Magnetic Resonance Imaging of Atherosclerotic Plaque With Ultrasmall Superparamagnetic Particles of Iron Oxide in Hyperlipidemic Rabbits

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Background—Based on the observation that ultrasmall superparamagnetic particles of iron oxides (USPIOs) are phagocytosed by cells of the mononuclear phagocytic system, the purpose of this study was to evaluate their use as a marker of atherosclerosis-associated inflammatory changes in the vessel wall before luminal narrowing is present.

Methods and Results—Experiments were conducted on 6 heritable hyperlipidemic and 3 New Zealand White rabbits. 3D MR angiography (MRA) of the thoracic aorta was performed on all rabbits by use of a conventional paramagnetic contrast agent that failed to reveal any abnormalities. One week later, all rabbits except 1 of the hyperlipidemic animals were injected with a USPIO contrast agent (Sinerem, Guerbet) at a dose of 1 mmol Fe/kg. 3D MRA data sets collected over the subsequent 5 days showed increasing signal in the aortic lumen. Whereas the aortic wall of the control rabbits remained smooth and bright, marked susceptibility effects became evident on day 4 within the aortic walls of hyperlipidemic rabbits. Ex vivo imaging of aortic specimens confirmed the in vivo results. Histopathology documented marked Fe uptake in macrophages embedded in atherosclerotic plaque of the hyperlipidemic rabbits. Electron microscopy showed multiple cytoplasmic Fe particles in macrophages. No such changes were seen in control rabbits or in the hyperlipidemic rabbit that had not received Sinerem.

Conclusions—USPIOs are phagocytosed by macrophages in atherosclerotic plaques of the aortic wall of hyperlipidemic rabbits in a quantity sufficient to cause susceptibility effects detectable by MRI. (Circulation. 2001;103:415-422.)

Key Words: atherosclerosis ■ magnetic resonance imaging ■ plaque ■ contrast media

Atherosclerosis represents a chronic inflammatory response to vessel wall injury, ending in an acute event induced by plaque rupture. Various injurious agents affecting the vessel walls cause an excessive inflammatory-fibroproliferative response resulting in progressive atherosclerotic plaque formation.1–3 Because the risks for thrombosis are more dependent on the particular plaque configuration than on the degree of luminal narrowing,4 the radiological assessment of atherosclerosis should extend beyond the mere depiction of luminal narrowing.

The uptake of intravenously administered superparamagnetic iron oxide preparations into cells of the mononuclear phagocytic system (MPS) results in hepatic, splenic, bone marrow, and nodal iron accumulation. Exploiting iron-associated T2 and T2* shortening effects, the select accumulation of iron particles in the MPS system has been successfully used for organ-specific MRI.5–7 In contrast to superparamagnetic iron oxide preparations composed of large particles (mean particle diameter, 72 nm) or magnetite albumin microspheres (mean particle diameter, 1 to 5 μm), ultrasmall particles of iron oxide (USPIOs) (mean diameter, 18 nm) are not immediately recognized by the hepatic and splenic MPS.8,9 The resulting prolongation of the intravascular half-life, together with inherent T1 shortening properties, has allowed USPIOs to be used as MR angiography (MRA) blood pool agents.10 In contrast to the large-particle superparamagnetic agents, the small USPIOs can extravasate through tight capillary pores characterized by diameters ranging between 5 and 100 nm.11 This capillary permeability permits USPIO uptake in MPS cells throughout the body.

Ross1 described the various stages of atherosclerotic genesis to represent different stages in a chronic inflammatory process affecting the arterial wall. The earliest lesion, the so-called “fatty streak,” can be found even in children12 and represents a primitive inflammatory response consisting of monocyte-derived macrophages and T lymphocytes.13 Because MPS cells are present in the atherosclerotic vessel wall, ultrasmall particulate iron oxide agents capable of navigating the very tight interstitial endothelial pores might be used to detect early atherosclerotic changes on MR images by means of USPIO-associated T2 and T2* shortening effects.
The purpose of this study was to evaluate the performance of USPIOs as a marker of macrophage activity in early atherosclerotic changes in the aortic wall of hyperlipidemic rabbits.

Methods
Experiments were conducted on 6 heritable hyperlipidemic rabbits (Harlan Interfauna Ltd, Wyton, Huntingdon Cambs, UK), a modified strain of Watanabe heritable hyperlipidemic (WHHL) rabbits, and 3 New Zealand White rabbits that served as a control group. The animals were 6 to 10 months of age. At this age, the animals are known to harbor active plaque formations within the aortic wall.14 All experiments were performed in full accordance with all regulations governing animal studies.

For each MRI session, the rabbits were fully anesthetized with ketamine (Ketasol 100, Dr. E. Graeub AG) 0.6 mL/kg body wt and xylazine (Rompun 2%, Bayer) 0.2 mL/kg body wt. All MRI was performed on a 1.5-T system (Signa Echospeed, GEMS). To maximize signal-to-noise ratio (SNR), a quadrature transmit-receive head coil was used. For 3D MRA, a 3D-enhanced fast gradient recall echo data set similar to that used for conventional 3D MRA was collected in the coronal plane with the following parameters: TR, 6.7 ms; TE, 1.6 ms; flip angle, 30°. A field of view of 28×19.6 cm was combined with a 256×192 matrix to provide an in-plane resolution of 1.1×1.0 mm. Two excitations were averaged. Thirty-two contiguous sections 1.4 mm thick were collected over 82 seconds. The use of zero interpolation in all 3 planes reduced voxel spacing by a factor of 2.

Experimental Design
The experiments were stacked to include 3D MRA imaging after the administration of conventional extracellular as well as USPIO contrast agents. Although 5 hyperlipidemic and all 3 control rabbits underwent the entire experimental protocol, 1 hyperlipidemic rabbit was not injected with the USPIO agent, thus skipping the second step of the outlined protocol:

1. Conventional 3D contrast-enhanced MRA of the thoracic aorta using conventional extracellular paramagnetic contrast material. During the acquisition of the 3D data set, 2 mL Gd-DOTA (Dotarem, Laboratoire Guerbet) diluted in 10 mL saline was injected intravenously by an automated injector at a flow rate of 0.1 mL/s.

2. After a 1-week delay to ensure excretion of all extracellular contrast material, the USPIO contrast agent (Sinerem, Laboratoire Guerbet) was injected intravenously at a dose of 1 mmol Fe/kg. 3D MRA imaging was performed daily up to 5 days after the intravenous Sinerem application.

3. After the 3D MRA imaging session on day 5, the rabbits were euthanized and the aortic specimen was removed. For ex vivo imaging, the aorta was tied at both ends, and the lumen was filled with water spiked with Gd-DOTA (1:50 dilution) to simulate the effect of intravascular contrast. The aortic specimens were placed in a small plastic container filled with saline for 3D MRA imaging.

4. Finally, the aortic specimen was subjected to histopathological evaluation. The vessel walls were inspected grossly for plaque protruding into the vessel lumen and subsequently scanned for the presence of iron after histochemical staining (Prussian blue staining). For electron microscopic analysis, a small portion of aortic wall of 2 hyperlipidemic rabbits was subsequently sampled: 1 that had received Sinerem and the 1 that had not.

Image Analysis
MRA data sets were postprocessed (Advantage Windows, GEMS). Maximum intensity projections (MIPs) were rendered. Rotated MIP displays ranging from −60° to +60° were documented on film. In addition, source images were available for analysis on a workstation.
which also allowed for interactive multiplanar reformatting of the data sets.

3D MRA data sets were analyzed by an observer blinded to the type of contrast agent administered as well as the type of animal regarding the ability to identify iron-induced susceptibility effects within the aortic wall.

For quantitative analysis, signal intensities were measured within regions of interest (ROIs) placed within the aortic lumen as well as within the aortic wall just beyond the confines of the vessel lumen. SNRs were calculated. To this end, individual source images were magnified on a workstation (Advantage Windows). SNR measurements were performed in a single large ROI (9 mm$^2$) placed within the aortic lumen and 3 small (1.1 mm$^2$) ROIs placed within the aortic wall, demonstrating marked USPIO uptake (hyperlipidemic rabbits) and corresponding regions in normal control rabbits. Measurements were performed on the precontrast image set as well as on the images collected on days 1, 2, 3, 4, and 5 after administration of Sinerem. Care was taken to ensure that ROIs of identical size were placed in identical locations on the different images. To compare the enhancement pattern and thus the uptake of USPIO in the aortic wall between hyperlipidemic rabbits and normal control rabbits, a paired $t$ test was performed on data points obtained on the precontrast images and those based on the day 5 post-Sinerem images.

### Results

3D MRA in conjunction with the conventional extracellular contrast agent Gd-DOTA failed to reveal any abnormality in either the hyperlipidemic or control rabbits. The aortic lumen was homogeneously bright, permitting easy assessment of the aortic lumen. The aortic wall appeared smooth, without any evidence of atherosclerotic plaque formations (Figure 1).

After the administration of USPIO, intravascular signal intensities were dramatically decreased. Reflecting a decrease of T2$^*$ effects induced by decreasing intravascular USPIO concentrations, luminal signal steadily increased over the 5-day imaging period, providing the best angiographic effect on day 5 for hyperlipidemic and control rabbits alike (Figures 2A and 3). Because of extensive susceptibility effects, delineation of the aortic wall and thus placement of ROIs were not reliably possible in any of the animals on the first 2 days after Sinerem administration. On day 3, visual inspection permitted delineation of the aortic wall in 2 of 5 hyperlipidemic and 1 of 3 normal control animals. On days 4 and 5 after contrast administration, the wall could be delineated in all animals. In the 3 control rabbits, the aortic wall was found to be smooth, void of any irregularities. The data sets obtained in the hyperlipidemic rabbits, conversely, began to exhibit irregularities first seen in 2 animals on day 3 and the remaining 3 rabbits on day 4. These irregularities, appearing as spotty signal voids, became more pronounced on day 5 and reflect susceptibility effects from iron deposits within the aortic wall (Figure 3).

Quantitative analysis based on SNR measurements of the vessel lumen confirmed the visual impression: Intraluminal signal measured in a single large ROI revealed a significant increase in SNR, with a maximum reached at day 5 after contrast administration. These changes reflect T2$^*$ effects, which decreased over time (Figure 2A). Similarly, the qualitative assessment of the aortic wall is mirrored by the quantitative analysis: although there was no significant difference in SNR values between the precontrast and 5 days postcontrast image sets obtained in normal control rabbits ($P>0.05$; paired $t$ test), a vast difference was evident in the hyperlipidemic rabbits ($P<0.01$: paired $t$ test) (Figure 2B). Thus, USPIO uptake was evident only in the aortic wall of hyperlipidemic rabbits.

The ex vivo data sets were very similar in appearance to the images collected in vivo on day 5 immediately before the rabbits were euthanized (Figure 4). Gross inspection of the aortic walls of hyperlipidemic as well as control rabbits did not reveal any appreciable irregularities. Histopathological analysis showed marked uptake of Fe particles in macrophages embedded in atherosclerotic plaque found in the aortic wall of all 5 hyperlipidemic rabbits that had received USPIOs (Figure 5). No such changes were seen in

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**Figure 2.** SNR measurements obtained in ROI in aortic lumen and wall of hyperlipidemic (n=5) and control rabbits (n=3) after intravenous administration of USPIO contrast agent Sinerem at a dose of 1 mmol Fe/kg. A, Intraluminal signal measured in single large ROI (9 mm$^2$) revealed significant increase in SNR, with maximum reached at day 5 after contrast administration. These changes reflect T2$^*$ effects, which decreased over time. B, SNR values based on 3 ROI measurements in aortic wall of each animal failed to reveal statistical difference between precontrast and 5 days post-Sinerem image sets in normal control rabbits. In hyperlipidemic animals, conversely, significant decrease in SNR corresponding to select USPIO uptake in plaque formations containing MPS cells was evident.
the control rabbits. In the 1 hyperlipidemic rabbit killed without having received USPIOs, plaque was identified without evidence of iron uptake.

Electron microscopy (Figures 6 and 7) demonstrated multiple foam cells containing an abundance of fatty vacuoles in a thickened subendothelial layer of the aortic wall of the 2 hyperlipidemic rabbits. However, only the atherosclerotic rabbit that received Sinerem showed multiple cytoplasmic Fe particles (Figure 6).

Discussion

The results of this preliminary animal study indicate that indeed, USPIOs are phagocytosed by MPS cells contained within atherosclerotic plaque in a quantity sufficient to be detected on T1-weighted 3D GRE images as susceptibility-induced signal voids. The implications of this observation are potentially vast, because they may profoundly affect future strategies for the diagnosis and therapy of atherosclerotic disease. The data presented underscore the limitations inherent to luminography: contrast-enhanced 3D MRA with conventional extracellular Gd-based contrast failed to identify plaque formation in the hyperlipidemic animals examined (Figure 1). Similar observations have been reported by other groups using digital subtraction angiography, which failed to detect wall abnormalities in hyperlipidemic rabbits 6 to 12 months old, although alternative imaging with high-resolution MRI and endovascular ultrasound confirmed extensive thickening of the aortic wall.

Recognizing the need to shift emphasis from the vascular lumen to the arterial wall, high-resolution MRI has been increasingly considered for assessing the vascular system. Reflecting the unsurpassed soft tissue contrast inherent to the MR experiment, MR images were found to be superior to intravascular ultrasound with regard to plaque characterization. MR-based visualization of the vascular wall does, however, require high spatial resolution. To achieve this, both external and intravascular surface coils were used. Limited signal and depth penetration allowed wall imaging with external coils only of peripheral vessels, such as the carotid, femoral, or popliteal arteries. Although intravascular coils can overcome this limitation, providing sufficient spatial resolution (117×156 μm) even to permit characterization of different plaque components, they do mandate an invasive approach.

The proposed USPIO method pursues a totally different approach. Instead of defining the morphological makeup of
atherosclerotic plaque, a functional strategy is pursued. Based on the assumption that regions of active plaque formation harbor phagocytic cells, the technique is based on the intravenous administration of ultrasmall iron particles with a long intravascular half-life. If we rely on the susceptibility effects associated with the accumulation of superparamagnetic iron particles (T2* effect), rather small amounts of iron are sufficient to induce vast changes on susceptibility-sensitive gradient echo MR images (Figure 3). The signal changes induced by the iron in the aortic plaque deposits were found to be statistically significant ($P < 0.05$).

The USPIO agent Sinerem has been designed for clinical lymph node imaging. It has successfully completed phase 3 clinical testing and has been registered by several health authorities for clinical use. Because of their small size and rather long half-life in the blood, the particles have the capability to migrate through interendothelial junctions and capillary pores with diameters ranging between 5 and 100 nm. On the T1-weighted fast 3D GRE sequence used, the USPIO agent is characterized by T1 shortening in lower concentrations, rendering the signal bright, and predominant T2/T2* shortening at higher concentrations, resulting in completely dark signal. Thus, the complete signal void in the aortic lumen after initial Sinerem administration reflects the high USPIO blood concentration at this time (Figure 2A). After the USPIOs are allowed to be taken up by the MPS over a 4- to 5-day period, an ideal situation for imaging the vascular wall is created: the iron concentration in the inflammatory cells contained within the plaque was sufficiently great for T2 and T2* effects to dominate (Figure 2B), whereas the iron concentration in the blood pool had decreased to levels at which the T1 shortening effects dominate (Figure 3). This combination of bright intraluminal signal with signal voids contained within the aortic wall permitted identification of regions of active inflammatory changes within the aortic wall at blinded analysis by a single observer at days 4 and 5 after the administration of Sinerem (Figure 3). The visual impressions are reflected by the quantitative analysis, which illustrates a dramatic signal decrease in regions of the aortic wall of hyperlipidemic rabbits (Figure 2), which at histological analysis corresponded to plaque formations (Figure 5).

Electron microscopy confirmed the intracellular presence of the iron particles (Figure 6). In addition, on the basis of the presence of myosin filaments, electron microscopy identified macrophages containing cytoplasmic Fe particles to be derivatives from smooth muscle cells (Figure 6). These actively phagocytosing cells were surrounded by inactive foam cells filled with fat vacuoles without cytoplasmic iron (Figures 6 and 7). These observations lend support to more recent reports favoring endothelial dysfunction rather than the response-to-injury hypothesis, with endothelial denudation representing the first step of atherosclerosis. Regardless of the cause, atherosclerosis represents an inflammatory process. Although the early fatty streak is made up of primitive macrophages and T lymphoctes, continued inflammation causes activation of more macrophages and lymphocytes, with release of hydrolytic enzymes, cytokines, and growth factors leading to necrosis. Further accumulation of mononuclear cells coupled with proliferation of smooth muscle cells and formation of fibrous tissue results in plaque growth. Further restructuring can lead to a so-called fibrous cap covering a core of lipid and necrotic tissue. This advanced stage is regarded as a complicated plaque lesion.
Because USPIO accumulation appears to directly reflect the presence of inflammation, it stands to reason that iron accumulation will occur only in plaque, subject to an active inflammatory reaction. On the assumption that the presence of MPS cells indicates the presence of active plaque, USPIOs may thus serve as a marker of active atherosclerotic plaque formation at a time long before luminal narrowing becomes evident. The technique therefore may not only detect atherosclerotic disease during the often lengthy preclinical phase, which may last decades, but instead should also aid in gauging the activity and thus the clinical relevance of older plaques. Because this study did not determine the physiological state of plaque, however, this conjecture, although likely, remains unproven. Further work will be directed at classifying plaque with high-resolution MRI and correlating the morphology of these formations with USPIO uptake.

Clearly, this animal study has limitations. The number of rabbits examined is small. To overcome this limitation, the study design encompassed examinations of 3 control rabbits as well as of 1 hyperlipidemic rabbit that did not receive the USPIO agent. The results leave little doubt as to the reproducibility of the observed changes affecting the aortic wall. A more severe limitation is associated with the fact that the rabbits were injected with 10 times the permitted clinical dose. Although it is quite possible that a reduced dose would produce the same effects, a dose-finding study has not yet been performed. Similarly, the imaging sequence has not been optimized: the sensitivity for iron-induced susceptibility effects could be enhanced by use of a more T2*-weighted GRE sequence with longer echo times. With such a sequence, even smaller accumulations of iron could be detected, thereby potentially reducing the required contrast dose. Finally, although several characteristics supported the choice of the heritable hyperlipidemic rabbit as a model for this study, the
observed imaging effects may be particular to this animal model. The documented similarity between rabbit and human atherosclerotic plaque formation makes this an unlikely scenario, however.

We conclude that the intravenous administration of USPIOs permits delineation of inflammatory changes accompanying the atherosclerotic disease process in hyperlipidemic rabbits. The medical, social, and economic potential associated with early detection and characterization of plaque activity warrants further investigation.

References

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