Accumulation of Ultrasmall Superparamagnetic Particles of Iron Oxide in Human Atherosclerotic Plaques Can Be Detected by In Vivo Magnetic Resonance Imaging

M.E. Kooi, PhD; V.C. Cappendijk, MD; K.B.J.M. Cleutjens, PhD; A.G.H. Kessels, MD, MSc; P.J.E.H.M. Kitslaar, MD, PhD; M. Borgers, PhD; P.M. Frederik, PhD; M.J.A.P. Daemen, MD, PhD; J.M.A. van Engelshoven, MD, PhD

Background—One of the features of high-risk atherosclerotic plaques is a preponderance of macrophages. Experimental studies with hyperlipidemic rabbits have shown that ultrasmall superparamagnetic particles of iron oxide (USPIOs) accumulate in plaques with a high macrophage content and that this induces magnetic resonance (MR) signal changes. The purpose of our study was to investigate whether USPIO-enhanced MRI can also be used for in vivo detection of macrophages in human plaques.

Methods and Results—MRI was performed on 11 symptomatic patients scheduled for carotid endarterectomy before and 24 (n=11) and 72 (n=5) hours after administration of USPIOs (Sinerem) at a dose of 2.6 mg Fe/kg. Histological and electron microscopical analyses of the plaques showed USPIOs primarily in macrophages within the plaques in 10 of 11 patients. Histological analysis showed USPIOs in 27 of 36 (75%) of the ruptured and rupture-prone lesions and 1 of 14 (7%) of the stable lesions. Of the patients with USPIO uptake, signal changes in the post-USPIO MRI were observed by 2 observers in the vessel wall in 67 of 123 (54%) and 19 of 55 (35%) quadrants of the T2*-weighted MR images acquired after 24 and 72 hours, respectively. For those quadrants with changes, there was a significant signal decrease of 24% (95% CI, 33% to 15%) in regions of interest in the images acquired after 24 hours, whereas no significant signal change was found after 72 hours.

Conclusions—Accumulation of USPIOs in macrophages in predominantly ruptured and rupture-prone human atherosclerotic lesions caused signal decreases in the in vivo MR images. (Circulation. 2003;107:2453-2458.)

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

Plaque instability, which is responsible for most of the complications of atherosclerotic disease, is morphologically characterized by a preponderance of macrophages, ulceration, intraplaque hemorrhage, and thrombosis.1 Using MRI, it is possible to differentiate between lipid, fibrous, and calcified plaque components.2 Recently, atherosclerotic plaques have been imaged by MR in hyperlipidemic rabbits using ultrasmall superparamagnetic particles of iron oxide (USPIOs).3,4 USPIOs are iron oxide nanoparticles stabilized with low molecular weight dextran with a mean diameter of 30 nm. These relatively small particles have a much larger half-life in blood than the conventional superparamagnetic iron oxide particles, with a mean diameter of 150 nm. Because of their long half-life in blood, USPIOs can be taken up by macrophages in the whole body. The animal studies indicate that the USPIOs are phagocytosed by macrophages in atherosclerotic plaques, which causes a signal decrease on MR images. Additionally, a retrospective analysis of MR images of 19 patients who had originally received USPIOs for cancer staging showed signal loss in the arterial wall of 7 patients on postcontrast compared with precontrast images.5 However, in this retrospective study, no material was available for histological or electron microscopical analysis of the plaques. The aim of the present study was to investigate the feasibility of in vivo MR imaging of USPIO accumulation in human atherosclerotic plaques. Additionally, we aimed to examine the relationship between MR signal changes in plaques after USPIO administration, the amount of USPIO uptake, macrophage content, and plaque stage.

Methods

Patients
Eleven patients with symptoms of recurrent transient ischemic attacks (TIAs) or small brain infarcts and an ultrasound-proven...
carotid stenosis between 70% and 99% who were scheduled for carotid endarterectomy were included in this single-center study. The institutional review board of the hospital approved the study, and written, unenforced consent was obtained from all patients. The patients were recruited consecutively. The age of the patients was 64±9 (mean±SD) years, and 9 of the patients were male. The procedures followed were in accordance with the institutional guidelines. Adverse events, defined as any untoward medical occurrence in a patient administered Sinerem, observed from the start of USPIO infusion (Sinerem, Guerbet) until the end of post-USPIO MRI (24 to 72 hours after the start of the infusion) were recorded in case report forms. No adverse events related to Sinerem were recorded in these 11 patients. Additional information about clinical safety can be found in the study by Sigal et al.6

Magnetic Resonance Imaging
The carotid atherosclerotic plaques of the patients were imaged in vivo on a 1.5-T whole-body scanner (Intera, Philips Medical Systems) using a surface radio frequency coil with a diameter of 47 mm. The patients were examined immediately before and after the USPIO's peak (Sinerem, Guerbet), which were administered at 2.6 mg Fe/kg at an infusion rate of 4 mL/min. The infusion time was typically 30 minutes. After a survey to determine the position of the carotid plaque, the following MRI sequences were used: (1) T1-weighted (T1w) gradient-recalled-echo, TR/TE: 41 to 44/8.0 to 9.2 ms; flip angle: 25 degrees; (2) T2*-weighted (T2*w) gradient-echo (GE), TR/TE: 1 heart beat/20 ms; flip angle: 40 degrees; and (3) proton-density–weighted fast spin-echo (PDw FSE), TR/TE: 2 to 3 beats/20 ms; echo train length: 5. Other parameters were transversal slices; field of view, 7056 mm; matrix size, 208 to 256×166 to 205; and slice thickness, 5.0 mm. A frequency-selective prepulse for fat suppression was used in the T2*w GE and the PDw FSE sequence. Note that the T1w GE images also contain some T2* weighting. Because the plaque was usually located just above the carotid bifurcation, for each sequence, typically 1 to 2 images of plaque in the common carotid artery and 3 to 4 images of plaque the internal carotid artery were obtained. Occasionally, the plaque extended in one image of the external carotid artery.

Image Evaluation
Pre- and post-USPIO MR images were matched, using the carotid artery bifurcation as a marker. The reading of the MR images was done irrespective of the histological results by 2 readers by consensus (J.M.A.v.E. and V.C.C.). The readers judged all images of atherosclerotic plaque with respect to signal changes in the vessel wall in the postcontrast compared with the corresponding precontrast image and the quadrant of the vessel wall in which these changes were present. Additionally, the relative signal intensity (rSI) in the quadrants with signal changes was determined by dividing the signal intensity of operator-defined regions of interest by that of surrounding muscle tissue. The operator who defined the regions of interest was unaware of the histological results. Signal changes were quantified by calculating the rSI ratio, which was defined as the quotient of the rSI in post- and pre-USPIO MRI. With the first 5 patients, the optimal delay for post-USPIO MRI was determined. To be able to compare the performance of the T2*w GE with the PDw FSE sequence, only those carotid segments of which images of both sequences were available were included for data analysis.

Histology and Immunohistochemistry
After surgery, the intact carotid artery segments were formalin-fixed, sectioned in 5-μm transversal slices (in plane with the MRI slices), decalcified for 30 minutes in 10% formic acid in PBS, and embedded in paraffin. Subsequently, 4-μm sections were subjected to histoimmunological analysis of plaque phenotype (HE staining), macrophage content (CD68 immunostaining), and USPIO uptake (Perl’s iron staining). Typically, for each patient, 2 tissue slices from the common carotid artery, 3 from the internal artery, and, occasionally, 1 from the external artery were obtained. In addition, a total of 10 atherosclerotic carotid artery samples consisting of 56 tissue slices derived at autopsy from patients who did not receive USPIO were analyzed for endogenous iron staining. Sections subjected to CD68 staining were pretreated with 0.1 mol/L pepsin and subsequently incubated with monoclonal CD68 mouse antibody (DAKO 1:100), biotinnylated swine–anti-mouse antibody (1:250, Amersham Life Science), and alkaline phosphatase–coupled ABC-reagent (1:200, Dako). Alkaline phosphatase activity was visualized using the Alkaline Phosphatase Kit I (Vector). Sections were counterstained with hematoxylin.

USPIO uptake (Perl’s iron staining) and the number of macrophages (CD68-positive cells) in the 4 quadrants were assessed by one investigator (K.B.J.M.C.) on a scale from 0 to 4, with 0 indicating no: 1, hardly any; 2, some; 3, many; and 4, very many iron/CD68-positive cells. In addition, for each tissue section, plaque stage was independently determined by 2 investigators (K.B.J.M.C. and R.J. van Suylen, University Hospital Maastricht, Maastricht, the Netherlands) according to the morphological criteria of the American Heart Association.1 Stable lesions (type IV and V) are characterized by an intact and thick fibrous cap. These plaques contain either a large lipid core (type Va), calcification (type Vb), or fibrous tissue (type Vc). A disrupted fibrous cap and the presence of a thrombus characterize the ruptured lesion (type VI). Type Va lesions with a very thin fibrous cap were considered as rupture-prone lesions.

To verify USPIO uptake by macrophages and to identify additional cell types containing USPIOs, carotid sections derived from patients who did receive USPIOs were double stained for Perl’s iron and CD68, α-smooth muscle actin (mouse monoclonal anti-ASMA antibody, Sigma 1:500) as a marker of vascular smooth muscle cells and myofibrolasts and CD31 (mouse monoclonal anti-CD31 antibody, DAKO 1:100) as a marker of endothelial cells.

Electron Microscopy and Energy-Dispersive X-Ray Analysis of Perl’s Positive Regions
For 2 of the specimens, tissue fragments of ~1 mm² were fixed overnight in a 2.5% glutaraldehyde solution. After several washes, tissue fragments were postfixed in 1% osmium tetroxide solution and routinely dehydrated and embedded in epoxy resin. Semi-thin (1-μm) serial sections were subjected to Perl’s iron staining. Subsequently, ultra-thin sections from Perl’s positive regions were mounted on Formvar (1595 E, Merck)-coated 75 mesh copper grids and counterstained with uranyl acetate and lead citrate before analysis in a Phillips CM12 transmission electron microscope. Subsequently, EDX spectrometry of the observed dense bodies was performed essentially as described previously.7,8

Statistical Analysis
A one-sample t test was used to test whether the rSI ratios of the MR images of each sequence were significantly (P<0.05) different from unity. The optimal delay for post-USPIO MRI was taken to be the time with the largest significant deviation of the rSI ratios from unity. A χ² test was used to test whether the proportions of USPIO uptake in stable versus ruptured and rupture-prone sections were significantly different.

Results

Histology and Electron Microscopy
The time between USPIO administration and surgery was 21 days for one patient, and therefore this patient was excluded from additional analysis. For the remaining 10 patients, time between administration and surgery was 4.7±3.0 (mean±SD; range, 2 to 11) days. Perl’s staining showed USPIO accumulation in the plaques of 9 of these 10 patients. Eight of the patients with USPIO accumulation showed a considerable USPIO uptake (total iron score ≥7 in all available quadrants), whereas 1 showed some uptake (total iron score of 4 in all available quadrants). The time between
by guest on April 15, 2017 http://circ.ahajournals.org/ Downloaded from

particles. Energy-dispersive X-ray (EDX) analysis of the observed that a single phagosome contained many USPIO in phagosomes of macrophages. In Figure 2b, it can be microscopy showed an intracellular granular iron distribution particle size corresponds to the size of Sinerem. Electron USPIO particles in a single macrophage. The observed microscopy image indicating the presence of numerous material of 2 patients. Figure 2a shows a typical electron 'microscopical analysis of Perl's positive regions in plaque carotid artery tissue sections derived from patients who did receive USPIOs showed no endogenous iron staining. In contrast, plaques of the 10 control patients who did not receive USPIO uptake. Perl's iron (USPIO) double staining of CD68 (a and b), Perl's (c and d), and CD68/Perl's (e) double staining of the endarterectomy specimen of a patient who received USPIOs. Double staining showed colocalization of macrophages and Perl's iron staining. The red coloring (a and b) and brown coloring (e) are indicative of macrophages, and the blue coloring (c, d, and e) is indicative of the accumulation of USPIOs. The double lumen is indicated with L, the surgical cut with C, and tears with T.

This finding was additionally substantiated by electron microscopical analysis of Perl’s positive regions in plaque material of 2 patients. Figure 2a shows a typical electron microscopy image indicating the presence of numerous USPIO particles in a single macrophage. The observed particle size corresponds to the size of Sinerem. Electron microscopy showed an intracellular granular iron distribution in phagosomes of macrophages. In Figure 2b, it can be observed that a single phagosome contained many USPIO particles. Energy-dispersive X-ray (EDX) analysis of the dense particles showed that these particles contain a large amount of iron, additionally confirming that they represent USPIOs (Figure 2c), whereas in the Perl's negative regions, no iron could be observed. Next to USPIO-positive macrophages, negative macrophages were also observed.

Although most USPIO staining was confined to macrophages, incidentally we observed ASMA/Perl’s iron and CD31/Perl’s iron double staining, which is indicative for limited USPIO uptake by smooth muscle cells, myofibroblasts, and endothelial cells (Figure 3).

In total, 50 histological sections were obtained. The stage of the plaques in each section was compared with the uptake of USPIO (Perl’s positive regions). Only 7% (1 of 14) of the stable lesions showed USPIO uptake, whereas this number was 75% (27 of 36) for the rupture-prone and ruptured lesions. These proportions were significantly different (P<0.0001). From the plaques of every patient, at least 2 sections were ruptured or rupture-prone.

In total, 235 quadrants were available for CD68 immunomacrophages and Perl’s iron reading. Note that occasionally sections consisted of material from both the carotid internal and external artery (8 quadrants), whereas in other sections, <4 quadrants were available because of incomplete intima removal during endarterectomy. Of these 235 quadrants, 86 showed Perl’s positive regions, indicating the uptake of USPIO. All quadrants that were Perl’s positive were also CD68 positive, but not vice versa. Perl’s positive cells were present in 3 of 35 quadrants with hardly any, 17 of 55 with some, 45 of 80 with many, and 21 of 39 with very many CD68-positive cells.

Magnetic Resonance Imaging

The rSI of the vessel wall on T2*w GE images acquired 24 hours after administration of USPIOs was decreased in comparison with the rSI on pre-USPIO images. From a comparison with histology, it becomes apparent that the decrease in rSI was confined to parts of the vessel wall that showed uptake of USPIO (Figure 4). Of the T2*w GE images of patients showing USPIO uptake acquired with a delay time of 24 and 72 hours, 67 of 123 (54%) and 19 of 55 (35%), respectively, showed signal changes. After 24 hours, the mean rSI ratio of the quadrants with signal changes was 0.76 (95% CI, 0.67 to 0.85), indicating a signal decrease of 24%. The mean rSI ratio after 72 hours was not significantly different from unity (P<0.05). In 4 images, a dark band adjacent to the lumen was observed in the post-USPIO MRI. Images with this or other artifacts were excluded from additional analysis.

In the T1w GE images, 24 hours after administration, in most cases the lumen was hyperintense. In these images, a narrow hypointense band adjacent to the hyperintense signal was observed (Figure 5). This band was not clearly confined to either the vessel wall or the lumen. This effect also occurred in other arteries. In the images acquired 72 hours after administration of USPIOs, the signal intensity of the lumen had decreased, but in some cases this artifact was still observed. Because this phenomenon also led to a signal decrease in the vessel wall, it was impossible to quantify
signal changes because of the uptake of USPIOs in the T1w GE MR images.

Of the PDw FSE images of patients showing USPIO uptake acquired with a delay time of 24 and 72 hours, 51 of 123 (41%) and 15 of 55 (27%), respectively, showed signal changes. However, the mean rSI ratios for these images, after both 24 and 72 hours, of the patients that showed uptake of USPIOs were not significantly different from unity.

**Discussion**

The results of the present study show that the tested USPIOs accumulate predominantly in macrophages in ruptured and rupture-prone human atherosclerotic lesions and that this can induce significant signal changes in the in vivo T2*w GE MR images acquired 24 hours after intravenous administration of USPIOs. The implications of this study could be vast, because it indicates that USPIO-enhanced MRI is a promising noninvasive method to identify high-risk plaques.

USPIO-enhanced plaque imaging could be combined with information about plaque composition obtained from nonenhanced MR plaque imaging. High-resolution MRI has a lot of potential for noninvasive in vivo plaque characterization.\(^9,10\) Therefore, one might be able to obtain information about plaque inflammation and composition using a single modality, which will be extremely valuable for the identification of patients at risk.

The composition and stage of atherosclerotic plaque rather than the severity of stenosis have emerged as being the most important determinants for the development of acute ischemic symptoms.\(^11\) These symptoms often occur as a result of erosion or uneven thinning and rupture of the fibrous cap. Lesions at risk for rupture usually show accumulation of macrophages.\(^12\) The tested USPIO contrast agent (Sinerem) is a relatively new contrast agent that is used for MR lymphography, where the accumulation of this contrast agent in macrophages reduces the signal intensity of normal functioning nodes attributable to the T2* and T2 shortening effects of USPIOs.\(^13\)

The exact route by which USPIOs enter the plaques is still unknown. However, the profound uptake of USPIOs by macrophages surrounding newly formed capillaries and the observation that USPIOs incidentally are located in endothelial cells suggests that the USPIOs may enter the plaque via transcytosis.

In the present study, electron microscopy showed unambiguously clustering of USPIO particles in phagosomes of macrophages. At high concentrations, USPIO decreases the signal intensity on T2*w images attributable to a shortening of T2 and T2*. Moreover, clustering of iron oxide particles in tissue may lead to additional T2* shortening.\(^14\) This study showed that the USPIO uptake induced significant signal decreases in the in vivo T2*w GE MR images acquired 24

**Figure 2.** Electron microscopy and EDX analysis of Perl's positive regions of an endarterectomy specimen of a patient who received USPIOs. a, Presence of numerous USPIO particles in a single macrophage (arrows). The plasma membrane is indicated with PL, the cell nucleus with N, and the extracellular space with ES. b, Detail of phagosomes in macrophages containing clusters of iron particles (arrows). c, EDX spectrum of particles in macrophages (dotted line). It is shown that these particles contain a large amount of iron. The solid line corresponds to an EDX spectrum of a reference area.
hours after administration of USPIOs but not in the images obtained after 72 hours. This suggests an active process of uptake and washout and therefore imaging 24 hours after USPIO administration is preferred above imaging after 72 hours.

The administration dose that can be used for human studies is much lower than the dose administrated in experimental animal models. Ruehm et al used a very high dose, namely 56 mg/kg, in hyperlipidemic rabbits. Schmitz et al showed, again in a study with hyperlipidemic rabbits, that at a dose of 11 mg Fe/kg compared with 2.8 mg Fe/kg, many more images showed a focal signal loss in the aortic wall (31±11% versus 3±5%). In the present human study, a similar dose as in MR lymphography of 2.6 mg Fe/kg was used. An advantage of human studies is that the half-life of USPIOs in blood is much larger than in rabbits. The half-life of USPIOs in blood in humans is ≈30 hours, whereas this is ≈6 hours for rabbits. This longer half-life will allow for more time for the uptake of the iron oxide particles by macrophages of plaques. Another difference is that in animal studies, usually a longer imaging time can be used.

The present study has several limitations. First, we experienced difficulties matching the 4.5-mm-thick MRI slices with the 4-μm-thick histological sections and could therefore not correlate signal changes in the MR images with USPIO uptake in the corresponding histological sections. Second, it is well known that GE sequences are most sensitive for iron detection, but unfortunately T2*w GE images have a relatively low signal to noise ratio. This has led to a somewhat limited image quality of the T2*w GE images in the present study. This needs to be improved for accurate detection of USPIO accumulation in human plaques by reducing the spatial resolution, by using other pulse sequences, by using a phased-array carotid coil, or by using higher magnetic field strength. Third, the time interval between USPIO administration and surgery was 4.7±3.0 days, whereas ideally this...
should be constant. Another complexing factor may be that in the post-USPIO T1w GE and some of the T2*w GE MR images, a dark band adjacent to the hyperintense signal of the lumen was observed. In T1w GE images, the signal of the lumen is hyperintense 24 hours after USPIO administration because of the T1-shortening effect of a low concentration of USPIOs in the vascular space. This band was not clearly defined to either the lumen or the vessel wall. The same phenomenon was also observed by Schmitz et al., who could not explain it as USPIO accumulation because iron was not demonstrated in corresponding histological sections. Images with this artifact were excluded from additional analysis.

Finally, a limitation of USPIO-enhanced MRI may be that the postcontrast imaging time needs to be rather long to allow uptake of iron particles by macrophages. Furthermore, we feel it is important to compare postcontrast with corresponding precontrast images to differentiate signal reductions attributable to USPIO uptake from other effects. For example, plaque calcification and blood degradation products can also have strong susceptibility effects and therefore cause signal loss. However, in contrast to signal loss attributable to USPIO uptake, these tissues will already cause signal loss in the precontrast images.

From this proof of concept study, we conclude that the tested USPIOs accumulate predominantly in macrophages in ruptured and rupture-prone human atherosclerotic plaques. This accumulation induces signal decreases in the postcontrast MR images. Because a preponderance of macrophages is an important feature of a high-risk plaque, USPIO-enhanced MRI is a promising method for the in vivo differentiation between low- and high-risk plaques. Larger studies are warranted to confirm these early encouraging results.

Acknowledgments

This work was supported by grants from the Dutch Heart Foundation (2000.173) and Guebert, Paris, France. We thank Dr. R.J. van Suylen (University Hospital Maastricht, Maastricht, the Netherlands) for determining plaque stage of the histological tissue sections.

References

Accumulation of Ultrasmall Superparamagnetic Particles of Iron Oxide in Human Atherosclerotic Plaques Can Be Detected by In Vivo Magnetic Resonance Imaging

Circulation. 2003;107:2453-2458; originally published online April 28, 2003; doi: 10.1161/01.CIR.0000068315.98705.CC

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/107/19/2453

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/