Ferumoxtran-10–Enhanced MRI of the Hypercholesterolemic Rabbit Aorta
Relationship Between Signal Loss and Macrophage Infiltration

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Objective—Ferumoxtran-10 is an MRI contrast agent, which accumulates in macrophages and induces magnetic susceptibility artifacts (MSAs). We evaluated the ability of ferumoxtran-10–enhanced MRI to quantify focal macrophage infiltration in the aortic wall of hypercholesterolemic rabbits.

Methods and Results—Six weeks after a double-balloon injury of the infrarenal aorta, 12 hypercholesterolemic rabbits underwent MRI of the aorta before (first MRI) and after (second MRI) intravenous injection of ferumoxtran-10 (n=10) or saline (n=2). A third MRI was performed 5 days later to detect ferumoxtran-10–induced MSA in the aortic wall. Aortas were subsequently processed for histology, immunohistochemistry, and gelatin zymography studies. Injured aortas displayed a macrophage-rich neointima with high-matrix metalloproteinase 2 and 9 activities. Iron stain of injured aortas showed massive accumulation of ferumoxtran-10 in neointimal macrophages. Five days after the injection of ferumoxtran-10, MSAs were detected only in the injured aortas by in vivo MRI and were quantified indirectly using the percentage reduction of luminal area attributable to the extension of these MSAs in the aortic lumen. This parameter correlated with macrophage infiltration on corresponding aortic cross-sections (r=0.82; P<0.05).

Conclusion—Ferumoxtran-10–enhanced MRI allows quantitative assessment of macrophage infiltration induced by balloon angioplasty in the aorta of hypercholesterolemic rabbits. (Arterioscler Thromb Vasc Biol. 2006;26:176-181.)

Key Words: MRI ▪ imaging agents ▪ atherosclerosis ▪ macrophages ▪ metalloproteinases

Despite important clinical advances in the prevention and treatment of atherosclerosis during the past 20 years, coronary artery disease remains the first cause of mortality in industrialized countries. Coronary angiography is the gold standard for diagnosis and quantification of coronary stenoses but does not reliably predict the occurrence of acute coronary syndromes associated with sudden destabilization of vulnerable atherosclerotic plaques. Hence, the development of new imaging techniques for the detection of vulnerable plaques is of paramount importance. High-resolution MRI has been extensively studied for characterization of atherosclerotic plaques in humans. However, MRI remains limited in its ability to identify the vulnerable components of the plaque, which may require the use of new contrast agents.

Macrophages accumulate in vulnerable plaques and secrete locally abundant quantities of fibrous cap–degrading matrix metalloproteinases (MMPs), proinflammatory cytokines, and tissue factor, which are involved in acute plaque destabilization and thrombus formation. Therefore, macrophage density is considered to be a surrogate for plaque vulnerability. Dextran-coated ultrasmall superparamagnetic nanoparticles of iron oxide (USPIO) have been designed for contrast-enhanced MRI. One of these USPIOs, ferumoxtran-10, accumulates in macrophages causing a strong T2*-shortening effect, which generates signal voids on MRI or magnetic susceptibility artifacts (MSAs). In contrast, low concentrations of ferumoxtran-10 in circulating blood induce a predominant T1-shortening effect, allowing for a contrast-enhanced (white signal) magnetic resonance angiogram.

Ferumoxtran-10–enhanced MRI has been used for in vivo imaging of macrophage infiltration in hyperlipidemic rabbit aortas and in human atherosclerotic carotid arteries. However, in these previous studies, no attempt was made to quantify macrophage infiltration in the arterial wall using ferumoxtran-10–enhanced MRI. To address this issue, we developed a model of focal macrophage infiltration induced by balloon angioplasty in the hypercholesterolemic rabbit aorta and evaluated the ability...
of MRI, performed immediately after and 5 days after the injection of ferumoxtran-10, to quantify macrophage infiltration in the aortic wall.

**Methods**

**Animal Model of Arterial Injury**

Animal protocols were approved by Bichat Hospital Institutional Animal Care and Use Committee. Male New Zealand white rabbits were fed a 0.3% cholesterol diet, which was started 14 days before the first angioplasty.

Twelve rabbits (age, 5 to 6 months; weight, 3.5 to 4.0 kg) were studied. Animals were anesthetized with intravenous pentobarbital, and a 5-French sheath was inserted into the right femoral artery. A 4-French Fogarty embolectomy catheter was advanced in the aorta under fluoroscopy and withdrawn, with the balloon inflated, from the ostium of the left renal artery to the iliac bifurcation. Fourteen days later, animals were anesthetized, and a 5-French sheath was inserted into the left femoral artery. A 4.5-mm-diameter angioplasty balloon catheter was advanced in the aorta and serial, oversized balloon inflations (total duration per site, 2×1 minute; inflation pressure, 14 atmosphere; final balloon diameter, 5 mm) were performed along the previously injured arterial segment. All of the animals received intravenous heparin (1000 IU) and aspirin (100 mg) before angioplasty procedures.

**In Vivo MRI Protocol**

Before each MRI session, animals were anesthetized with intravenous pentobarbital. All of the MRIs were performed on a 1.5-T system (General Electric Healthcare) with a quadrature transmitreceive knee coil.

Preliminary studies were performed to determine the optimal time to echo (TE), so that the T1-shortening effect (white signal), associated with low-circulating ferumoxtran-10 concentrations, prevailed in the aortic lumen; and the T2*-shortening effect (MSA, signal void), associated with tissue accumulation of ferumoxtran-10, could be easily detected in the aortic wall. The same TE was used in all of the in vivo MRI studies thereafter.

Six weeks after the second angioplasty, a 3D fast gradient recall echo was collected in the axial plane before (first MRI) and immediately after (second MRI) an intravenous bolus of a low dose (2.8 mg Fe/kg) of ferumoxtran-10 (Sinere; kindly provided by Guerbet, Aulnay, France; n=10) or saline (n=2). An additional intravenous injection of ferumoxtran-10 was performed afterward in 10 rabbits, so as to reach a total dose of 56 mg Fe/kg, as described.10 Five days later, a third MRI using the same parameters as for the first MRIs was performed. The following parameters were used for all of the MRIs: repetition time, 9.4 ms; TE, 2.1 ms; flip angle, 30°; field of view, 10×10 cm; 256×192 matrix; in-plane axial resolution, 0.4×0.5 mm; number of excitations, 4; 48 partitions (3-mm-thick); overlap 1.5 mm; and acquisition time, 5 minutes.

Artificial luminal narrowing, because of encroachment of parietal, ferumoxtran-10–induced MSA into the aortic lumen, was quantified by the percentage reduction in aortic luminal area and volume between the 2 MRIs performed immediately after and 5 days after the injection of ferumoxtran-10 and was used as an indirect measure of MSA size (Figure 1 and see MRI section in Results for rationale). MRI acquisitions were postprocessed on a dedicated workstation (Advantage Windows, GE Healthcare). Axial views of the aortic segments, obtained immediately and 5 days after the injection of ferumoxtran-10, were matched using the origin of the left renal artery as an anatomic landmark and used for quantitative analyses of ferumoxtran-10–induced MSA. Aortic luminal area and luminal volume were measured automatically using the integrated Lumen MP (GE Healthcare) edge-detection software.15 Automated edge-detection was aberrant in <5% of studied axial views, which could be corrected manually. Therefore, all of the MRI measurements were performed in duplicate by 2 independent operators (F.H. and J-P.L.) who were blinded to histology, and the means of the 2 values were used in the analysis. Luminal areas were measured on an axial slice in the injured aorta (1 cm distal to the left renal artery) and the noninjured aorta (1 cm proximal to the left renal artery). Luminal volumes in the injured and noninjured aortas were measured over a 30-mm-long aortic segment (20 partitions), distally and proximally to the left renal artery, respectively.

Maximum intensity projections of the studied aortas were used to construct longitudinal views of the aortic lumen, which allowed a qualitative comparison of full-length segments of injured and noninjured aortas.

**Histology, Immunohistochemistry, and Morphometry**

Rabbits were euthanized by intravenous pentobarbital overdose immediately after the third MRI. A bolus of heparin was injected before euthanization to prevent clot formation. In each rabbit, 5-mm-long rings were cut from the injured aorta (1 cm distal to the left renal artery) and the noninjured aorta (1 cm proximal to the left renal artery), flushed with saline, fixed in 4% paraformaldehyde, and embedded in paraffin. Four adjacent 5-μm-thick, arterial cross-sections were cut and stained with the following: (1) hematoxylin-phloxin-safran; (2) Perl iron stain; and (3) monoclonal antibodies against RAM-11, a marker of rabbit macrophage cytoplasm (dilution 1:50; Dako, Trappes, France), and smooth muscle α-actin (dilution 1:50; DAKO, Trappes, France), and smooth muscle α-actin (dilution 1:50; DAKO), a marker of smooth muscle cells, as described.14 Sections were observed with a light microscope.

The Histolab software (Microvision) was used for digital planimetry of arterial cross-sections.15 Measurements of the luminal area, as well as the 2 areas bounded by the internal and external elastic laminae, served to compute intimal and medial areas and the intima/media ratio. Iron-rich (Perl-positive) and macrophage-rich (RAM-11-positive) areas were measured digitally using an automated, contrast-based, area analysis function of the Histolab software.

**SDS-PAGE Zymography**

In 8 rabbits euthanized 5 days after USPIO injection, a 5-mm-long ring was cut, in each rabbit, from the injured and noninjured aorta (15-mm distal and proximal from the left renal artery, respectively) and incubated for 24 hours in 1 mL of serum-free DMEM (Biomedia) at 37°C in humidified 5% CO2/95% air. Gelatinolytic activities of conditioned media were measured as described.16 Densitometric analysis of scanned gelatinolytic bands was performed with National Institutes of Health Image 1.55 software. Results are expressed in densitometric units per milligram of wet weight.

**Statistical Analysis**

Data are expressed as mean±SD. Injured and noninjured aortas were compared with the Student paired t test. To assess the interpreter variability in measures of percentage reduction of luminal area and volume, we compared the 2 sets of individual measurements (per studied aortic segment) provided by the 2 operators using a paired Student t test and a Pearson test. Linear regression analysis was used to study the relationships between the following: (1) RAM-11-
positive and Perl-positive areas; and (2) RAM-11-positive (or Perl-positive) areas and MSA-induced luminal narrowing. Statview 5.0 (SAS Institute Inc) was used for statistical analysis. A value of P<0.05 was considered significant.

Results

Histology

Six weeks after the second balloon injury, a concentric neointima had developed in injured aortas whereas there was no significant neointima in noninjured aortas (cross-sectional intimal areas: 1.85±0.67 mm² versus 0.18±0.03 mm²; P<0.001; intima/media ratio: 1.13±0.11 versus 0.13±0.05; P<0.001). Immunohistochemistry studies showed that neo-intimal lesions in injured aortas were composed of lipid-laden macrophages, which accumulated in the deeper layers of the intima, and of a smooth muscle cell-rich fibrocellular reaction, which was visible in the superficial layers of the intima.

Perl and RAM-11 staining of adjacent aortic cross-sections showed massive accumulation of ferumoxtran-10 in intimal macrophages of all (10 of 10) injured aortas 5 days after the injection of ferumoxtran-10 (Figure 2). In contrast, ferumoxtran-10 deposition was limited to rare, scattered subendothelial macrophages in noninjured aortas (n=10). No iron stain was observed in injured and noninjured aortas of control rabbits injected with saline (n=2, data not shown). Morphometric analysis indicated a linear relationship (r=0.98; P<0.05) between RAM-11-positive and Perl-positive cross-sectional areas (Figure I, available online at http://atvb.ahajournals.org).

Gelatinolytic Activity

Gelatinolytic activities were measured in the conditioned media after 24-hour incubation of aortic rings obtained from the injured and noninjured aortas of 8 rabbits. Intense pro-MMP-9 (98 kDa), pro-MMP-2 (70 kDa), and MMP-2 (60 kDa) activities were detected in the conditioned media of injured aortas (Figure II, available online at http://atvb.ahajournals.org). In contrast, a lower, constitutive pro-MMP2 activity, but no or marginal MMP-2 and pro-MMP-9 activities, were found in the conditioned media of noninjured aortas.

MRI

Magnetic resonance signal intensities were similar in the aortic wall and lumen (both in injured and noninjured aortas) before the injection of ferumoxtran-10 (Figure 3A and 3B). Axial (Figure 3C and 3D) and longitudinal (Figure 4A) views, acquired immediately after low-dose injection of ferumoxtran-10, showed an intraluminal white signal, with diffuse luminal irregularities over the length of the injured aorta, which were not observed in the noninjured aorta. No MSA was observed in the arterial wall on MRIs performed either before or immediately after the injection of ferumoxtran-10. In contrast, strong MSAs (signal void) were observed in the arterial wall and adjacent luminal areas of all (10 of 10) injured aortas on the third MRI performed 5 days after the injection of ferumoxtran-10 (Figures 3E and 4B). No MSA was present in noninjured aortas of rabbits injected with ferumoxtran-10 (n=10; Figures 3F and 4B) and in aortas (either injured or noninjured) of rabbits injected with saline (n=2; data not shown). Of note, dark semicircular artifacts were observed at the water-fat interface in the frequency-encoding direction around the aorta (either injured or noninjured) on MRIs performed before, immediately after, and 5 days after the injection of ferumoxtran-10 (arrowheads in Figure 3), precluding direct measurement of the MSA area.

Typically, MSAs extended outside the anatomic borders of the aortic wall, both perivascularly and intraluminally. Intraluminal MSAs resulted in a “pseudostenotic” luminal encroachment of the injured aorta, which could be clearly delineated on both axial (arrows on Figure 3E) and longitudinal (arrows on Figure 4B) views. In contrast, it was difficult to discriminate between MSA and dark perivascular artifacts. Hence, the percentage reduction in luminal area and volume, measured on axial views acquired immediately and 5 days after the injection of ferumoxtran-10, were used as indirect measures of MSA size.

Individual measures of the percentage reduction in luminal area and volume did not differ significantly between the 2
operators (12.7±3.3% versus 12.9±2.9% and 8.9±1.9% versus 8.5±1.8%, respectively; not significant for both) and strongly correlated ($r=0.98$ and $r=0.89$, respectively; $P<0.001$ for both). Both percentage reduction in luminal area (Figure 5A) and volume (Figure 5B) were significantly higher in injured versus noninjured aortas 5 days after the injection of ferumoxtran-10. In addition, the percentage reduction in luminal area in the injured aorta strongly correlated with the Perl-positive ($y=172.1x+0.6$; $r=0.78$; $P<0.05$; Figure 5C), as well as the RAM-11-positive ($y=178.5x+1.9$; $r=0.82$; $P<0.05$; Figure 5D) area on corresponding arterial cross-sections ($n=10$).

Discussion

The main result of the present study is that ferumoxtran-10–enhanced MRI allows a noninvasive detection and indirect quantification of macrophage infiltration in the arterial wall in a model of focal inflammation induced by balloon injury in the aorta of hypercholesterolemic rabbits.

It is increasingly recognized that the development of new imaging techniques that may identify vulnerable atherosclerotic plaques is a major objective in the search for vulnerable patients, that is, patients at high risk of acute coronary syndromes and sudden cardiac death. Hence, the list of imaging modalities aimed at characterizing plaque structure, rather than plaque volume and resultant luminal stenosis, has been growing at a rapid pace. However, most of these techniques rely on invasive approaches. High-resolution MRI is a noninvasive technique that has been used successfully for in vivo and ex vivo plaque imaging in animal models
and in patients. Contrast agents, which either enhance the contrast in tissues in which they reside or target a specific biological feature associated with plaque vulnerability, may be required for a more accurate identification of the vulnerable components of the plaque with MRI. Ferumoxtran-10 belongs to the latter group of agents and has been proposed as a candidate for functional imaging of vulnerable atherosclerotic plaques.

Previous experimental and clinical studies suggested that ferumoxtran-10 accumulates in plaque macrophages and induces MSAs because of a potent T2* shortening effect. In the present study, we developed a model of focal aortic inflammation induced by endothelial abrasion followed by overstretched balloon angioplasty in hypercholesterolemic rabbits. We documented that the resulting injury-induced neointima was particularly rich in macrophages and secreted abundant amounts of MMP, which are important features of vulnerable atherosclerotic plaques. In contrast to previous studies performed in animal models with diffuse atherosclerosis, our model allows straightforward anatomic localization of the inflammatory arterial lesion and a direct comparison of diseased versus normal arterial segments in the same rabbit. We found that ferumoxtran-10 colocalized with intimal macrophages and induced MSAs, which were readily detectable in vivo in the arterial wall 5 days after intravenous injection. These MSA extended both inward, into the lumen, and outward, in the periaortic region. Of note, MSAs were observed in all of the injured aortas, but in no case in noninjured aortas and in control rabbits injected with saline, suggesting that these MSAs are related to the presence of ferumoxtran-10-rich macrophages in the injured arterial wall.

However, the precise extension of MSAs in the aortic wall could not be quantified directly because of the presence at the water-fat interface of dark perivascular artifacts, which were difficult to discriminate from MSAs. Dark perivascular artifacts have been observed in previous studies of ferumoxtran-10–enhanced MRIs in vivo and may result in reduced specificity and overestimation of the true MSA area. Interestingly, these artifacts were present on the first MRI performed before ferumoxtran-10 injection and, hence, do not seem to be related to ferumoxtran-10.

To quantify MSA size, we developed an indirect approach, in which the percentage reduction in luminal area and volume, measured on axial slices immediately and 5 days after the injection of ferumoxtran-10, were used as surrogates for MSA size. A better contrast between aortic wall and lumen was obtained immediately after the injection of a low dose of ferumoxtran-10. Therefore, we used this second MRI acquisition (rather than the first acquisition) as a baseline, to which the third MRI performed 5 days after the injection of ferumoxtran-10 was compared. We found a strong correlation between the percentage of luminal area reduction and Perl-positive/RAM-11-positive areas on corresponding arterial cross-sections.

There are, indeed, 2 distinct approaches for quantitative measurement of ferumoxtran-10–related MSAs in models of arterial inflammation. Ruehm et al propose a direct measure of signal:noise ratio in subjectively defined regions of interest. The principal advantage of the approach by Ruehm et al stems from its focus on the MSA itself. However, SNR measurements are performed only in predefined regions of the arterial wall, and, therefore, the technique does not provide a mean intensity of the entire MSA, nor does it provide an estimate of the MSA spatial extension. In contrast, we propose an indirect measure of MSA size based on the observation that MSAs extend inward into the lumen and, thereby, reduce the luminal area. This approach does not focus directly on the MSA and, hence, underestimates the size of MSAs, which impinge minimally on the arterial lumen (small MSA or MSA extending outward the arterial wall) and overestimates the true size of the macrophage-rich area in the arterial wall (a 0.1-mm² macrophage area induces an ∼20% luminal narrowing 5 days after ferumoxtran-10 injection). However, MSA-related luminal reduction provides a measure of MSA over the entire aortic circumference (percentage of luminal area reduction) and aortic length (percentage of luminal volume reduction). Another advantage of this technique is the objective nature and the good reproducibility of computer-based delineation of the luminal area as opposed to subjectively defined regions of interest. Finally, the presence of perivascular artifacts in our experimental study, as well as in recent clinical studies, is a limitation for direct measurements of MSA intensity and/or spatial extension but might be circumvented by comparison of pre-ferumoxtran and post-ferumoxtran-10 MRI data.

Whether our results may be used in atherosclerotic patients to study the natural history of plaque inflammation over time and the efficacy of treatments aimed at plaque stabilization will require additional studies addressing several limitations. First, this model of arterial wall inflammation is not a model of atherosclerosis and does not replicate the complex nature of advanced atherosclerotic plaques. In particular, it is unknown whether the luminal narrowing induced by MSA in highly cellular lesions in the hypercholesterolemic rabbit can be replicated in hypocellular lesions in humans. Second, the 5-day interval between the injection of ferumoxtran-10 and the detection of MSAs by MRI may be clinically impractical. Interestingly, MSAs have been detected optimally in the carotid arteries of patients 24 to 48 hours after the injection of ferumoxtran-10, suggesting that shorter intervals may be considered. Third, it cannot be excluded that some of the MSAs present in the arterial wall result from ferumoxtran-10 uptake not only by arterial wall macrophages, but also by periaortic lymph nodes. However, no periaortic lymph node was observed on the first MRI (before the injection of ferumoxtran-10) or on macroscopic and microscopic examination of studied aortas. Finally, the use of ferumoxtran-10 to identify macrophage-rich atherosclerotic plaques has been tested, both experimentally and clinically, in the aorta and carotid arteries. Whether ferumoxtran-10 can be used to identify vulnerable plaques in human coronary arteries remains to be determined.

In summary, ferumoxtran-10–enhanced MRI allows an indirect, quantitative assessment of macrophage infiltration after balloon injury in the hypercholesterolemic rabbit aorta. Although ferumoxtran-10–enhanced MRI is a promising technique for the detection of aortic inflammation in the hypercholesterolemic rabbits, it will need to be tested clinically for the detection of vulnerable atherosclerotic plaques.
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Figure legends

Figure I. Correlation between RAM-11- and Perl’s-positive areas on aortic cross-sections of injured aortas. Five days after ferumoxtran-10 injection, adjacent cross-sections from injured aortas (n=10) were stained with Perl’s reagent (iron stain indicative of ferumoxtran-10 uptake) or an anti-RAM-11 monoclonal antibody (specific of rabbit macrophages). Note the linear relationship between Perl’s- and RAM-11-positive areas in injured aortas (r = 0.98, p<0.05).

Figure II. Gelatinolytic activities of injured and non-injured aortas. SDS-PAGE gelatin zymography was performed on conditioned medium, after 24-hour incubation of aortic rings obtained from injured and non-injured aortas (n= 8). A: Representative gelatinolytic activities of the injured and non-injured aorta in the same rabbit. Note the intense pro-MMP2 (70 kDa), MMP2 (60kDa) and pro-MMP9 (98kDa) activities in the conditioned medium of the injured aorta, whereas only constitutive pro-MMP2 activity was visible in the conditioned medium of the non-injured aorta. B-D: Bar-graphs showing pro-MMP2 (B), MMP2 (C) and pro-MMP9 (D) activities in injured and non-injured aortas. Results are expressed in densitometric units (DU)/mg wet weight. * p<0.05.
Figure I