In Vivo Detection of Macrophages in Human Carotid Atheroma
Temporal Dependence of Ultrasmall Superparamagnetic Particles of Iron Oxide–Enhanced MRI

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Background—It has been suggested that inflammatory cells within vulnerable plaques may be visualized by superparamagnetic iron oxide particle–enhanced MRI. The purpose of this study was to determine the time course for macrophage visualization with in vivo contrast–enhanced MRI using an ultrasmall superparamagnetic iron oxide (USPIO) agent in symptomatic human carotid disease.

Methods—Eight patients scheduled for carotid endarterectomy underwent multisequence MRI of the carotid bifurcation before and 24, 36, 48, and 72 hours after Sinerem (2.6 mg/kg) infusion.

Results—USPIO particles accumulated in macrophages in 7 of 8 patients given Sinerem. Areas of signal intensity reduction, corresponding to USPIO/macrophage–positive histological sections, were visualized in all 7 of these patients, optimally between 24 and 36 hours, decreasing after 48 hours, but still evident up to 96 hours after infusion.

Conclusions—USPIO-enhanced MRI of carotid atheroma can be used to identify macrophages in vivo. The temporal change in the resultant signal intensity reduction on MRI suggests an optimal time window for the detection of macrophages on postinfusion imaging.

Key Words: magnetic resonance imaging • carotid arteries • atherosclerosis • macrophages

Conventional angiographic measurements of luminal stenosis from digital subtraction techniques do not adequately reflect disease burden in carotid atherosclerosis. Vessel wall remodeling may produce normal luminal measurements despite a large atheromatous plaque in situ. Histological studies have identified features that may predict rupture in “high-risk” plaques. Such plaques have thin/disrupted fibrous caps overlying large necrotic lipid cores, together with an abundance of macrophages.

Studies in hyperlipidemic rabbits revealed that superparamagnetic iron oxide particles are taken up by macrophage-laden aortic plaques as intracellular inclusions and reveal areas of focal signal loss on T2*-weighted MRI within the vessel wall when compared with precontrast images. More recently, the ultrasmall superparamagnetic iron oxide (USPIO) agent Sinerem has been used in the evaluation of human carotid atheroma, in which areas of focal signal loss on in vivo MR images corresponded to accumulation of iron particles in ex vivo specimens.

The authors described optimal results with postcontrast imaging at 24 hours with a T2*-weighted 2D gradient echo (GE) sequence, although data on the time course and duration of the in vivo MRI signal intensity (SI) effect were not fully reported. These factors are likely to be important in understanding and interpreting in vivo macrophage labeling using USPIO enhanced MRI, particularly because intracellular clustering of these particles can have a profound result on signal effect. In this study, we determined the in vivo temporal relationship of SI reduction on MRI after Sinerem-enhanced in vivo MRI of human carotid atheroma in symptomatic patients scheduled for carotid endarterectomy.

Materials and Methods

Patients

The carotid arteries of 8 consecutive symptomatic patients with severe internal carotid artery stenosis (6 males and 2 females; overall mean age 66, range 63 to 79 years) scheduled for carotid
endarterectomy were imaged. The average time from symptom onset to surgery was 5.3 months (range 0.5 to 7 months). Approval for the study was obtained from the Local Ethics Research Committee. All patients gave fully informed written consent.

**Contrast Agent**
Sinerem (Guerbet) was supplied as a dry powder in 20-mL vials. The injection concentration of 2.6 mg/kg was obtained after suspension in 10 mL sodium chloride. The physiochemical properties and the safety data of the agent have been described previously. The contrast agent was infused during a period of 30 minutes through an indwelling large-bore intravenous cannula.

**MRI**
All the imaging studies were conducted on a 1.5-T whole body system (CV/i; GE Medical Systems) using a customized 4-channel phased array coil (Flick Engineering Solutions BV) wrapped around the neck. Imaging was performed before and 24, 36, 48, and 72 hours after the administration of Sinerem. The following ECG-gated, fat-suppressed pulse sequences using double-inversion blood suppression were used on each occasion: 2D T₁-weighted fast spin-echo (repetition time [TR]/echo time [TE]=1 1 R-7/8.8 ms; voxel size 0.4×0.4×3 mm; echo train length=12 matrix: 256×256 and 2 signal averages) and 2 2D T₂*-weighted spiral acquisitions using spectral–spatial excitation pulses, 1 with a TE of 5.6 ms and 1 with a TE of 15 ms: both sequences used a TR of 1 R-R. The inversion time of the postinfusion T₁-weighted sequence was adjusted to counter the USPIO shortening effect in blood. The multishot spiral sequence involved the acquisition of 22 spiral interleaves, each of 4096 data points, resulting in an effective in-plane pixel size of 0.42×0.42 mm; 2 signal averages were performed.

**Image Analysis**

**Histology and MR Coregistration**
Axial MR images were acquired with reference to the carotid bifurcation from a planning time-of-flight sequence. The same anatomical markers were used for each postinfusion study, and accurate coregistration between MR images was further facilitated by use of soft cervical collar around the neck to minimize movement. Histological sections cut from blocks with reference to the carotid bifurcation of the ex vivo specimen were matched to the axial MRI slices by visually coregistering for orientation. There was little difficulty in matching the axial slices to histology section with this method.

**Qualitative**
All MR images were analyzed by 2 independent attending neuroradiologists. The readers were blind to the histological data as well as the time points and sequences of the postinfusion images, which were compared with those from the preinfusion study. The sequence order of images on the preinfusion images was maintained on each set of postinfusion images, but the latter were viewed in a random order. The readers were asked to identify on which set of postinfusion images the signal effect was best visualized, if at all. Images were deemed acceptable for analysis only if both readers agreed that the entire border of the carotid vessel wall was clearly visible and the lumen free of flow artifacts. The presence of the major plaque components (lipid core, fibrous cap, calcium) were characterized by both readers on preinfusion images on the basis of previously validated work. Previous preclinical and early human studies demonstrated that USPIO particles in atheroma caused signal loss in the subendothelial region of the vessel wall. To avoid misclassifying plaque calcification, which can give similar appearances to USPIO on T₁, proton density, and T₂-weighted sequences, we attributed only new areas of signal loss to USPIO accumulation.

**Quantitative**
Changes in relative SI (rSI) between preinfusion and postinfusion images within the entire noncalcified portion of the atheromatous plaque were determined by an independent reader unaware of the qualitative or histological analysis using Analyze (BIR). The rSI was defined as the quotient of the SI in the user-defined plaque area divided by that of the adjacent sternoclavomastoid for each MR image. To ensure that SI measurements from postcontrast images were being taken from comparable areas, the area of the entire noncalcified plaque was used for SI analysis rather than vessel quadrants because after review of the report using the latter approach, it was felt that this approach was more susceptible to error, particularly if image quality was variable.

**Histological Analysis**
Intact atheromatous plaques were obtained after surgery and fixed in 10% formalin solution. Plaques underwent decalcification in EDTA solution to facilitate specimen sectioning. Subsequently, 3-mm-thick transverse sections were cut and embedded in paraffin blocks. From these blocks, 4-μm sections were obtained from the caudal end. Sections were dehydrated and underwent hematoxylin and eosin, Perls reagent, elastin van Gieson stains, and immunostaining for macrophages (MAC 387). Histological sections were reviewed independently by an experienced reader. USPIO accumulation was confirmed by Perls-positive staining, and histological sections were rated as positive or negative accordingly. Perls/MAC 387 double staining was used to confirm the colocalization of USPIO with macrophages.

**Electron Microscopy**
Electron microscopy was performed on 1-mm slices adjacent to Perls-positive sections in 2 patients. These were rinsed, dehydrated, and embedded in Spurr epoxy resin. Ultrathin sections (100 nm) were taken from Perls-rich regions and mounted on nickel grids. They were viewed unstained with a CM100 transmission electron microscope (Phillips) operated at either 60 or 80 kV using a 10-μm lens. Structures with similar electron scattering properties to a Sinerem/gelatin standard were considered to be accumulated Sinerem particles.

**Data Analysis**
Cohen’s k-statistic with 95% CI was computed to quantify the agreement between the readers to identify new areas of signal loss, and the time point and sequence image on which this was best visualized was measured. In the event of disagreement, a decision was reached by consensus. A value of k≥0.70 was used to indicate a high level of agreement. Statistical comparisons of rSI between the postinfusion images were performed, using a paired t test with a probability value of <0.05 indicating statistical significance. Agreement on the size of area defined for rSI analysis in preinfusion and postinfusion images was determined by calculating the intraclass correlation coefficient. The data were analyzed using SAS 6.12 software (SAS Institute) and SPSS for Windows (version 10.1; SPSS).

**Results**

**Histological Analysis**
The time from USPIO infusion to endarterectomy ranged from 40 hours to 18 days (mean±SD, 6.9±4.8 days). There were 44 histological sections containing plaque available for analysis. Perls-positive staining was seen in 35 sections from 7 of 8 patients given USPIO in multiple nonconfluent locations within the plaque, including the fibrous cap, lipid core, and adventitia of the vessel wall. Macrophage immunostaining, with the MAC 387 antibody, colocalized with Perls-positive regions in all 35 sections from the 7 patients. MAC 387/Perls double staining suggested uptake of USPIO into macrophages (Figure 1), which was confirmed by electron microscopy, demonstrating electron dense particles of comparable size to Sinerem within phagolysosomes (Figure 2). However, there were regions with large populations of macrophages where there was no Perls staining seen. In the 1 patient given USPIO, in whom no Perls staining was seen, there was MAC 387 positivity in multiple regions.
Qualitative MRI Analysis
A complete set of images was obtained for 7 of 8 patients who underwent USPIO-enhanced MRI. In 1 patient, expedited surgery terminated the imaging protocol after 36 hours. There were 6 matched MR images and histological sections for each time point up to and including 36 hours in this study. In 1 patient, the 72-hour imaging time point was delayed until 96 hours after infusion because of his lack of availability. After excluding images of poor quality and those without plaque, there were 132 (44 axial slices × 3 sequences) matched postinfusion images per time point available for analysis. Overall, Cohen’s $\kappa$-value between the classification obtained by reader 1 and that of reader 2 was 0.88 (95% CI, 0.74 to 1.00), corresponding to good agreement. In 7 of 8 patients, there was a positive finding on the MR images (area of new signal loss in the subendothelial region) observed by both MRI readers. This finding was seen in only those images corresponding to Perls-positive stained sections (35/44). There was no signal loss seen in any of the MR images matched to tissue sections with no Perls-positive staining (9/44). This positive finding was visualized most easily on the “short TE” $T_2^*$-weighted spiral sequence but also evident on the “long TE” $T_2^*$-weighted spiral and 2D $T_1$-weighted fast spin-echo sequences at all time points after infusion. In 3 of the 7 patients, this signal loss was seen as a focal area, whereas in the remainder, the signal loss was diffuse within the plaque. The size of the visualized area of signal loss varied between the patients for any given time point, but there was a distinct temporal variation in the size of this area between images from any 1 patient. The earliest discernible signal loss was evident by 24 hours, becoming visually more obvious at 36 hours after infusion and remaining so at 48 hours after infusion. The area of signal loss began to decrease after 48 hours but was still visible on images taken 96 hours after infusion. This area of signal loss localized to the fibrous cap region (Figure 3).

Quantitative MRI Analysis
Quantitative SI analyses of MR images confirmed a reduction in rSI in only those images rated positive by qualitative and histological analysis ($n=35$). There was a reduction in mean rSI at 24, 36, and 48 hours on images from all 3 MR sequences, being greatest on the long TE blood-suppressed $T_2^*$-weighted spiral sequence at 24 (7%, range 1.2% to 36%) and 36 (9%, range 0.2% to 40%) hours. The magnitude of reduction in rSI decreased after 48 hours and was minimal or reversed at 72 hours (Figure 4). There was no statistically significant difference
in the magnitude of rSI reduction between 24 and 36 hours \( (P=0.2499) \), but there was between 36 and 48 hours \( (P/H_{11005} 0.035) \).

The intraclass correlation coefficient, for precontrast and post-contrast user-defined areas, was 0.75 (95% CI, 0.69 to 0.83), indicating that the regions used for rSI analysis were comparable. For the patient in whom there was no qualitative signal loss visualized on axial MRI, rSI analysis revealed no change from preinfusion images, and histology revealed no Perls-positive staining in any of the matched sections.

**Discussion**

High-resolution MRI has been used to visualize the major components of carotid atheromatous plaque. Both the necrotic lipid core and overlying fibrous cap give characteristic SIs on MRI.\(^{10}\) The evolution of the vulnerable plaque has been attributed to ongoing active inflammation, a theory supported by the finding of a large number of inflammatory cells within vulnerable plaques.\(^{11}\) Contrast-enhanced MRI with iron oxide particles has been shown in animal studies to give characteristic SI changes that correlate with iron accumulation within intraplaque macrophages.\(^{3,5}\) A recent study of a similar contrast agent, Sinerem, has shown that this might prove to be a useful in vivo marker of inflamed human atheromatous plaques.\(^{7}\) Sinerem particles have a small diameter (30 nm)\(^{8}\) and a long half life in blood (30 hours), thereby allowing phagocytosis by cells of the monocyte–macrophage system throughout the whole body, including those within the atheromatous plaque.

In this blinded analysis, we found a new area of signal loss in 7 of 8 patients who had Sinerem-enhanced MRI. This positive finding was most easily visualized using a blood-suppressed 2D \( T_2^* \)-weighted spiral sequence (TE 5.6 ms) between 24 and 48 hours after infusion. Quantitative analysis further supported that imaging up to 48 hours after infusion can identify macrophages in vivo, although imaging between 24 and 36 hours may be better. This finding adds to the report by Kooi et al\(^ {7} \) that suggested the optimal postinfusion time to be 24 hours based only on the magnitude of rSI reduction. Although those authors reported a larger reduction in rSI than we observed, this effect can be attributed partially to the fact that they used a GE sequence with a longer ET of 20 ms. This reflects in vitro data demonstrating a “blooming” effect of the USPIO agent on \( T_2^* \)-weighted imaging, with increasing ETs\(^ {12} \) rather than peak accumulation. Although defining the entire noncalcified plaque as the region of interest to measure SI changes would ensure measurement accuracy, this might underestimate the magnitude of signal change. We chose to implement a blood-suppressed spiral imaging sequence that we found to provide better image quality than ECG-gated \( T_2^* \)-weighted conventional GE because of the improved time efficiency of spiral acquisitions. This allowed us to reliably determine the lumen/plaque boundary. Increasing the TE to 15 ms increased the magnitude of the rSI reduction at the expense of a decrease in image signal to noise. Furthermore, because the signal reduction properties of USPIO are attributable in some part to intracellular clustering of particles rather than absolute number alone, it is difficult to determine the relationship be-

**Figure 3.** Axial short TE spiral \( T_2^* \)-weighted images through the same level of the internal carotid artery of a patient before infusion (a) and at 24 hours (b), 36 hours (c), 48 hours (d), and 96 hours (e) after infusion of Sinerem, and the matched histological section obtained 8 days after infusion, stained with elastin van Gieson to demonstrate plaque morphology (f). a, The fibrous cap is visualized as showing no signal loss on the preinfusion image. b, An area of signal loss is evident in subendothelial region (arrow), c and d, The area of signal loss has increased in size (arrow), e, The size of the area of signal loss has decreased but is still visible. f, The excised plaque shows typical features of the “vulnerable” plaque, a thin fibrous cap (arrowhead) overlying a large lipid core (arrow).

**Figure 4.** The temporal change in rSI after Sinerem infusion. The rSI measurement is depicted as a change from preinfusion and plotted for each time point and sequence. A drop in rSI is seen as early as 24 hours but is apparent as late as 48 hours after infusion for all 3 sequences. The rSI value approximates that for the preinfusion images by 72 hours.
between magnitude of SI reduction and macrophage burden within the plaque, and consequently, this necessitates caution when sequence and timing optimization are based solely on SI reduction. From this we would consider a T2*-weighted sequence with a minimum ET of 5.6 ms to be sufficient to detect the T2*-weighted effect of USPIOs.

In addition, it is difficult to be sure of the accuracy of precontrast and postcontrast coregistration of the areas used by Kooi et al in their rSI analyses because lumen/plaque boundaries were not reliably discernible in all the images they presented, a fact that might account for the larger reduction in rSI that they measured. We have shown that the regions used in our preinfusion and postinfusion rSI analyses were comparable, and consequently, although the magnitude of SI reduction is less than that has been reported, we consider this to be highly accurate.

We observed a relative increase in rSI after 48 hours compared with earlier postinfusion time points, and almost no change in rSI at 72 hours, which mirrored the depression in USPIO effect seen on qualitative analysis. We think this most likely represents a “washout” of iron particles from the plaque by diffusion or by physiological recirculation of the endocytosed iron, which would be supported by the relative increase in rSI measured after 48 hours. Despite an average time interval between USPIO infusion and surgery of less than 7 days, there was a wide range (up to 18 days) because of pressures on the operating department, which made the relationship between MRI signal effect and USPIO accumulation difficult to interpret.

The way in which USPIOs enter the atheromatous plaque remains unknown. Analysis of our histological data seems to suggest either recruitment of USPIO particles directly into plaque or recruitment of macrophages loaded peripherally with USPIO via the vaso vasora or neovascularization because iron particles were found in the adventitial wall as well as within the plaque substance itself. Alternatively, entry may be through widened endothelial junctions within the surface of the fibrous cap overlying the plaque, thereafter becoming phagocytosed by intraplaque macrophages. From this, we would consider a lack of USPIO uptake in 1 patient, operated on 48 hours after USPIO infusion, who had the longest lag time from symptoms to surgery (7 months), may be explained by a lack of intraplaque neovascularization or minimal to no endothelial dysfunction, factors that are likely to be important in determining plaque accessibility to either USPIO directly or peripherally loaded macrophages. Because it was not possible to validate the MRI appearance of the contralateral vessel, these vessels were not included in the analysis.

We have demonstrated the safe use of Sinerem and shown that it has the potential to be used as an in vivo MRI contrast agent to identify inflamed atheromatous plaques. Furthermore, we have shown by the temporal variability of MRI signal loss that the process of in vivo macrophage visualization is dynamic and not sustained. USPIO-enhanced MRI between 24 and 48 hours using an ECG-gated, blood-suppressed T2*-weighted spiral imaging sequence with a minimum ET appears to be sufficient to detect this effect and preserve image quality. In the future, larger prospective studies of USPIO-enhanced MRI could be undertaken to identify vulnerable plaques at high risk of rupture as well as to evaluate the effect of novel therapies on functional activity within atheromatous plaques.

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