MRI Monitoring of Neuroinflammation in Mouse Focal Ischemia

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Background and Purpose—A growing body of evidence suggests that inflammatory processes are involved in the pathophysiology of stroke. Phagocyte cells, involving resident microglia and infiltrating macrophages, secrete both protective and toxic molecules and thus represent a potential therapeutic target. The aim of the present study was to monitor phagocytic activity after focal cerebral ischemia in mice.

Methods—Ultrasmall superparamagnetic particles of iron oxide (USPIO) were intravenously injected after permanent middle cerebral artery occlusion and monitored by high resolution MRI for 72 hours.

Results—We here present the first MRI data showing in vivo phagocyte-labeling obtained in mice with focal cerebral ischemia. USPIO-enhanced MRI kinetic analysis disclosed an inflammatory response surrounding the ischemic lesion and in the contralateral hemisphere via the corpus callosum. The imaging data collected during the first 36 hours postinjury suggested a spread of USPIO-related signal from ipsi- to contralateral hemisphere. Imaging data correlated with histochemical analysis showing inflammation remote from the lesion and ingestion of nanoparticles by microglia/macrophages.

Conclusions—The present study shows that MR-tracking of phagocyte cells is feasible in mice, which may have critical therapeutic implications given the potential neurotoxicity of activated microglia/macrophages in central nervous system disorders. (***Stroke***. 2007;38:131-137.)

Key Words: animal model ■ focal ischemia ■ inflammation ■ magnetic resonance imaging
■ ultrasmall superparamagnetic particles of iron oxide

A growing body of evidence suggests that inflammatory processes are involved in the pathophysiology of stroke.[1] Phagocyte cells, involving resident microglia and infiltrating macrophages, secrete both protective and toxic molecules and thus represent a potential therapeutic target.[2] To date, most studies on microglia/macrophage response to cerebral ischemia have used cell culture or ex vivo histology. New imaging techniques enabling in vivo assessment of phagocytic activity, therefore, need to be developed. In addition, the growing availability of transgenic mice has raised a lot of interest in mouse models of ischemic stroke.[3] The small size of the mouse brain and the surgical complexity of creating ischemia in mice make imaging of murine brain infarction technically challenging. Only a few in vivo positron-emission tomography or MRI studies have reported phagocyte imaging in the rat or human brain.[4–9]

In this context, the aim of the present study was 2-fold: (1) to investigate the feasibility of in vivo phagocyte labeling by ultrasmall superparamagnetic particles of iron oxide (USPIO) in mice, and (2) to monitor phagocyte cells after focal cerebral ischemia. USPIO-enhanced MRI kinetic analysis disclosed an inflammatory response surrounding the ischemic lesion and in the contralateral hemisphere via the corpus callosum. Imaging data correlated with histochemical analysis showing inflammation remote from the lesion and ingestion of nanoparticles by microglia/macrophages.

Materials and Methods

All animal experimentation was performed in accordance with institutional guidelines. Twenty-one male Swiss mice were included in the study (weight: 25 to 30 g; Charles River, France).

Subjects and Protocol

	Group I pMCAO+USPIO (n=14)			Group II Control pMCAO (n=4)		Group III Control USPIO (n=3)
	n=4	n=4	n=6	n=3	n=1	n=3
D0	5 h, 6 h, 12 h	5 h, 6 h			5 h	
D1	24 h, 36 h		24 h†			
D2		48 h	48 h†	48 h		48 h
D3	72 h*	72 h*		72 h	72 h	72 h

Times correspond to MRI scanning time postischemia except †(time of animal euthanasia without MRI). USPIO were injected IV 5 hours postischemia after precontrast imaging (5 hours in the Table). Postcontrast imaging was performed 1 hour after injection (6 hours in the Table).

*n=3, one mouse having died before 72 hours postischemia; †n=3 euthanized animals out of 6 at each timepoints.

Induction of Focal Cerebral Ischemia

Focal ischemia was induced in 18 mice under anesthesia with 12 mg/kg xylazine and 90 mg/kg ketamine, by permanent occlusion of the distal middle cerebral artery (pMCAO) using an operating microscope as described previously.[10] Briefly, the right MCA was exposed by subtemporal craniectomy and occluded by electrocoagulation. Wounds were then sutured and the mice allowed to recover. Body temperature was held at 37°C by a feedback-regulated heating pad.

Contrast Agents

The Ferumoxtran-10 (Sinerem) USPIO contrast agent was provided by Guerbet. It is composed of a 4- to 6-nm crystalline iron oxide core coated in dextran; the mean hydrodynamic diameter was 35 nm (range: 20 to 50 nm). A dose of 2 mmol Fe/kg body weight was injected intravenously into the tail vein 5 hours post-pMCAO,[5] based on a pilot study that demonstrated relevance of this protocol. Gadolinium (Dotarem, Guerbet) was administered at a dose of 0.5 mmol/kg before USPIO administration to assess blood–brain barrier (BBB) integrity at injection (n=3).

MRI

MRI experiments were carried out on a Bruker Biospec 7T/12-cm system using a birdcage head-coil of 72 mm inner diameter for RF transmission and a 15-mm diameter surface coil for reception. Gradient-echo (GRE) images were acquired using a FLASH sequence with the following parameters: time of echo/time of repetition (TE/TR)=3.5/157.5 ms; flip angle=50°. T2-weighted images were acquired using a RARE sequence with TE/TR=75/3000 ms. T2 maps were obtained from a multiple spin-echo sequence (TE [interecho delay]/TR=11.4/4000 ms; 8 echoes; number of experiments 2) by fitting a monoexponential function to the data. Diffusion-weighted spin-echo images were acquired with TE/TR=14/2000 ms using 3 b-values (138, 554, and 1060 s/mm^2 in slice direction). Apparent diffusion coefficients (in mm^2/s) were calculated by fitting to a monoexponential model function on a pixel-by-pixel basis. For all scans, the field of view was 20×20 mm^2, slice thickness 1.0 mm, number of slices 15, and matrix size 256×256, except for the diffusion sequence where the matrix size was 128×128. During the MRI experiments, anesthesia was maintained with isofluorane (1.5% in oxygen/nitrous oxide 1:2). The mice were placed in a cradle equipped with a stereotaxic holder, an integrated heating system to maintain the body temperature at 37±1°C, and a pressure probe to monitor the respiration.

Protocol

Nanoparticles of iron oxide (USPIO) were administered to 14 of the 18 operated mice (Group I). The 4 other operated mice did not receive USPIO and served as controls (Group II). Three nonoperated mice received USPIO the same day and at the same dose as in Group I (Group III). Fifteen mice (Group I: n=8, Group II: n=4 and Group 3, n=3) were imaged between 4 and 72 hours post-pMCAO. Six mice of Group I were euthanized without imaging (n=3 at 24 hours post-pMCAO and n=3 at 48 hours). The Table summarizes the protocol for each subgroup.

Image Analysis

All MR image data were transferred to a personal computer (1.6 GHz, 256 Mbytes) and analyzed using homemade software written in C++ (*CreaTools*). T2-weighted images revealed a sharp transition from normal to abnormal values (either hyperintensities without USPIO or hypointensities with USPIO) at all timepoints post-pMCAO, and therefore they were used to manually draw regions of interest. To avoid overestimation of volume attributable to edema, the lesion area was normalized by the ratio of ipsilateral to contralateral hemisphere areas. The volumes were calculated by summation of lesion areas of all brain slices showing brain damage and integrated by slice thickness.

Histology/Immunohistochemistry

After the last MR examination, animals were anesthetized by halothane inhalation and killed by intracardiac perfusion with 50 mL of 4% paraformaldehyde in 100 mmol/L pH=7.4 phosphate buffer. The brains were then dissected out, immersed overnight in fixative at 4°C and kept in PBS containing 30% sucrose at 4°C until use. When needed, tissues were then frozen in dry ice, and the blocks embedded in polyethylene glycol and cut into 20-μm-thick sections with a cryostat. Brain sections were rinsed 3 times in PBS and incubated for 30 minutes at room temperature with a blocking solution consisting of 1% bovine serum albumin diluted in PBS and supplemented with 0.3% triton. Brain sections were then incubated overnight at 4°C with rat antimouse F4/80 antibody (clone MCA497, Serotec, Oxford, UK) diluted 1/50 in blocking solution. Sections were then rinsed 3 times in PBS and incubated with a biotinylated goat anti-rat secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa) for 2 hours at room temperature. After 3 rinses, the sections were treated with avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 90 minutes at room temperature, followed by 0.2 mg/mL of diaminobenzidine in 50 mmol/L pH=7.6 Tris-HCL buffer in PBS with 0.01% hydrogen peroxide until stained. Finally, sections were counterstained with nuclear red so as better to visualize the cytoplasm, and the iron nanoparticles were stained by Prussian blue.

Results

pMCAO induced infarction in the cortex and part of the striatum within 24 hours after surgery (Figure 1A), with no significant change in lesion size between 24, 48, and 72 hours: respectively 42±7 mm^3 (n=4), 45±5 mm^3 (n=7) and 41±6 mm^3 (n=10). Preliminary data showed that tissue resorption started after day 3 (data not shown). In order to
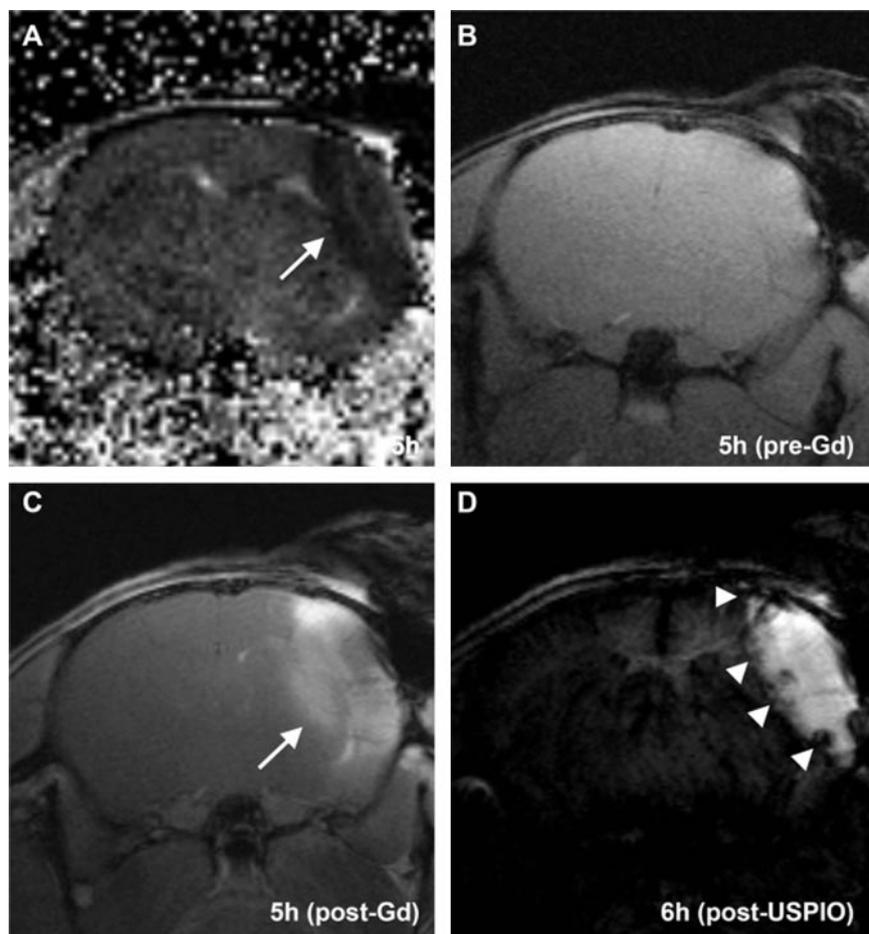
Figure 1. Multicontrast MRI at the acute stage of cerebral ischemia in a transverse slice through the mouse brain (Bregma 0.74 mm, according to Franklin and Paxinos's atlas[33]). Time is given postischemia. A, Apparent diffusion coefficient map before gadolinium and USPIO injections. The lesion is defined by reduced apparent diffusion coefficient (arrow). B, GRE imaging before gadolinium (Gd) and USPIO injections. C, GRE imaging post-Gd injection. Note T1 enhancement of the entire lesion (arrow), indicative of BBB disruption. D, GRE imaging 1 hour post-USPIO. Note T2* decrease in the perilesional area (arrow heads).

correlate the kinetics of macrophage activation with lesion development, we imaged mice brains during the first 72 hours post-pMCAO.

Time Course of USPIO-MR Signals in Focal Ischemia of the Mouse Brain

At the time of USPIO administration (5 hours postinjury), gadolinium injection resulted in T1 enhancement of the entire lesion, indicative of BBB disruption (Figure 1). GRE images acquired 1-hour post-USPIO injection (6 hours postinjury) allowed visualization of vessels detected by organized signal loss (Figure 2B) attributable to USPIO compartmentalization within the vascular space. At the same timepoint, GRE and T2 maps revealed a marked signal drop-out, demonstrating the presence of USPIO at the border of the lesion (Figure 2B). Signal changes remained confined to the periphery of the lesion during the first 12 hours postinjury (Figure 2C).

Then, quite surprisingly, a strongly contrasted line was seen to migrate along the corpus callosum from the ipsi- to contralateral hemisphere (Figure 2D through 2G). This line, moreover, while hypointense in T2 maps, was simultaneously transiently hyperintense in GRE images (Figure 2D through 2F). In imaging performed at later timepoints, 48 hours and 72 hours postinjury, rim-like signal loss was observed around the lesion (Figures 3B and 3C, and 4A). The ipsilateral area of decreased T2 extended beyond the perilesional area to the whole cortex and striatum (Figure 3B and 3C). Furthermore,

the contralateral corpus callosum was hypointense in both GRE and T2-weighted images (Figures 3B and 3C, and 4A). These signal changes were observed in all mice. However, in 1 mouse, we missed the early ipsilateral enhancement in the corpus callosum. None of the described signal changes were present in control mice (Figure 2H).

USPIO-MR Signals Are Correlated to Remote Inflammation

To confirm that USPIO imaging correlated to inflammation, immunohistochemical analyses were performed at 24, 48 and 72 hours postischemia. There was no difference between Groups I (pMCAO+USPIO) and II (pMCAO) in the distribution of activated microglia as detected by F4/80 immunostaining, whereas in Group III (USPIO) only a few plexus-associated Kolmer cells were stained. At 24 hours postischemia, only few F4/80+ cells were found in the perilesional area and in the ipsilateral corpus callosum (Figure 3A). At 48 and 72 hours, a larger number of F4/80+ cells were observed in the perilesional area, the striatum adjacent to the lesion, and the whole corpus callosum, including the contralateral part (Figure 3 B and 3C). The areas where F4/80+ cells localized corresponded spatially to regions of T2 decrease outside the lesion (Figure 3). Areas of T2 decrease were slightly larger than areas covered by macrophage/microglia infiltration, probably because of partial volume effects. Finally, it is noteworthy that part of the T2 hypointense signals observed inside the infarct might not be linked to
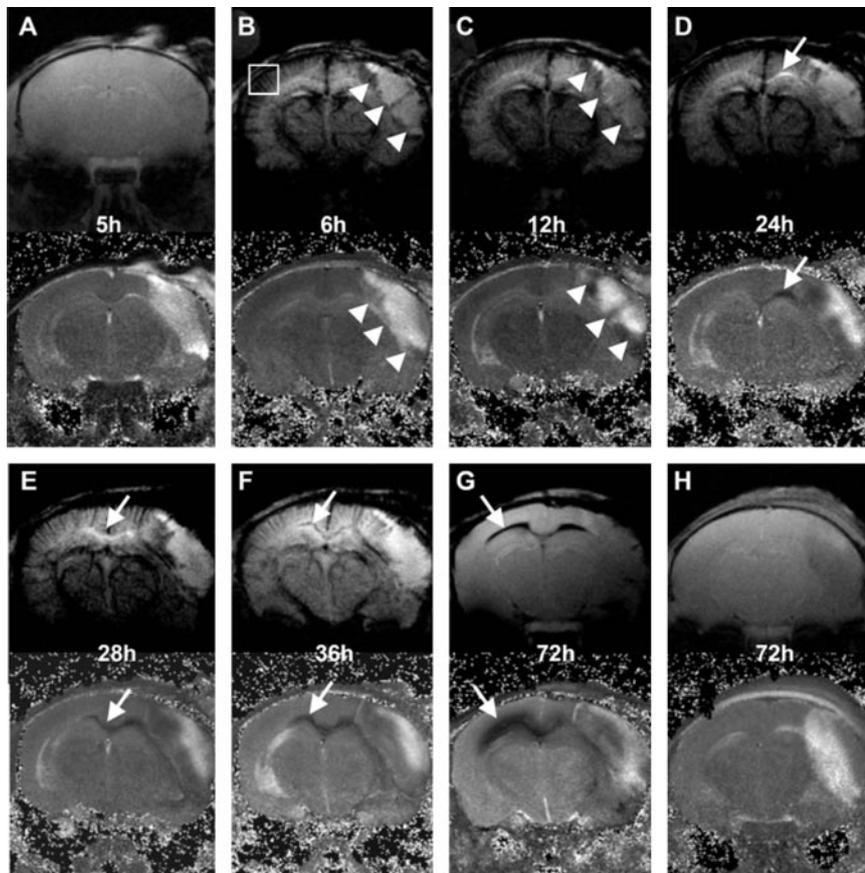
Figure 2. Spatiotemporal pattern of USPIO distribution over 3 days in a transverse slice through the mouse brain (Bregma −1.28 mm, according to Franklin and Paxinos's atlas[33]) as observed on GRE (top) and T2 maps (bottom). A, Before USPIO injection (5 hours postischemia). B, 1 hour after USPIO injection (6 hours postischemia). Note the marked signal decrease in the perilesional area (arrow heads). Box shows pial vessels appearing in hyposignals because of USPIO compartmentalization. C, The USPIO-hyposignal remains confined to the periphery of the lesion (arrow heads). D, Note the hyperintense signal in the ipsilateral corpus callosum (arrow) on day 1 on GRE imaging and the corresponding signal decrease on T2 maps. E, During the next hours, the observed signal migrated from the ispi- to the contralateral hemisphere (arrow). F, Note the transition from hyper- to hyposignal at the end of day 1 on GRE imaging. G, The whole corpus callosum was hypointense on day 3 both on GRE and on T2-weighted imaging (arrow). H, Corresponding control slice in a mouse with pMCAO but without USPIO.

USPIO-laden macrophages, because the area appeared highly necrotic and contained very few F4/80+. Distally to the lesion, F4/80+ cells showed ramified processes, in contrast to the round morphological features at the lesion edge (Figure 3C). Activation of brain-resident microglia is accompanied by a morphological transition, which makes it indistinguishable from blood-derived macrophages. Consequently, immunostaining at 72 hours postischemia fails to discriminate phagocytic microglia from infiltrating macrophages. There was an overlap between microglia and astrocyte activation in the ipsilateral hemisphere only (data not shown). Iron+ cells, stained by Prussian blue, were found in the corpus callosum, commissures, ipsilateral subarachnoid spaces, choroid plexus and periventricular areas (Figure 4). A double-staining experiment showed Prussian blue-stained cells with the typical ramified morphology of reactive F4/80+ phagocytes, demonstrating USPIO intracellularity (Figure 4F).

Discussion

The present study shows that MR tracking of phagocyte cells is feasible in mice. Most importantly, this noninvasive technique allowed us to demonstrate an inflammatory response remote from the lesion site, which to our knowledge had never been monitored in living animals. USPIO-laden macrophages were detected in the perilesional area as well, in accordance with findings of other investigators in stroke-induced rats.[5,6,9]

Microglial response-spread beyond the primary lesion site to the nonischemic ipsilateral cortex and striatum has been reported in animal models of focal ischemia with the help of invasive techniques[10,11] and recently in long-term follow-up of stroke patients using positron-emission tomography scan.[12] The pathophysiological process underlying this phenomenon needs to be further investigated. It has been suggested that cortically spreading depression partly accounts for remote ipsilateral microglial and astrocytic reaction in focal ischemia.[11,13] Other causes of remote change with focal brain lesion include: (1) remote change caused by brain edema; (2) remote change in projection areas; and (3) remote change attributable to reactive plasticity and systemic effects.[14,15] Acute transcallosal deafferentation might be responsible for microglia/macrophage activation in the corpus callosum. The final impact of contralateral inflammation on residual brain damage should be explored because the observed remote effects may critically determine the process of recovery and compensation.[16] Noninvasive cell-tracking procedures are urgently needed to study these mechanisms. Our results describing the spatiotemporal patterns of USPIO from 5 hours until 72 hours postischemia in correlation with microglia/macrophage activation demonstrate that USPIO-enhanced MRI might be a good candidate for such studies, provided that the reported immunomodulatory effects of USPIO labeling[17] are proved to be negligible.

The main difficulty in validating the USPIO-enhanced MRI technique as a marker of neuroinflammation is to rule out the possibility of nonspecific USPIO brain uptake. Three mechanisms have been proposed to explain how USPIO enters the mouse brain[18]: (1) blood-stream USPIO may be
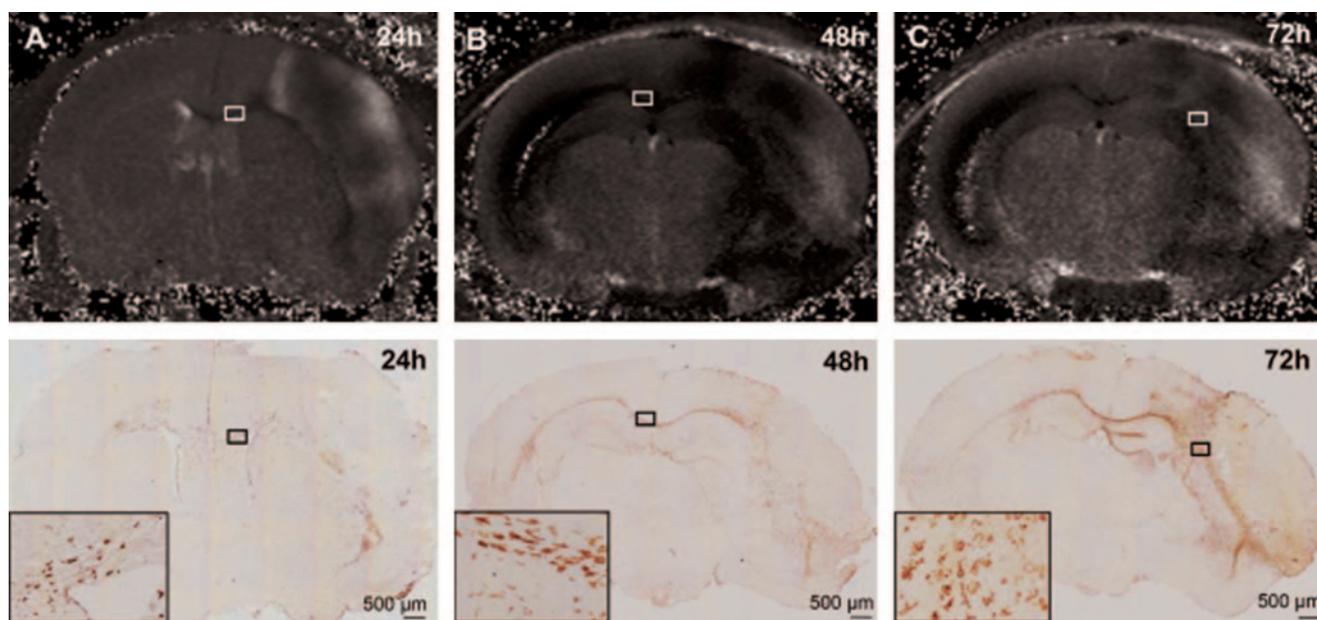
Figure 3. Correlation of T2 maps (top) with F4/80 immunostaining (bottom) at 24 hours (A, Bregma 0 mm according to Franklin and Paxinos's atlas[33]), 48 hours (B, Bregma −1.28 mm) and 72 hours (C, Bregma −1.28 mm) post-pMCAO and IV injection of USPIO. At 24 hours, there were some F4/80 positive cells in the perilesional area as well as in the corpus callosum (insert), in accordance with T2 decrease at this early time-point. F4/80 immunostaining was increased at 48 and 72 hours compared with 24 hours, with a marked staining in the perilesional area and in the adjacent striatum, as well as in the whole corpus callosum (B and C, insert). F4/80+ cells found at the boundary of the lesion had the typical round-shaped morphology of phagocytic macrophages (C, insert).

phagocytosed by circulating monocytes migrating toward the brain parenchyma in response to inflammatory chemoattractant stimuli; (2) USPIO may cross the brain endothelium by transcytosis; or (3) they may diffuse passively across a disrupted BBB. Exclusion of nonspecific T2 decrease was especially critical within the infarct where macrophages were scarce according to F4/80 immunostaining. In particular, small hemorrhagic transformations inside the lesion (as detected by histology) resulted in focal asignals. Furthermore, the presence of free USPIO in the necrosed tissue cannot be ruled out. Therefore, caution should be used when interpretating MR signals inside the lesion with the current model of focal cerebral ischemia.

We were surprised to observe a rim-like signal loss around the lesion as early as 1-hour post-USPIO injection. Indeed, 35-nm-diameter USPIO are not immediately recognized by the mononuclear phagocytic system, making the rate of monocyte-labeling probably very low at this stage. Moreover, monocyte/macrophage infiltration is thought to occur days rather than hours after focal ischemia.[11,19] Therefore, at the acute stage, signal void around the lesion is more likely to be attributable to USPIO crossing the endothelium, whether passively or actively, than to USPIO-laden macrophage infiltration. The relative mismatch between gadolinium and USPIO enhancement on postcontrast imaging may argue in favor of active uptake rather than passive leakage, but the hypothesis of a differential BBB leakiness to low versus high molecular weight agents remains to be tested (in progress). In the same mouse model as in the current study,[10] activated microglia was detected at the periphery of the lesion as early as 30 minutes after onset of pMCAO. After entering the brain parenchyma, USPIO may thus be captured by perilesional microglia forming a shield around the damaged

area, and phagocytosing debris and leaked blood components.[20] At later stages, however, (72 hours postischemia), the perilesional hyposignals could be explained in part by migration of hematogenous macrophages labeled with USPIO.

Mechanisms leading to USPIO-labeling of macrophages/microglia outside the perilesional area should also be clarified. Although the microglial shield around the infarct is thought to protect nonischemic tissue from leaky components,[20] there is assumed to be no cellular infiltration or BBB disruption remote from the lesion.[11] At low concentrations, extracellular iron oxides exert a marked T1 effect in T1-weighted sequences at various field strength (0.5 to 3T), caused by interactions with surrounding protons.[21–24] The r1 of the nanoparticles decreases with increasing field strength but is higher at 7T than r1 values for gadolinium chelates.[25] The T1 enhancement after Gd-DTPA leakage in the cerebral tissue post-pMCAO showed that the GRE sequence was sensitive to T1-effects of gadolinium at 7T. To cause the transient T1-effect observed in the corpus callosum at 24 hours post-pMCAO, USPIO needed to interact with protons, therefore suggesting an extracellular distribution of USPIO in the edematous interstitium of the corpus callosum. This interpretation is in line with recent works showing an early T1-effect of interstitial USPIO in inflammatory arthritic tissues.[23] Microglial shielding of the lesion might have failed, letting USPIO escape to migration-friendly regions. However, additional experiments should be performed to confirm these hypotheses.

A T2/T2*-effect (signal drop) gradually replaced the T1-effect on GRE images in the corpus callosum. This could be attributable to an increase of free USPIO concentration because T2-effects are known to overpower T1-effects at
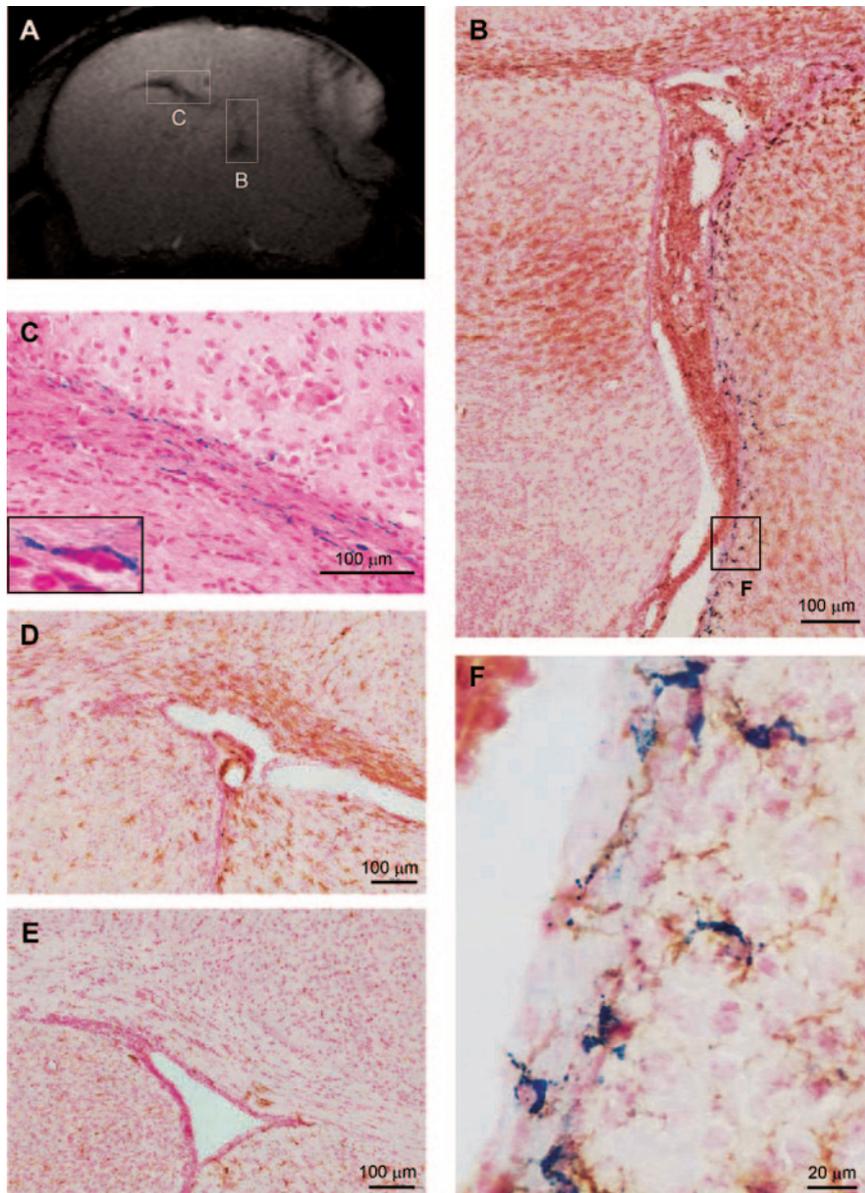
Figure 4. Correlation of GRE signals with immunohistology (Bregma 0 mm according to Franklin and Paxinos's atlas[33]). A, GRE MRI 72 hours post-pMCAO and IV injection of USPIO (same mouse as in Figure 3C). Note the hyposignal around the lesion, the contralateral corpus callosum and the ipsilateral periventricular area. B, Double staining with Prussian blue and F4/80 of the right (ipsilateral) ventricle. Iron-stained microglia/macrophages are clearly visible along the lateral wall, in correlation with the hyposignal observed in GRE images (A). C, Prussian blue staining for iron in the contralateral corpus callosum of the corresponding slice. Positive staining was observed around cell nuclei, which suggests cytoplasm uptake (insert). D, F4/80 immunostaining for mouse microglia/macrophages in the contralateral corpus callosum. Note the F4/80+ brown cells, in spatial agreement with iron+ cells (C) and MRI hyposignal (A). E, F4/80 immunostaining of a nonoperated control mouse in the left corpus callosum, with no positive staining. F, Double staining with Prussian blue and F4/80: magnification of B. Microglia/macrophages as identified by their brown color and typical ramified shape, were also blue-stained, suggesting USPIO intra-cellularity.

high concentration on T1-weighted images.[26] Alternatively, this observation could be explained by slow phagocytosis of USPIO by macrophages. The intracellular compartmentalization in macrophages causes restriction of iron oxide interactions with surrounding protons, which leads to a decline in T1-effect, as well as an increase in susceptibility effect and, consequently, T2*-effect.[27] As sensitivity to susceptibility artifacts augments with field strength, the signal drop observed on gradient echo images at 7T likely reflected cell uptake of USPIO. The intracellularity of iron oxides was confirmed at 72 hours postischemia by double immunostaining with F4/80 for macrophages and Prussian Blue for iron.

Redistribution of USPIO within the cerebrospinal fluid attributable to leakage from pial vessels into the subarachnoid space[28] or disruption of the blood/cerebrospinal fluid barrier at the choroid plexus[29] may also play a role in delivery to secondary sites such as the lateral wall of the ipsilateral ventricle and adjacent striatum.[30] T2 decrease was particularly marked in the striatum region where microglia/macrophages activation was intense, but Prussian blue staining did not show iron within the parenchyma in this area. Thus, the MRI sequences developed specifically for this application might be more sensitive to the presence of iron than histological techniques.[31]

The disappointing results in clinical stroke trials with inflammation inhibitors[32] have resulted in a clear need to develop surrogate markers for monitoring the clinical response to therapy. In this report, we propose an MRI method to assess phagocytic activity in the living mouse, based on intravenous USPIO injection. If USPIO are approved for use in humans, this method could easily be applied in clinical practice.[8] Most importantly, this technique could become a powerful tool for studying the molecular mechanisms that are ultimately responsible for the expansion of ischemic lesions in genetically engineered mice.

Acknowledgments

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MRI Monitoring of Neuroinflammation in Mouse Focal Ischemia

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Correction

In the article entitled "MRI Monitoring of Neuroinflammation in Mouse Focal Ischemia" by Wiart et al,[1] an author name in the byline is incorrect. "Samir Moucharaffie" should read "Samir Moucharrafie." The authors regret this error.

The corrected version of this article can now be viewed online at http://stroke.ahajournals.org.