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FULL PAPER

MRI study of atherosclerotic plaque progression using ultrasmall superparamagnetic iron oxide in Watanabe heritable hyperlipidemic rabbits

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Objective: The purpose of this study was to evaluate plaque progression by using MRI with ultrasmall superparamagnetic iron oxide (USPIO) and by histopathological studies.

Methods: We divided 12 Watanabe heritable hyperlipidemic (WHHL) rabbits into 4 groups based on their age (3, 9, 14 and 26 months) and injected them intravenously with 0.8 mmol (Fe) kg⁻¹ of USPIO (size, 32 nm; concentration, 15 mg dl⁻¹). On the fifth post-injection day, they were again given an intravenous injection with 40 µmol kg⁻¹ of the same USPIO, and MR angiography (MRA) was performed. The signal-to-noise ratio (SNR) in regions of interest in the wall of the upper abdominal aorta was calculated on coronal images. Specimens from the same level of the aorta were subjected to iron staining and RAM-11 immunostaining and used for histopathological study. For statistical analysis of

the MRA and histopathological findings, we used analysis of variance [Tukey's honest significant difference (HSD) test].

Results: In 9-month-old rabbits, the SNR was significantly lower than in rabbits of the other ages ($p < 0.01$), and the area of RAM-11 (DAKO Corporation, Glostrup, Denmark) and iron uptake in the aortic wall was significantly larger (RAM-11, $p < 0.01$; iron, $p < 0.05$). These areas were the smallest in 3-month-old rabbits.

Conclusion: Histopathologically, the number of macrophages was the greatest in 9-month-old rabbits. Our findings indicate that the SNR on MRI scans reflects the number of macrophages in the aortic wall of WHHL rabbits.

Advances in knowledge: USPIO-enhanced MRI visualized the accumulation of macrophages in early atherosclerotic plaques of WHHL rabbits in the course of natural progression.

INTRODUCTION

Atherosclerosis is a chronic inflammation of the arterial wall and a leading cause of diseases such as acute coronary syndrome and myocardial and cerebral infarction. Macrophages play an important role in the progression of atherosclerosis; they produce molecules that promote inflammation and trigger plaque disruption and subsequent thrombus formation.^{1–3} Therefore, the evaluation of target molecular components in atherosclerotic plaques is important.

The uptake of superparamagnetic iron oxide particles (SPIOs) by the mononuclear phagocytic system (MPS) results in hepatic, splenic, bone marrow and nodal iron accumulation.⁴ Unlike SPIOs with a diameter of a few hundred nanometres, ultrasmall iron oxide particles (USPIOs), whose mean diameter is 30 nm, are not immediately recognized by the hepatic and splenic MPS.^{4–6} The capillary permeability of USPIOs facilitates their uptake by macrophages and they have been used in the study of multiple sclerosis, arthritis and

atherosclerotic plaques.^{7–9} The blood half-life of USPIOs is longer than that of SPIOs and their T_1 -shortening property has led to their use in low concentrations in MR angiography (MRA) studies. Owing to their T_2/T_2^* -shortening effect, at high injection concentrations, the accumulation of USPIO in the aortic wall results in a very low signal after 4–5 days, and this facilitates the acquisition of excellent vascular wall images.⁴

The aim of this study was to evaluate spontaneous atherosclerosis plaque progression in the aorta of Watanabe heritable hyperlipidemic (WHHL) rabbits using MRI and USPIOs. We also compared imaging and histological findings made at identical aortic wall sites in rabbits of different ages.

METHODS AND MATERIALS

All experimental protocols were approved by our animal experimentation committee, and all experiments were conducted

in accordance with the Animal Care Guidelines of Shiga University of Medical Science, Otsu, Shiga, Japan.

Iron oxide nanoparticles

USPIO (particle diameter, 32 nm; iron concentration, 15 mg dl⁻¹) was supplied by Meito Sangyo, Kiyosu, Aichi, Japan.

Animal model

We obtained 12 (WHHL) rabbits from the Institute for Experimental Animals at Kobe University, Hyogo, Japan. They were 3–26 months old and weighed approximately 3 kg. They were divided into four equal groups based on their age (3, 9, 14 and 26 months).

MRI studies

The animals were anesthetized with ketamine (Ketalar 50; Sankyo Yell Yakuhin, Tokyo, Japan) and medetomidine (Domitor; Meiji Seika, Tokyo, Japan) (25 and 0.1 mg kg⁻¹ body weight, respectively). USPIOs were injected into an ear vein (0.8 mmol Fe kg⁻¹), and 5 days later, the animals were again injected with USPIO (40 µmol Fe kg⁻¹). The low dose, 40 µmol Fe kg⁻¹, was administered for its *T*₁-shortening effect that renders the signal bright in the aortic lumen.

MRA scans were obtained on a 1.5-T MRI system (Signa® Excite HDx; GE Healthcare, Milwaukee, WI) (maximum amplitude, 33 mT m⁻¹; slew rate, 120 mT m⁻¹ ms⁻¹) using a single-channel

Figure 1. Curved planar reconstruction of ultrasmall superparamagnetic iron oxide-enhanced MR images obtained under a three-dimensional fast spoiled gradient echo protocol. The aortas of four Watanabe heritable hyperlipidemic rabbits aged 3 (a), 9 (b), 14 (c) and 26 months (d) are shown. The aortic wall was relatively smooth in 3-month-old rabbits. Some irregularities are apparent in 14- and 26-month-old rabbits (c, d). The insets show regions of interest placed in the aortic wall.

birdcage head coil. Scanning was performed with a three-dimensional fast spoiled gradient echo protocol; the scanning parameters were TR, 9.0 ms; TE, 1.4 ms; receiver bandwidth, 62.5 kHz; slices, 106; flip angle, 25°; field of view (FOV), 320 × 240 mm; matrix size, 256 × 256; slice thickness, 1 mm; average, 1; acquisition time, 159 s.

MR image analysis

The MR images were analysed on a workstation (Ziostation2; Ziosoft, Inc., Tokyo, Japan) and post-processed. Multiplanar reformatting images were acquired.

For quantitative analysis, the signal-to-noise ratio (SNR) was calculated on coronal images at three regions of interest (ROIs) in the wall of the upper abdominal aorta, the same areas used in the histopathological studies. The ROI (2 × 10 pixels) was set in the aortic wall on coronal images (Figure 1) using Photoshop CS6 (Adobe Systems Inc., San Jose, CA), and the signals were recorded. We measured the aortic segments 2 cm down from the celiac artery level, using the celiac and the superior mesenteric artery as markers. Image background noise was also evaluated by measuring the standard deviation of the ROI. For each animal, the SNR of the aortic wall was calculated as $SNR = SI/BN$, where SI is the signal

intensity of the aortic wall, and BN the background noise. At least 5 months after the first evaluation, this analysis was performed twice by a single radiologist blinded to the pathological data.

Care was taken to ensure that ROIs of identical size, as large as possible, were placed at identical locations in the different animals. The thinness of the arterial wall of our relatively small rabbits dictated the use of large ROIs.

In addition, USPIO-induced magnetic susceptibility artefact was quantified by the size of signal loss in the aortic walls. The lengths of signal loss area were measured in the reconstructed transverse plane (Figure 2). The lengths were measured in two directions that crossed perpendicularly. Two radiologists (CK and NN, with 16 and 26 years' experience, respectively) measured levels below the celiac artery in three different slices of the aorta. The mean values of each group ($n = 12$ per one animal) were then calculated.

Histological examination

The rabbits were euthanized and the aortic specimens were removed. The abdominal aorta, 2 cm down from the diaphragm, was fixed in 10% paraformaldehyde. For general morphologic

Figure 2. Axial MR images of the abdominal aorta in rabbits obtained after ultrasmall superparamagnetic iron oxide injection. The white lines indicate the measurement of lengths of the vessel wall area. The aortas of four Watanabe heritable hyperlipidemic rabbits aged 3 (a), 9 (b), 14 (c), and 26 months (d) are shown. Note that the area of signal loss of the aortic wall in 9-month-old rabbits is relatively diffuse and in 14- and 26-month-old rabbits is relatively spotty.

analysis, we stained 4- μm thick serial sections (sagittal plane; mean, 22.5 slices per animal) with haematoxylin–eosin and Elastica van Gieson stains. We used Prussian blue staining to visualize accumulated iron oxide. Immunostaining was with monoclonal antibodies against rabbit macrophages (RAM-11; DAKO Corporation, Denmark). For quantitative assessments, we measured the Prussian blue and RAM-11 stained areas in one FOV (magnification $\times 200$) in three different lesions on each section and calculated the average values. Histology sections corresponding to the MRI-scanned regions were located on the MRI scans using the celiac artery and superior mesenteric artery as references.

Statistical analysis

Differences in the SNR values among the four age groups and in the stain-positive areas were analysed by one-way analysis of variance and by Tukey's honest significance difference test. To assess the variability in the recorded SNR of the vessel wall, we compared the two sets of measurements using the Pearson test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

MRI findings

With respect to luminal SI, the low-dose ($40 \mu\text{mol Fe kg}^{-1}$) injection of USPIO produced an angiographic T_1 -shortening effect. The aortic lumen was bright, and the aortic walls were clearly visualized. While curved planar reconstruction revealed no aortic wall abnormalities in 3- and 9-month-old rabbits, in the older animals, we observed contour irregularities with some degree of stenosis visualized as spotty signal voids (Figure 1).

The high-dose ($0.8 \text{ mmol Fe kg}^{-1}$) injection of USPIO resulted in T_2^* effects that led to a signal loss in the aortic wall. The MRI-derived SNR and the lengths of vessel wall areas of 3-, 9-, 14- and 26-month-old rabbits are summarized in Table 1.

Quantitative assessment of USPIO showed that all images provided a sufficient SNR for atherosclerotic lesions. The SNR of the aortic wall was significantly lower in 9-month-old rabbits than in rabbits of the other ages ($p < 0.01$) (Figure 3). As shown in Table 1, there were significant differences when we compared the SNR in 3- and 26-month-old animals ($p = 0.002$) and in 14- and 26-month-old rabbits ($p = 0.003$). The Pearson correlation coefficient calculated to evaluate interobserver variability in the SNR measurements was 0.77 ($p < 0.01$).

The lengths of signal loss area in the aortic wall were significantly lower in 9-month-old rabbits than in rabbits of 14- and 26-month-old animals ($p < 0.01$). There were significant differences when we compared the lengths in 3- and 14-month-old animals and in 3- and 26-month-old rabbits ($p < 0.05$, respectively).

Histological findings

Prussian blue and RAM-11 staining confirmed the uptake of iron- and RAM-11 by the arterial wall of all 12 rabbits. The localization of iron coincided with the observed accumulation of macrophages in the neointima of the aortic wall (Figure 4). In 3-month-old rabbits, thickening of the intima was mild and their aortic wall was smooth. As the age of the animals increased, so did intimal thickening and the uptake of USPIOs. In 14-month-old rabbits, intimal thickening was advanced while the uptake of USPIOs and the number of macrophages declined. Calcification and plaque disruption were evident in 26-month-old rabbits; RAM-11 and iron were taken up in a small area, and the degree of intimal thickening was greater than in 14-month-old animals.

By quantitative analysis, the iron- and RAM-11-positive areas were significantly larger in 9-month-old rabbits than in animals of the other ages (RAM-11, $p < 0.01$; iron, $p < 0.05$); they were the smallest in 3-month-old rabbits (Figures 5 and 6).

DISCUSSION

Our study shows that USPIOs were phagocytosed by macrophages in the early atherosclerotic plaques in the abdominal aorta of WHHL rabbits.

Assessment on images of USPIOs in the arterial wall and histopathological and immunopathological study of the areas of USPIO uptake showed a significant accumulation of macrophages in atherosclerotic plaques of 9-month-old rabbits. These animals did not manifest marked intimal thickening. While no aortic wall abnormalities were observed on images of 3-month-old rabbits, in 14- and 26-month-old animals, we observed aortic wall irregularities and the older rabbits harboured several ruptured plaques. Our findings suggest that the neointimal USPIO uptake and the accumulation of macrophages observed in 9-month-old rabbits may be predictive of vulnerable plaques.

Previous experimental^{4,10} and clinical^{6,11} pathological studies revealed that USPIOs are taken up by macrophage-laden atherosclerotic plaques. Those findings coincide with ours. However, no

Table 1. Signal-to-noise ratio (SNR) and length of vessel wall area studied by MRI in four different age groups ($n = 3$ each)

Watanabe heritable hyperlipidemic rabbit age (months)	3	9	14	26
SNR	$25.43 \pm 14.15^{a,b}$	$6.19 \pm 2.72^{c,d,b}$	$25.92 \pm 3.26^{a,b}$	$40.82 \pm 7.80^{c,a,d}$
Length of vessel wall area (mm)	$0.98 \pm 0.36^{d,b}$	$1.06 \pm 0.34^{d,b}$	$0.72 \pm 0.46^{c,a}$	$0.75 \pm 0.31^{c,a}$

All measurements are the mean $\pm$ standard deviation.

^aSignificantly different ($p < 0.05$) from 9-month-old rabbits.

^bSignificantly different ($p < 0.05$) from 26-month-old rabbits.

^cSignificantly different ($p < 0.05$) from 3-month-old rabbits.

^dSignificantly different ($p < 0.05$) from 14-month-old rabbits.

Figure 3. Signal-to-noise ratio (SNR) in the aortic wall of rabbits injected with ultrasmall superparamagnetic iron oxides. SNR was significantly lower in 9-month-old rabbits than in rabbits of the other age groups (* $p < 0.01$).

MRI data using USPIOs have been published on the natural progression of atherosclerosis in WHHL rabbits. Wang et al,¹² who evaluated the natural progression of atherosclerosis in WHHL rabbits on unenhanced MRI scans by measuring the abdominal aortic wall thickness and the vascular lumen, reported

good agreement between their MRI observations and pathological findings on the wall thickness and plaque components reported by others.^{13,14} We noted many macrophages in the aortic wall of rabbits up to the age of 9 months. This confirms that macrophages play an important role in early atherosclerosis.

Figure 4. Histology of the abdominal aorta of four rabbits aged 3 (a, e), 9 (b, f), 14 (c, g) and 26 months (d, h). Prussian blue (a-d, blue) and RAM-11 (DAKO Corporation, Glostrup, Denmark) staining (e-h, red) at identical sites. The localization of iron coincided with the accumulation of macrophages in rabbits of all ages. Note intimal hyperplasia and moderate ultrasmall superparamagnetic iron oxide (USPIO) uptake in the neointima of the 3-month-old rabbits (a, e). In 9-month-old animals, there is greater USPIO uptake and progressive intimal thickening (b, f). The USPIO uptake and the number of macrophages were lower in 14-month-old rabbits (c, g). RAM-11 staining and the uptake of USPIOs were seen in small areas in 26-month-old rabbits (d, h). Intimal thickening progressed with increasing age. Bars = 100 μ m (magnification, $\times 200$).

Figure 5. The area of iron uptake in the aortic wall of rabbits injected with ultrasmall superparamagnetic iron oxides was significantly larger in 9-month-old rabbits than in the other age groups (* $p < 0.05$; ** $p < 0.01$).

Ruehm *et al*⁴ documented the presence of USPIOs in the aortic wall of 6-month-old WHHL rabbits. According to Wang *et al*,¹² the aortic wall thickness of WHHL rabbits increased rapidly as their age increased from 20 to 50 weeks; it continued to increase slowly up to an age of 73 weeks. Their and our observations are indicative of active atherosclerosis at around 9 months; however, they did not address changes in the activity of macrophages. As

the evaluation of intimal thickening and of the vascular stenosis rate alone does not help to identify vulnerable plaques, the quantification of macrophages, cells that play an important role in the development of atherosclerosis, may be more useful.

The developmental process of atherosclerosis is similar in rabbits and humans,¹⁵ and our use of WHHL rabbits, in whom the disease

Figure 6. The RAM-11-positive areas (DAKO Corporation, Glostrup, Denmark) in the aortic wall of rabbits injected with ultrasmall superparamagnetic iron oxides were significantly larger in 9-month-old rabbits than in the other age groups (* $p < 0.01$).

progresses in the course of a few months, made it possible to observe its progression over a relatively short time. We followed the natural course of atherosclerosis progression on USPIO-enhanced images of rabbits without balloon-induced vessel wall injury who were fed a normal diet.

There are several techniques for the quantitative analysis of USPIO signal changes. We chose the SNR because it can be used even when the imaging methods and the measured areas are different.^{6,11,16,17} Hyafil et al¹⁰ reported that magnetic susceptibility artefacts, which generate signal voids on MRI scans, extended periaortally and intraluminally to outside the anatomic borders of the aortic wall. Sigovan et al¹⁸ used the area of signal loss for their quantitative analysis. We hypothesized that we could use the vessel wall areas for USPIO quantification. Millon et al¹⁹ prepared R2* maps to calculate the mean R2* value in rabbit plaques. Positive-contrast approaches after USPIO injection, e.g. gradient echo acquisition of superparamagnetic particles (GRASP), have been studied.^{20,21}

Atherosclerosis can also be evaluated with invasive imaging techniques such as X-ray angiography and intravascular ultrasound. Because MRI is non-invasive and does not require ionizing radiation, USPIOs can be used in patients with renal

dysfunction. USPIO-enhanced MRI is therefore more suitable in clinical settings. In fact, the ability of USPIO-enhanced MRI to detect vulnerable human atherosclerotic plaques has been well documented.^{6,22,23}

Our study has some limitations. First, the number of rabbits was too small to assess interindividual differences. Second, signal loss owing to motion, partial voluming, calcification and haemorrhage rendered SNR measurements difficult. Third, our imaging sequence was not optimized. We are in the process of evaluating the usefulness of other sequences such as a more T₂* weighted GRE sequence, T₂ weighted spin-echo axial images of high-resolution MRI and positive-contrast techniques such as the GRASP sequence.^{20,21}

In conclusion, USPIO-enhanced MRI visualized the accumulation of macrophages in the early atherosclerotic plaques of WHHL rabbits. We consider it a promising technique for the detection of vulnerable plaques and for the assessment of the effects of therapy.

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