Identifying Inflamed Carotid Plaques Using In Vivo USPIO-Enhanced MR Imaging to Label Plaque Macrophages

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Background—Inflammation within atherosclerotic lesions contributes to plaque instability and vulnerability to rupture. We set out to evaluate the use of a macrophage labeling agent to identify carotid plaque inflammation by in vivo magnetic resonance imaging (MRI).

Methods and Results—Thirty patients with symptomatic severe carotid stenosis scheduled for carotid endarterectomy underwent multi-sequence MRI of the carotid bifurcation before and after injection of ultrasmall superparamagnetic particles of iron oxide (USPIOs). USPIO particles accumulated in macrophages in 24 of 30 plaques (80%). Areas of signal intensity reduction, corresponding to USPIO/macrophage-positive histological sections, were visualized in 24 of 27 (89%) patients, with an average reduction in signal intensity induced by the USPIO particles of 24% (range, 3.1% to 60.8%).

Conclusions—USPIO-enhanced MRI can identify plaque inflammation in vivo by accumulation of USPIO within macrophages in carotid plaques. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: carotid ■ inflammation ■ MRI ■ USPIO ■ vulnerable plaque

Although conventional angiographic measurements of luminal stenosis do not reflect disease burden in carotid atherosclerosis,1 they are still used as the primary criteria for definitive surgical therapy. Histological studies have identified features that may better predict rupture in “high-risk” plaques; these plaques have thin/eroded fibrous caps that overly large necrotic lipid cores and have an abundance of inflammatory cells (macrophages).2 Inflammation within atherosclerotic plaques increases vulnerability to rupture and subsequent thromboembolism and presents itself as a target for plaque stabilization therapies.

Animal studies of atherosclerosis have shown that superparamagnetic iron oxide (SPIO) particles are taken up by inflamed plaques rich in macrophages as intracellular deposits3 that induce areas of signal loss on T₂*-weighted magnetic resonance imaging (MRI) within the vessel wall.4 More recently, in vivo human studies using the ultrasmall SPIOs (USPIO) agent, Sinerem, have confirmed these findings and also refined optimal MRI parameters to detect inflamed plaques.5,6 These pilot studies, while demonstrating the potential of USPIO enhanced MRI to visualize plaque macrophages, are limited by sample size and methodology issues. This report describes the findings of the largest in vivo human study evaluating Sinerem-enhanced MRI to identify inflammation within atherosclerotic plaques with histological correlation.

Materials and Methods

Patients
The carotid arteries of 30 nonconsecutive (patients with contraindications to MRI or for whom MRI slots could not be obtained were excluded) symptomatic patients (22 males, 8 females; median age, 70; range, 48 to 83 years) with severe internal carotid artery (ICA) stenosis1 (mean (±SD) ICA stenosis 77% (±7%), measured by digital subtraction angiography), recruited from a specialist neurovascular clinic, scheduled for carotid endarterectomy were imaged. The overall median time from symptom onset to surgery was 3.5 months (range, 0.5 to 7 months). Approval for the study was obtained from the Local Research Ethics Committee. All patients gave informed consent.

MR Contrast Agent
The USPIO contrast agent, Sinerem (Guerbet, Roissy, France) consisting of ferromagnetic iron oxide particles with an overall size of ~30 nm, was suspended in normal saline and given as an intravenous infusion (2.6 mg/kg) over 30 minutes.5

MRI
All the imaging studies were conducted on a 1.5-Tesla system (CV/I; GE Medical Systems, USA) using a customized 4-channel phased array coil (Flick Engineering Solutions, the Netherlands) wrapped
around the neck. Images were acquired through the carotid bifurcation using the following EKG-gated, fat-suppressed pulse sequence using double inversion blood suppression before and 36 hours after USPIO injection: 2-dimensional T$_1$-weighted spiral acquisitions using spectral-spatial excitation pulses (echo time/repetition time 5.6/1 R-R). The multi-shot spiral sequence involved the acquisition of 22 spiral interleaves each of 4006 data points, resulting in an effective in-plane pixel size of 0.42×0.42 mm, 2 signal averages were performed. The field of view was 12-cm×12-cm and slice thickness was 3 mm for all sequences. Typically between 4 to 6 plaques containing images were generated for each vessel, covering the length of the plaque. The time from USPIO infusion to endarterectomy ranged from 40 hours (1 patient) to 18 days (1 patient) with a mean (±SD) interval of 6.9 (±4.8) days.

**Histological Staining**

Histology sections underwent H&E, Elastin van Gieson, and immunostaining for macrophages CD68 (mature macrophages), MAC387 (immature macrophages), smooth muscle cells (α-SMA), and endothelial cells (CD31). Perls reagent was used to identify the contrast agent. Double immunostaining and Perls staining was performed on serial sections from several plaques that had evidence of strong Perls positivity to determine the distribution and localization of Sinerem.

**MRI Analysis**

Images were viewed on a standard computer workstation attached to a high-resolution display screen using an image analysis software package (CMRTools, Imperial College, UK). Images were viewed at 200% magnification and pre-infusion and post-infusion images from any individual were adjusted to ensure identical window/level settings. Coregistration of histological sections and MR images was performed in a similar manner to that previously described. In brief, the internal carotid bifurcation was used as reference marker for both MR and histology section localization and corresponding images were re-oriented according to gross morphological features, such as lumen position.

**Qualitative Image Analysis**

Images were deemed acceptable for analysis if the entire border of the carotid vessel wall was visible and the lumen free of flow artifacts. The presence of USPIO within the plaque was confirmed by noting whether the matched post-infusion image contained a new region(s) of low signal intensity (SI) within the vessel wall (plaque). The nature of any new area of SI reduction was noted as “focal,” if the region of signal change was localized to 1 well-circumscribed area, as “multi-focal” if there was >1 such nonconfluent area or as “diffuse,” for any other pattern of signal change. The location (quadrant) of the region of signal change was also noted, which was determined by constructing an imaginary set of perpendicular axes with their inter-section at the center of the vessel lumen. This approach was taken for pre-infusion and post-infusion images. Then each pair of matched quadrants (right upper, left upper, right lower, left lower) was viewed in turn and the presence or absence (and nature where present) of USPIO effect noted.

**Quantitative Image Analysis**

The maximum SI change within a matched region of interest (ROI) was determined, because this was thought to better reflect the Sinerem load within plaques. The following method was used to define the ROI from which the SI was measured. On the post-infusion image, the ROI was defined to include and circumscribe the region of SI change (focal or diffuse). The delineated ROI was then copied and transposed to the appropriate location on the pre-infusion image to provide a “mirror” location for comparative analysis. The SI of these ROIs was then measured. The SI within the ROI (SI$_{max}$) was then normalized to that of a similar sized ROI in the adjacent sternocleidomastoid muscle (SI$_{max}$ (we had previously observed that the SI of such a ROI varied minimally in relation to its position). The relative signal intensity (rSI) was calculated as follows: SI$_{max}$/SI$_{max}$. The magnitude of SI change was quantified separately for the “diffuse” and “focal” effect groups; images that were classified as “multi-focal” had the area with the most pronounced signal effect used as the ROI. If there was no such area, then each “focus” was used as a separate ROI and the mean value used to describe the magnitude of signal change in this image.

**Histological Image Analysis**

**Determination of USPIO Accumulation Within Plaques**

Perls reagent uptake was used as a surrogate marker for the presence of USPIO. Previous analysis of Perls reagent stained sections from subjects not given USPIO injections revealed only sporadic Perls positivity in a few plaque sections. Using low-power magnification (×1.6 lens), which allows the majority of the vessel cross-sections to be viewed in one field of view, the overall distribution pattern of Perls positive cells was determined in a similar manner to that used for the USPIO-induced MR signal effect (focal, multi-focal, diffuse, absent). At higher magnification (×40 lens) cell counts were performed on sections stained for USPIO (Perls) and macrophages (CD68/MAC 387). For each section, the total from 10 randomly selected high-power fields (hpf) was determined. Only positively stained material with the morphological appearance of cells (nucleus, cytoplasm) was counted as cells.

**Colocalization of USPIO Particles**

USPIO particles were deemed to have colocalized with a particular cell type (macrophage, smooth muscle cell, endothelial cell) if both the typical blue appearance of the Perls reagent and the brown appearance of the antibody revealing chromogen were present in the same cell.

**Plaque Characterization**

Plaque sections were classified by an independent histopathologist as either vulnerable/ruptured or stable by a grading system loosely based on the American Heart Association criteria: plaques that had evidence of plaque erosion, fissure, or rupture or had a thin fibrous cap and/or had large necrotic lipid cores were considered “vulnerable/ruptured” and other morphological types were considered “stable.” When there was uncertainty, the plaques sections were considered “unclassifiable.”

**Statistical Analysis**

The magnitude of change in signal intensity induced by Sinerem was quantified as the percent change in SI within the defined ROI. For statistical comparisons of rSI between pre-infusion and post-infusion images, the data were analyzed separately for those images where the signal effect was diffuse and where it was focal (where the effect was multi-focal, the mean value was used). Significance in any differences in pre- and post-infusion rSI was measured using the Wilcoxon-Rank test, with P<0.05 indicating statistical significance. The Mann-Whitney U test was used to determine whether there were any significant differences in SI change between the “focal” and “diffuse” effect groups. The relationship between % change in SI and the number of Perls positive and CD68/MAC387 cells was determined by calculating Pearson’s correlation coefficient. Agreement between location of signal loss on MR and location of Perls positive cells on histology was measured by computing Cohen’s kappa. The relationship between the number of Perls positive cells and macrophages in individual slices was determined by calculating Spearman’s rho coefficient. A χ² test was performed to determine whether Sinerem positivity was associated with vulnerable/ruptured plaques.

**Results**

**Clinical Details**

All patients were either current or former smokers and had 1 other risk factors for cerebrovascular disease (22 had hypertension, 11 had diabetes, 20 had hypercholesterolemia). Seven patients had ischemic heart disease; no patients had other systemic inflammatory conditions. All subjects had been in sinus rhythm for the previous 6 months and were...
taking anti-platelet therapy; half were taking cholesterol-lowering medication; 12 were taking an ACE inhibitor. No patients had evidence of aortic atherosclerotic disease on DSA.

**Image Analysis**

There were 210 matched MR and histology image pairs generated following the coregistration process from 27 patients; 3 patients had excessive movement artifacts during the post-infusion MR study resulting in incomplete image acquisition, and these were therefore excluded. After review of the coregistered image pairs, 54 were excluded from further analyses because MRI revealed no vessel wall thickening (and consequently no signal change within the wall was detectable) and histological sections revealed vessels with normal intima with no Perls staining (concordant absence). Because the endarterectomy specimen always included the region of the carotid bifurcation, to allow ex vivo coregistration, if the atheromatous plaque was distant to this reference landmark, then it was foreseen that there would be disease-free vessel cross-sections among the histology–MRI pairs. This left a total of 156 image pairs available for further comparative analyses.

**Histological Analysis**

**Distribution of Perls Stain**

There were 97 (62%) histological sections from 23 patients that demonstrated Perls positivity. At low magnification, the distribution of the Perls stain in 45 (46%) of these was thought to be diffuse, with another 45 (46%) slices showing a focal accumulation and the remainder a multi-focal distribution. At high magnification, Perls staining appeared to be both intracellular and extracellular in location. In those plaques with focal Perls staining, this was almost always in the subendothelial fibrous cap region. In particular, Perls staining appeared to colocalize to macrophages in the shoulder regions of the plaque (Figure 1). In those sections where Perls staining was diffuse, this was visualized at every depth of the atheromatous plaque; in the peri-luminal region of the fibrous cap, within the thickened intima in close proximity to the necrotic lipid core, at the intima/media border (region of the internal elastic lamina), and in a few sections in the adventitial region of the vessel wall. In all of these latter sections, there was also staining in the fibrous cap region (Figure 2).

Frequently, Perls staining was observed in close proximity to areas of neovascularization within deep portions of the
intima and distant from the peri-luminal region. In the individual in whom there was a short interval between USPIO infusion and surgery, there appeared to be a greater concentration of Perls staining in areas of neovascularization than in other sites (Figure 3).

Colocalization of USPIO Particles
In the majority of the 97 Perls positive sections, there were macrophage accumulations located in similar regions of the plaque. There were areas in a few plaques where macrophages were not identified, but where Perls staining was evident; serial sections, however, stained for smooth muscle or endothelial cells also revealed an absence of these cell types in these locations. Conversely, there were locations within plaque sections, where there were abundant macrophages, but where Perls staining was absent, including several sections from the 4 subjects in whose plaques no Perls staining was seen whatsoever. These later 4 subjects had plaques harvested within 5 days of the USPIO infusion.

Double immunostaining/Perls staining performed on serial sections of randomly selected Perls positive sections confirmed that there was colocalization of the Perls stain with macrophages (Figure 1) but not with either smooth muscle or endothelial cells (not shown).

Relationship of Perls Stain to Macrophage Content
A poor but significant correlation between the total number of Perls positive cells and MAC 387-stained macrophages (Spearman rho=0.24, P<0.001) and a slightly stronger correlation with CD68-stained macrophages (Spearman rho=0.29, P<0.001) was found, suggesting that either not all plaque macrophages were internalizing Sinerem or that the Perls stain was an inadequate method for detecting Sinerem.

MRI Analyses
Detection of Sinerem-Induced Signal Effect
Comparison between the pre- and post-Sinerem infusion image pairs resulted in 128 (82%) post-infusion images being deemed positive for USPIO-induced signal effect, from a total of 26 individuals. In 56 (44%) of these images, the induced signal loss was described as focal, whereas it was seen as multi-focal in nature in 12 (9%) and diffuse in 60 (47%) slices. There was no USPIO effect observed in the remainder images (28, 18%).

Location of Sinerem Signal Within Plaque
In the “focal” effect images the induced signal effect was consistently in the peri-luminal region corresponding to the fibrous cap region (Figure 4). In the “diffuse” effect images the extent of the induced signal loss extended between the peri-ventinal and peri-luminal regions (Figure 5).

Quantification of Sinerem-Induced Signal Effect
Comparison of the rSI between pre- and post-infusion images of the “focal” effect group revealed a significant difference between the 2, attributable to Sinerem (median change in rSI=−24.1%; range, −3.1% to −60.8%; P<0.0001). In the “diffuse” effect group, the overall reduction in rSI was not statistically significant (median change in rSI=−3.5%; range, −65.1% to 58.7%; P=0.06), with an increase in rSI being measured between some image pairs. There was a significant difference in the magnitude of the USPIO-induced signal change between the “focal” and “diffuse” effect groups (median difference in signal change between “focal” and

![Figure 3](Image)

**Figure 3.** Proximity of Perls stain to areas of neovascularization (CD31/Perls double stain, ×30). Dense Perls positive staining (a) (blue) near vascular channels (white arrowheads) compared with scant Perls positivity (b) (black) in other regions in the same cross-section.

![Figure 4](Image)

**Figure 4.** Pattern of USPIO induced signal loss. Axial MR images (T2*W [TE=5.6 ms]) through the common carotid artery. Diffuse USPIO signal effect (a,b). Focal USPIO signal effect (c,d). Pre-USPIO infusion (a,c). 36 hours after infusion (b,d) (*lumen).
“diffuse” groups = −17.7%; range, −10.8% to −27.6%; P < 0.0001).

Comparative Analyses Between MR and Histology
Qualitative MRI analysis was highly sensitive (92.5%) and moderately specific (64%) for detection of Sinerem particles within atheromatous plaques. In addition, good agreement between location of Perls stain and Sinerem signal effect was observed (Cohen kappa = 0.60); however, the agreement between MRI and histology for characterizing the nature of the Sinerem signal effect and Perls stain was only moderate (Cohen kappa = 0.48).

There was a significant correlation between magnitude of Sinerem effect and Perls staining in the “focal” effect group (Spearman rho = −0.63, P < 0.001) but not in the “diffuse” effect group (Spearman rho = 0.17, P > 0.05). There was a significant correlation between the magnitude of Sinerem effect and macrophage count in the “focal” effect group (Spearman rho = −0.49 [CD68] and −0.60 [MAC 387], P < 0.001 for both). There was no statistically significant correlation in the “diffuse” effect group.

There was a stronger association between the “focal” signal effect group and histologically “unstable/vulnerable” plaques than with the “diffuse” signal effect group (72.9% versus 27.6%, χ² = 20.7, 2 df, P < 0.001).

Discussion
It has been difficult to visualize atheromatous plaque inflammation in vivo because of limitations in the resolution obtainable with standard imaging technology. Contrast-enhanced MRI with USPIO particles has been shown in animal models to overcome these issues by exploiting the physico-chemical properties of this new genre of MR contrast agent. A recent study of such a contrast agent, Sinerem, prompted exploration of whether this might prove to be a useful in vivo marker of inflammation in human atherosclerosis. The present report suggests that Sinerem-enhanced MRI of human carotid atherosclerotic plaques is a sensitive way of identifying inflamed plaques in symptomatic individuals.

Interpretation of Sinerem-Induced Signal Effect
The finding of a significant correlation between SI reduction and number of macrophages/Perls-positive cells within plaques would suggest that a linear relationship exists between Sinerem accumulation and macrophage burden. However, whereas a linear relationship appears to exist between magnitude of SI reduction and macrophage or Perls positive cells independently, there was no strong correlation between the Perls stain and macrophage counts. Because the size of the area of SI reduction observes a nonlinear relationship with Sinerem particle content, and because the SI change induced by these particles is heavily dependent on dense packing within intracellular organelles, it is difficult to know how to interpret the magnitude of SI change in terms of macrophage burden. Furthermore, this linear relationship did not hold true for the “diffuse” group where occasionally SI increases were measured. This group is likely to represent mainly extra-cellular Sinerem, and the increase in SI may be explained by the known T₁ effect of Sinerem. From the data presented here, it is probable that only a “focal” area of signal loss represents USPIO accumulating in macrophages.

Suitability of Perls Stain for Detecting Sinerem Uptake
The qualitative and quantitative histological analyses suggest no consistent pattern of Perls stain and macrophage accumulation. There are several possible explanations for this. First, it is possible that there is a heterogeneous population of macrophages with differing phagocytic capacities for USPIO. Second, labeling of macrophages by Sinerem is a dynamic process, dependent on individual cell kinetics and, as has been recently suggested, the kinetics of Sinerem differs to those of other USPIO. It is possible that Sinerem may have slower kinetics in inflammatory tissue than in other tissue types. Thus, performing a detection stain at any given time (time form imaging to surgery) may result in a significant number of cells being classified as negative, either because the iron moiety has not yet become detached from the dextran coating or because it has become sequestered by intracellular proteins such as ferritin. A more likely explanation, we feel, is that the Perls stain may not be a very sensitive marker for USPIO, in keeping with others who have used an enhanced Perls stain to increase their detection at the expense of specificity. The individual in whom no Sinerem effect/Perls stain was observed had tandem carotid stenosis. The failure to detect Sinerem or induced signal loss could be explained by the fact that the more proximal lesion (acute plaque) was just out of range of the imaging coil and could not be visualized and was not the lesion that was excised. The more distal lesion appeared heavily calcified (plaque characterization sequences) and made any smaller accumulation of Sinerem more difficult to visualize.

Specificity of Sinerem for Macrophage Labeling
There are some who have described Sinerem uptake in smooth muscle and/or endothelial cells, although no colocalization with either of these cell types was found in this study.
This might be because of a sampling bias, however, smooth muscle cells are not naturally phagocytic and it is possible that pinocytosis alone might not be sufficient to allow detectable amounts of Sinerem to be internalized and subsequently visualized within these cells.

It is more surprising that there was no colocalization with endothelial cells, because Sinerem entry within plaques may be mediated by entry across dysfunctional endothelium. Explanations for this lack of uptake come from knowledge of the endothelial cell physiology. Endothelial cells have a known capacity to transcytoses particles across from the luminal surface. This process is likely to be rapid, if endothelial cell contraction occurs, because this will result in disruption, albeit transient, to the integrity of the endoluminal barrier. Also, plaque endothelium is not fenestrated, unlike in glands, and this therefore limits internalization.

**Safety of Sinerem MRI**

It has been speculated that iron-borne free radicals might contribute to atherosclerotic plaque instability, thereby raising some concerns about the use of an iron-based contrast agent to visualize inflammatory cells. Sinerem has been extensively evaluated in both the preclinical and clinical setting, with only mild adverse events being reported with the slow intravenous infusion method, as was used in this study. In this larger study of carotid atherosclerosis, 1 subject reported a transient alteration to taste, which resolved within a few seconds, and did not require cessation of the USPIO infusion. There were no other adverse events reported in the period up to the 1 year postoperative visit, confirming the safety of this compound.

In summary, this report suggests that high-risk individuals with inflamed plaques may be identified on the basis of a “focal” area of signal loss visualized on MRI after Sinerem infusion.

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**References**


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