superparamagnetic Nanoparticles Measures Macrophage Burden in Atherosclerosis

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Background—Macrophages contribute to the progression and acute complications of atherosclerosis. Macrophage imaging may serve as a biomarker to identify subclinical inflamed lesions, to predict future risk, and to aid in the assessment of novel therapies.

Methods and Results—To test the hypothesis that nanoparticle-enhanced, high-resolution magnetic resonance imaging (MRI) can measure plaque macrophage accumulation, we used 3-T MRI with a macrophage-targeted superparamagnetic nanoparticle preparation (monocrystalline iron oxide nanoparticles-47 [MION-47]) in cholesterol-fed New Zealand White rabbits 6 months after balloon injury. In vivo MRI visualized thickened abdominal aortas on both T1- and T2-weighted spin-echo images (T1 spin echo, 20 axial slices per animal; T2 spin echo, 28 slices per animal). Seventy-two hours after MION-47 injection, aortas exhibited lower T2 signal intensity compared with before contrast imaging (signal intensity ratio, aortic wall/muscle: before, 1.44±0.26 versus after, 0.95±0.22; 164 slices; P<0.01), whereas T1 spin echo images showed no significant change. MRI on ex vivo specimens provided similar results. Histological studies colocalized iron accumulation with immunoreactive macrophages in atheromata. The magnitude of signal intensity reduction on T2 spin echo in vivo images further correlated with macrophage areas in situ (150 slices; r=0.73). Treatment with rosuvastatin for 3 months yielded diminished macrophage content (P<0.05) and reversed T2 signal intensity changes (P<0.005). Signal changes in rosuvastatin-treated rabbits correlated with reduced macrophage burden (r=0.73). In vitro validation studies showed concentration-dependent MION-47 uptake by human primary macrophages.

Conclusion—The magnitude of T2 signal intensity reduction in high-resolution MRI after administration of superparamagnetic phagocytosable nanoparticles can assess macrophage burden in atheromata, providing a clinically translatable tool to identify inflamed plaques and to monitor therapy-mediated changes in plaque inflammation. (Circulation. 2010;122:1707-1715.)

Key Words: atherosclerosis — inflammation — macrophages — magnetic resonance imaging — nanoparticles

Accumulation of activated macrophages influences clinical outcomes of cardiovascular diseases such as atherosclerosis and its acute complications, cardiac remodeling after acute infarction, and arterial and valvular calcification. In atherosclerosis, macrophage accumulation initiates lesion development and triggers clinical events by elaborating various molecules that promote inflammation, plaque disruption, and subsequent thrombus formation.1–4 However, conventional anatomic measures of advanced lesions (eg, plaque size or luminal stenoses) do not necessarily correlate with macrophage content. Imaging of macrophages should therefore offer a much-needed strategy for identifying inflamed plaques, often precursors of acute thrombotic events, enabling pathophysiological investigations, and assessing the effects of interventions.

Clinical Perspective on p 1715

Molecular imaging visualizes specific cell types or biological processes.5–9 Magnetic resonance imaging (MRI) noninvasively produces tomographic images with high soft-tissue contrast and excellent spatial resolution. Previous studies, including ours, reported that MRI with a 1.5-T scanner detects the progression and regression of aortic atherosclerosis in rabbits,10,11 characterizes human plaque components,12 and monitors changes in plaques in patients during drug interventions.13 The introduction

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of macrophage-targeted superparamagnetic nanoparticles improves the capabilities of MRI to image vascular targets specifically.\textsuperscript{14,15} Macrophages internalize such nanoparticles, altering the local magnetic field and thus producing the T2 shortening effect as visualized by signal reduction.\textsuperscript{16–19} The present study used a 3-T clinical MRI scanner in combination with recently developed biocompatible monocrystalline iron oxide nanoparticles-47 (MION-47) to test the specific hypothesis that high-resolution MRI enhanced with superparamagnetic nanoparticles can measure macrophage accumulation in atherosclerotic plaques and monitor changes in inflammatory burden during statin treatment.

**Methods**

See the online-only Data Supplement for detailed methods.

**Animal Protocol**

Aortic atherosclerosis was induced in 9 male New Zealand White rabbits through consumption of an atherogenic diet and mechanical injury, as previously described.\textsuperscript{11,20} In 3 rabbits, the atherogenic diet was halted at 3 months, and rosuvastatin (1 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}, AstraZeneca, London, UK) was administered mixed in a regular diet; the 6 other rabbits continued the atherogenic diet.

**Superparamagnetic Nanoparticles**

MION-47 has a ∼5-nm diameter core of superparamagnetic iron oxide coated with a ∼10-nm-thick dextran layer and has a long blood half-life, which facilitates its accumulation in macrophages of atherosclerotic plaques. The characteristics of MION have been described previously.\textsuperscript{17,21}

A previous study rigorously evaluated toxicity of a similar class of nanoparticles.\textsuperscript{22} Researchers in this study infused rats and dogs with up to 168 mg Fe/kg AMI-25. Peak concentrations of Fe were found in the liver after 2 hours and in the spleen after 4 hours. Fe slowly cleared from the liver (half-life, 3 days) and spleen (half-life, 4 days) and was incorporated into the hemoglobin of erythrocytes in a time-dependent fashion. Histological and serological studies detected no acute or subacute toxic effects. In addition, MION-47 has similar size and biological properties as ferumoxtran-10, a Food and Drug Administration–approved agent.\textsuperscript{23}

MION-47 was infused via an ear vein (10 mg Fe/kg, ∼40 mg Fe per rabbit). This dose was chosen on the basis of a preliminary in vivo MRI study comparing the effects of 2.6, 10, and 30 mg Fe/kg on changes in T2 signal intensity (SI) on spin-echo (SE) images. The recommended dose of ferumoxtran is 2.6 mg Fe/kg and of ferumoxtol is 7 mg Fe/kg, with higher doses possible in severely anemic patients. Although 2.6 mg Fe/kg produced no significant T2 effect on the aorta, 30 mg Fe/kg resulted in overwhelming signal reduction in various tissues surrounding the aorta, eg, back muscles. Seventy-two hours after injections of 10 mg Fe/kg MION-47, the signal loss in back muscles had already returned to baseline while reliably producing T2 signal reduction of the atherosclerotic aorta in these preliminary experiments.

**MRI Procedure**

Rabbits were sedated and imaged supine in a GE 3-T MRI system using a 4-channel knee coil. Twenty-eight axial slices on T2-weighted SE sequence (echo time, 34 ms; repetition time, 1800 ms) and 20 axial slices on T1-weighted fast SE (echo time, 13 ms; repetition time, 800 ms) of the abdominal aorta were acquired. Other general conditions included the following: number of excitations, 6; field of view, 6×6 cm; slice/gap, 2.0/1.0 mm; data matrix, 256×192; and in-plane resolution, 234×312 μm, plus spatial/spectral saturation pulses to null blood and peri-adventitial fat signal.

**Results**

**Human Primary Macrophages Internalize MION-47 In Vitro**

Initial validation studies examined in vitro whether macrophages internalize MION-47. After incubation with MION-47, human primary macrophages differentiated in culture media containing 5% human serum for 14 days tended to accumulate iron particles in a concentration-dependent manner at greater levels than did day 1 monocytes (Figure 1A and 1B). Iron nanoparticle uptake, if any, in cultured human smooth muscle and endothelial cells fell below the threshold of detection of the assay.

**3-T MRI Detects Progression of Atherosclerosis**

Normocholesterolemic rabbits usually have an abdominal aortic wall thinner than 200 to 300 μm. The 3-T MRI system used in the present study, which afforded resolutions of 234×312 μm per pixel, barely visualized the normal aortic wall (Figure 2A). After mechanical injury and cholesterol supplementation for 3 or 6 months, however, this instrumentation readily detected the thickened wall (Figure 2B). The aortic wall area on T2SE correlated well with the histomorphometric measurements (Figure 2C). The vessel wall appeared isointense to muscle on T1SE, whereas it generally appeared brighter than muscle on T2SE (Figure 2D).

**MION-47 Produces T2 Signal Reduction in Atherosclerotic Aortic Wall MRIs**

Plasma Fe concentrations in rabbits increased dramatically immediately after MION-47 injection as expected (10 mg Fe/kg), and returned to the levels of control rabbits within 72 hours (Figure 3A). MRI showed vessel wall signal reduction on T2SE in vivo at 24 hours (not shown) and 48 hours after MION-47 injection (Figure 3B). T2-weighted signal also declined in other tissues, including back muscle (Figure 3B). This SI decline in atherosclerotic aortas persisted at 72 hours; however, other organs, including back muscles, rebounded to their initial SIIs (Figure 3C). Iron nanoparticles did not appear to alter the aortic wall SI on T1SE (Figure 3C). Superimposing subtraction pseudocolor images (Figure 4A, top) illustrates the local-
ization and magnitude of T2 signal reduction. Reconstructing the axial MRIs 3-dimensionally into the coronal views demonstrated that T2 signal reduction occurred in the entire abdominal aorta, although its magnitude varied (Figure 4A, bottom).

Quantitative analysis of 164 MRI slices from 6 hypercholesterolemic rabbits on an atherogenic diet without rosuvastatin treatment demonstrated that MION-47 administration (10 mg Fe/kg) reduced the T2-SI in the aortic wall 72 hours after injection, whereas T2-SI in back muscles did not differ substantially between preadministration and postadministration images (Figure 4B). These quantitative data agree with representative images shown in Figures 3C and 4A. Despite the lack of apparent change in muscle T2-SI, further normalizing the aortic wall SI by the muscle SI on each MRI (SI ratio = aortic wall SI/back muscle SI) enabled the measurement of net T2-SI reduction in the aorta and compensated for the superior-inferior inhomogeneity of the MRI receiver coil. The data demonstrated that MION-47 produced significant T2-SI changes in all 28 slices of an individual rabbit (Figure 4C). Notably, all 6 rabbits studied showed similar results (164 slices; Figure 4D), which correlates with the data on the aortic wall T2-SI (Figure 4B).

Figure 2. MRI (3 T) detects atherosclerotic plaque burden in hypercholesterolemic rabbits. A, MRI (3 T) barely visualized normal rabbit abdominal aorta in vivo. B, MRI (3 T) detected thickened aortic wall on a T2-weighted sequence (T2SE) in vivo 3 or 6 months after the initiation of cholesterol feeding and balloon injury. MRIs further exhibited the progression of maximal wall thickness from 0.47 mm at 3 months to 0.94 mm at 6 months in the same rabbit. C, Quantitative analysis closely associated T2SE MRI and the histological measurements of aortic wall area (164 slices in 6 rabbits). D, Vessel wall was isointense to muscle on T1SE, whereas it generally appeared brighter than muscle on T2SE at 6 months after initiation of cholesterol feeding and balloon injury.

Figure 3. Superparamagnetic iron nanoparticles (MION-47) produced T2-signal reduction in atherosclerotic plaques. A, In vivo kinetics of MION-47 in rabbits. Plasma concentrations of iron after MION-47 injection were determined by the quantitative colorimetric iron detection assay. Plasma iron concentrations decreased at 72 hours after MION-47 injection to the level of control rabbits. The data represent 3 independent experiments that provided similar results. B, MION-47 decreased T2-SI in the aortic wall of hypercholesterolemic rabbits 48 hours after injection. Other organs (eg, kidney, back muscle, and bone marrow, indicated by asterisk) also showed the signal reduction. C, top, MION-47–mediated T2-SI reduction in aortic wall lasted 72 hours after the injection with minimal reduction of SI in other organs. Back muscles had similar SI in preinjection and postinjection MRIs. Bottom, MION-47 produced no substantial change in the SI on T1SE.
T2-SI Changes In Vivo Correlate With Macrophage Accumulation In Situ

Macrophage immunostaining and iron staining corroborated the mechanisms of T2-SI loss in the atherosclerotic aorta of hypercholesterolemic rabbits in vivo and ex vivo (Figure 6A through 6C). Conversely, segments of the aorta that exhibited little (if any) T2-SI reduction contained sparse macrophages or iron (Figure 6D).

The Magnitude of T2-SI Reduction Quantitatively Reflects Plaque Macrophage Burden

The present study tested the specific hypothesis that MRI can quantify macrophage burden in atherosclerosis by examining whether T2-SI changes correlate with macrophage burden. Macrophage content in atherosclerotic plaques represented by percent immunoreactive area in situ (absolute positive area/absolute aortic wall area) correlated closely with the magnitude of T2-SI reduction in vivo (Figure 7A). These data agree with the representative images shown in Figure 6. In contrast, the magnitude of T2-SI reduction correlated poorly with the wall area (Figure 7B). Collectively, these results suggest that nanoparticle-enhanced MRI can measure the extent of macrophage accumulation and does not merely reflect anatomic plaque burden, affirming our study hypothesis.

T2-SI Reduction Correlates With Pre–MION-47 T2 SI Levels

Accumulating evidence suggests that hyperintensity on T2-weighted MRI is associated with inflamed lesions that contain superparamagnetic nanoparticles (MION-47) reduced signal on T2SE consistently in atherosclerotic aortas. A, top, Axial MRIs (left and middle) showed the T2 signal reduction in the abdominal aorta of a cholesterol-fed rabbit 72 hours after MION-47 injection (Pre vs Post). Top right, T2-SI reduction was further demonstrated as superimposed pseudocolor of image subtraction. Bottom, Three-dimensionally reconstructed coronal view of the same aorta showed that T2-SI reduction occurred throughout the aortic wall, although its magnitude varied. Arrows indicate the level of the axial views shown in the top row. B, MION-47 administration produced statistically significant reduction in T2-SI at 164 levels of the aortic wall of cholesterol-fed rabbits 72 hours after injection (P<0.001). Notably, T2-SI in back muscles did not differ substantially. C, The average T2-SI ratio (aortic wall T2-SI/back muscle T2-SI) in all in vivo axial slices of a representative rabbit decreased after MION-47 injection, although the magnitude varied. D, Quantitative analysis of 164 axial slices from 6 cholesterol-fed rabbits further indicated that MION-47 administration produced a statistically significant reduction in the T2-SI ratio in the aortic wall (P<0.01). E, MION-47 injection did not substantially affect the T1-SI ratio (120 slices, 6 rabbits).
increased free water content (water not bound by macromolecules or organelles). Aortic wall with higher SI on T2SE before MION-47 administration produced greater T2-SI changes after injection (Figure 7C). Similarly, precontrast T2-SI levels correlated with macrophage accumulation (Figure 7D), indicating that precontrast T2-SI may also reflect, indirectly via water content, macrophage burden in atherosclerosis.

**Statin Administration Decreases Macrophage Accumulation and T2-SI Reduction in Atherosclerosis**

We further tested whether an intervention could alter this imaging index of macrophage content. Rosuvastatin administration was started in rabbits 3 months after mechanical injury and initiation of a high-cholesterol diet. The 3 rabbits treated with rosuvastatin added to a standard diet for 3 months had lower total cholesterol levels than the 6 untreated rabbits (control group, 1122±89 mg/dL; rosuvastatin group, 63±18 mg/dL; P<0.01). Rosuvastatin-treated rabbits exhibited reversal of T2-SI changes in atherosclerosis in vivo and ex vivo, validated by histological analyses exhibiting less macrophage burden and iron accumulation within the intima (Figure 7A and 8B) compared with rabbits that did not receive rosuvastatin (Figure 6). MION-47 did not significantly reduce T2-SI in rosuvastatin-treated rabbits (Figure 8C). The magnitude of T2-SI reduction in rabbits treated with rosuvastatin was significantly lower than that of controlled rabbits that continued on the atherogenic diet (Figure 8D). Furthermore, these 2 factors, T2-SI changes and macrophage burden, were closely correlated (Figure 8E; see Figure 7A for the untreated rabbits). Although precontrast T2-SI was not substantially decreased in rosuvastatin-treated rabbits (Figure 8F), this metric remained positively correlated with T2-SI reduction (Figure 8G).

**Discussion**

Macrophage burden influences clinical outcomes of various inflammatory diseases, including atherosclerosis. Therefore, development of novel circulating or imaging biomarkers targeting macrophages should help identify patients with subclinical inflamed lesions and provide new and important insights into preventive cardiovascular medicine. The present study demonstrated in vivo and validated ex vivo and in situ that the magnitude of T2-SI reduction, resulting from the presence of superparamagnetic iron nanoparticles (MION-47), reflects the extent of macrophage accumulation in the aorta of hypercholes-

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**Figure 5.** MRI validated ex vivo the T2 signal reduction effect of MION-47 administration. Before MION-47 injection, the ex vivo atherosclerotic wall showed high T2-SI (top left; ex vivo, pre). The image localizer indicates levels of slices 1 and 2 (bottom). Both in vivo and ex vivo images of slice 1 exhibited the T2-SI reduction in the whole layer of aortic wall. Slice 2 showed in vivo and ex vivo T2-SI reduction in the intimal layer close to the luminal surface.

**Figure 6.** The magnitude of MION-47–mediated T2-SI reduction is associated with the extent of macrophage accumulation in rabbit aortic atherosclerosis. A and B, MRIs show similar patterns of T2-SI reduction in vivo and ex vivo. C, Histological analyses of colocalized iron accumulation (DAB-enhanced Perl Prussian blue) in macrophages (RAM-11) in the luminal surface of atherosclerotic aorta at the level shown in the in vivo and ex vivo MRIs. The rectangle in the ex vivo MRI (B) indicates the area shown in C. D, A representative MRI that exhibited less T2-SI reduction associated with few, if any, macrophages identified by immunostaining. Bars=100 μm.
terolemic rabbits, supporting the ability of this technique to measure inflammatory burden in atherosclerosis. Such a noninvasive imaging approach might also monitor changes in inflammation during therapeutic interventions.

Previous studies, including our own, proved MRI useful in detecting plaque size changes during the progression or regression of experimental atherosclerosis. Yet, anatomic assessments of atherosclerosis may not reflect the pathophysiology underlying acute clinical events. We and others established the role of macrophage products in the onset of acute thrombotic complications of atherosclerosis. Recent contributions have linked clinical evidence, macrophage biology, and molecular imaging research to establish the feasibility of visualizing macrophage burden or activation in cardiovascular diseases. The present results affirm that an MR approach combined with ultrasmall superparamagnetic iron oxide particles can quantitatively assess inflammatory burden within the plaque. This imaging approach, when translated to humans, should aid the study of patients with subclinical high-risk lesions.

Clinical evidence established that cholesterol lowering reduces acute coronary events. Preclinical studies, including our own, further demonstrated that dietary cholesterol lowering and statin treatment can limit inflammation, improve endothelial activation/dysfunction, and limit monocyte/macrophage infiltration in arteries. Diminished macrophage burden should limit proteolytic and thrombogenic activities in atheromata, reducing cardiovascular risk. Therefore, establishing methods that can noninvasively monitor changes in plaque macrophage content or activity during interventions will likely provide new insights into preventive cardiovascular medicine and offer powerful tools for choosing doses and assessing efficacy in pilot clinical trials on novel antiinflammatory therapies. Indeed, the present results shed mechanistic light on recent clinical studies that indirectly suggested that an antiinflammatory effect of statins may contribute to their clinical benefit.

Accumulating evidence suggests that macrophages internalize superparamagnetic iron nanoparticles to a greater extent than do endothelial and smooth muscle cells. Recent contributions have linked clinical evidence, macrophage biology, and molecular imaging research to establish the feasibility of visualizing macrophage burden or activation in cardiovascular diseases. The present study shows in vitro that MION-47 uptake by macrophages depends on their state of differentiation. The underlying mechanisms of macrophage uptake of such MRI contrast agents remain incompletely understood and may involve multiple mechanisms (eg, fluid-phase versus receptor-mediated endocytosis). Recent studies suggested the role of several molecules in this process, including macrophage-scavenger receptor A and integrin Mac-1 (CD11b/CD18). Activation of macrophages may also enhance the uptake of superparamagnetic nanoparticles. Although the mechanisms of endocytosis remain uncertain, the T2-SI reduction caused by iron accumulation in macrophages reflects metabolic activity of these proinflammatory phagocytes, providing direct relevance to complications of atherosclerosis.

Increased iron accumulation in inflamed plaques may involve other factors. Enhanced vascular permeability resulting from injured endothelium or leaky microvessels may promote nanoparticle penetration into atherosclerotic lesions, augmenting access of the iron oxide to macrophages. The long half-life of the latest class of ultrasmall superparamagnetic iron oxide particles, including MION-47 and ferumoxtran-10, also favors efficient accumulation within plaques. In addition, hypointense appearance on T2-weighted MRI without any enhancing agents likely results from increased free water. Thus, the

Figure 7. Quantitative analyses correlate T2-SI changes and precontrast T2-SI levels with macrophage burden. A, Quantitative analysis demonstrated that in vivo magnitude of T2-SI reduction correlated with macrophage content in situ (percent macrophage-positive area in total aortic wall area) (r=0.73; n=150 MRI slices in 6 rabbits). B, The in vivo T2-SI reduction correlated poorly with plaque area measured histologically (r=0.26; n=164). C, T2 changes correlated with precontrast T2-SI in vivo (r=0.67; n=164). D, Correlations between precontrast T2-SI and percent macrophage-positive area (r=0.53; n=164).
association of inflammation in atherosclerosis with local edema may lead indirectly to its detection. Oxidative stress mediated by reactive oxygen species may also affect T2-SI.39 In support, a previous study correlated T2-SI with serum levels of biomarkers of inflammation in humans.40 Extensive quantitative analyses in the present study positively correlated precontrast T2-SI and plaque macrophage content (Figure 7C). Precontrast T2-SI further associated with the magnitude of T2-SI reduction in atherosclerotic aortas (Figure 7D). Intervention with rosuvastatin, an agent with lipid-lowering and likely antiinflammatory actions, further supported these findings. As discussed above, cholesterol lowering improves aspects of endothelial cell dysfunction and reduces macrophage accumulation.20,26,31 The present study demonstrates that statin treatment reduced total cholesterol levels in hypercholesterolemic rabbits and substantially reduced T2-SI in both precontrast and postcontrast images (Figure 8).

This investigation used a 3-T high-resolution clinical MRI scanner and consistently produced satisfactory in vivo plaque images. The spatial resolution produced in this study (234×312 μm) should suffice for imaging macrophage burden in human carotid or proximal coronary arteries. MRI combined with the superparamagnetic iron nanoparticles documented here presents a favorable method for screening and monitoring arteries in patients because of its noninvasive nature and the absence of iodine-based contrast or radioactive agents. Although recent developments now enable positive contrast with superparamagnetic iron nanoparticles,41 the techniques used here permit clinical translation without modification of the software on the MRI scanner.

Recent efforts have also attempted to establish noninvasive MRI as a tool for detecting other aspects of plaque inflammation, including cell growth or apoptosis, thin fibrous caps,43 intraplaque microvessels or hemorrhage,44,45 thrombus formation,46 and lipids.47,48 Ongoing studies are further exploring the feasibility of visualizing plaque macrophages in a manner more specific to the activated state using small-molecule conjugates49 or detecting specific molecules associated with acute complications of atherosclerosis such as myeloperoxidase, a product of macrophages and other inflammatory leukocytes, and vascular cell adhesion molecule 1.50
The present study expands more than a decade of fundamental research into the inflammatory mechanisms that prevail in atheromata and promote its clinical expression.\textsuperscript{1,2} Gauging plaque macrophage accumulation as a well-validated indicator of local inflammatory status has several clinical applications, including the assessment of the inflammatory burden, rather than the mere anatomy, of atherosclerotic plaques in individual patients before clinical complication occurs. Visualizing macrophage accumulation or function as a biomarker thus offers a potentially powerful strategy in personalized preventive medicine. This method will also provide a research tool to further dissect the role of vascular inflammation in clinical outcomes. Finally, noninvasive imaging of macrophage burden directly implicated in plaque pathobiology may furnish an important functional biomarker for the evaluation of novel antiinflammatory therapies in humans.

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Disclosures

Dr Aikawa has received a research grant from AstraZeneca. Dr Ganz has received honoraria from Pfizer for his speakers’ bureau appointments. Dr Libby has served as an unpaid consultant to and member of the steering committees of investigator-initiated clinical trials for AstraZeneca, including A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden (ASTEROID), Justification for the Use of statins in Primary prevention: an intervention Trial Evaluating Rosuvastatin (JUPITER), and Study of Coronary Atheroma by Intravascular Ultrasound: Effect of Rosuvastatin Versus Atorvastatin (SATURN). The other authors report no conflicts.

References

Accumulation of macrophages influences clinical outcomes of various inflammatory diseases, including atherosclerosis. Particularly, these proinflammatory phagocytes promote not only development of atherosclerosis but also its acute thrombotic complications. Development of novel imaging or imaging biomarkers targeting macrophages should thus help to identify patients with subclinical inflamed lesions and to provide new and important insights into preventive cardiovascular medicine. Molecular or functional imaging, a rapidly emerging technology, visualizes biological or pathological processes in specific organs or disease contexts, in addition to providing anatomic information. The present study represents modern molecular imaging that can assess vascular inflammation. We demonstrate that high-resolution 3-T MRI enhanced with superparamagnetic iron nanoparticles can measure inflammatory burden in atherosclerosis of hypercholesterolemic rabbits. Macrophages internalize such iron particles, most likely via phagocytosis, thus changing the magnetic field in inflamed tissues. The present study demonstrates that the magnitude of T2-weighted signal intensity loss, reflecting phagocytic activity of macrophages, is associated positively with the extent of accumulation of these immune cells in atherosclerotic plaques. Such a noninvasive imaging approach might also offer a powerful tool to monitor the effects of antiinflammatory therapies in clinic practice or during clinical trials for new drugs. Our study indeed reports that the magnitude of T2-weighted signal intensity loss significantly decreased after statin treatment and correlated well with the reduction of lesional macrophage accumulation. Furthermore, such macrophage-targeting molecular imaging should provide novel insight into the mechanisms of atherosclerosis and its acute complications.
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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 rosvastatin (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 ~5-nm diameter core of superparamagnetic iron oxide, coated by a ~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
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, Carpineria, 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**


