Noninvasive Magnetic Resonance Imaging Evaluation of Endothelial Permeability in Murine Atherosclerosis Using an Albumin-Binding Contrast Agent

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Background—Endothelial dysfunction promotes atherosclerosis and precedes acute cardiovascular events. We investigated whether in vivo magnetic resonance imaging with the use of an albumin-binding contrast agent, gadofosveset, could detect endothelial damage associated with atherosclerosis in apolipoprotein E–deficient (ApoE $$^{-/-}$$) mice. Furthermore, we tested whether magnetic resonance imaging could noninvasively assess endothelial function by measuring the endothelial-dependent vasodilation in response to acetylcholine.

Methods and Results—ApoE $$^{-/-}$$ mice were imaged at 4, 8, and 12 weeks after commencement of a high-fat diet. Statin-treated ApoE $$^{-/-}$$ mice were scanned after 12 weeks of a high-fat diet. Wild-type mice were imaged before and 48 hours after injection of Russell’s viper venom, an endothelial toxin. Delayed enhancement magnetic resonance imaging and T1 mapping of the brachiocephalic artery, 30 minutes after injection of gadofosveset, showed increased vessel wall enhancement and relaxation rate ($$R_1$$) with progression of atherosclerosis in ApoE $$^{-/-}$$ ($$R_1$$ [s$$^{-1}$$]: $$R_1$$ weeks 2.42±0.35, $$R_1$$ weeks 3.45±0.54, $$R_1$$ weeks 3.83±0.52) and Russell’s viper venom–injected wild-type mice ($$R_1$$=4.57±0.86). Conversely, wild-type ($$R_1$$=2.15±0.34) and statin-treated ApoE $$^{-/-}$$ ($$R_1$$=3.0±0.65) mice showed less enhancement. Uptake of gadofosveset correlated with Evans blue staining, morphological changes of endothelial cells, and widening of the cell-cell junctions, suggesting that uptake occurs in regions of increased vascular permeability. Endothelial-dependent vasomotor responses showed vasoconstriction of the arteries of the ApoE $$^{-/-}$$ (−22.22±7.95%) and Russell’s viper venom–injected (−10.37±17.60%) mice compared with wild-type mice (32.45±12.35%). Statin treatment improved endothelium morphology and function (−8.12±8.22%).

Conclusions—We demonstrate the noninvasive assessment of endothelial permeability and function with the use of an albumin-binding magnetic resonance contrast agent. Blood albumin leakage could be a surrogate marker for the in vivo evaluation of interventions that aim to restore the endothelium. (Circulation. 2012;126:707-719.)

Key Words: atherosclerosis ■ endothelial dysfunction ■ gadofosveset ■ magnetic resonance imaging ■ permeability

Atherosclerosis is a chronic disease of the vessels and a major cause of death in Western societies. Dysfunction of the vascular endothelium triggers leukocyte transmigration, platelet activation, smooth muscle cell proliferation, and vasoconstriction, which collectively promote the development of atherosclerosis.1 Additionally, damaged endothelium can precipitate the complications of atherosclerosis through vasospasm and thrombosis, causing life-threatening cardiovascular events.

Clinical Perspective on p 719

Transport across the normal endothelium occurs between endothelial cells (ECs) (intercellular pathway) and/or through the ECs (transcytosis). Intercellular junctions with a diameter of ≈2 nm allow transport of small water-soluble molecules up to that diameter,2 whereas breaks in the tight junctions with a diameter of ≈20 nm3-4 accommodate the influx of albumin (diameter of ≈6 nm) and Evans blue dye (EBD).

Received January 10, 2012; accepted June 22, 2012.

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The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.112.092098/-/DC1.

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Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.112.092098
However, under pathological conditions and with aging, open/leaky junctions (diameter >25 nm) increase endothelial permeability and allow access to all solutes and low-density lipoprotein particles and transmigration of leukocytes that are critical in atherosclerotic plaque formation. Hypercholesterolemia changes the types of endothelial junctions, increases endothelial permeability, and alters EC morphology. At the molecular level, oxidized low-density lipoprotein (1) decreases nitric oxide availability, (2) increases endothelin-1 expression, and (3) increases the production of reactive oxygen species. Functionally, endothelial damage is associated with a paradoxical vasoconstriction in response to acetylcholine in both humans and apolipoprotein E–deficient (ApoE−/−) mice and precedes angiographic evidence of coronary artery disease.

Magnetic resonance imaging (MRI) is a noninvasive imaging modality that offers high spatial resolution for the evaluation of the vessel wall. MRI of atherosclerosis has improved significantly with the use of gadolinium-DTPA, whereas targeted contrast agents allowed imaging of biological processes. Recently, MRI has also been used to assess endothelial function. Gadofosveset is a clinically approved gadolinium-based contrast agent that reversibly binds to serum albumin, resulting in a prolonged circulation half-life (r1). Gadofosveset is predominantly present within the neovessels and mechanically damaged endothelium. Although it has long been known that endothelial dysfunction is the primary event in atherogenesis, noninvasive assessment of endothelial permeability has not yet been established in vivo. We therefore sought to investigate whether contrast-enhanced in vivo MRI with the use of gadofosveset could detect changes in endothelial permeability at different stages of atherosclerosis and in response to statin therapy in high-fat diet (HFD)–fed ApoE−/− mice in the absence of neovascularization or mechanical endothelial damage. Furthermore, we investigated whether increased endothelial permeability is associated with local endothelial dysfunction as measured by the endothelial-dependent vasodilation in response to acetylcholine with the use of in vivo cine MRI.

Methods

Animals

Homozygous male ApoE−/− mice (B6.129P2–ApoE10132c/J) and wild-type (WT) C57BL/6J were purchased from Charles Rivers Laboratories (Edinburgh, UK). Eight-week-old old ApoE−/− mice were switched to a HFD containing 21% fat from lard and 0.15% (wt/wt) cholesterol (Special Diets Services, Witham, UK). Three groups of ApoE−/− mice were imaged at 4, 8, and 12 weeks after commencement of the HFD (n=8 per group) before and 30 minutes after injection of gadofosveset (0.03 mmol/kg). In the treatment group, ApoE−/− mice received pravastatin (40 mg/kg per day) (Kemprotec Ltd, Middlesbrough, UK) administered in the drinking water simultaneously with the HFD and for 12 weeks (n=8). WT mice were fed a normal chow diet for 12 weeks (n=8). A subgroup of WT mice was injected with Russell’s viper venom (RVV) (0.15 mg/kg IP; Enzyme Research Laboratories, Swansea, UK) twice within 24 hours (n=8). These animals were imaged 48 hours after the first injection. RVV is an endothelial toxin and was used to induce endothelial damage in the absence of atherosclerosis. Additional control mice fed a normal chow and statin-treated and untreated ApoE−/− mice fed a HFD for 12 weeks were used to test the effects of a high dose (0.84 mmol/kg) of gadofosveset (n=6 per group). Each animal was imaged at a single time point, and ex vivo tissues were used for additional analysis. The experimental protocol is illustrated in Figure I in the online-only Data Supplement. All procedures used in these studies were performed in accordance with the guidelines of the UK home office.

In Vivo MRI Protocol at 3 T

In vivo vessel wall imaging was performed with the use of a Philips Achieva MR scanner (Philips Healthcare, Best, Netherlands) equipped with a clinical gradient system (30 mT/m, 200 mT/m per millisecond) and a single-loop surface coil (diameter=23 mm). Mice were imaged in the prone position before and 30 minutes after intravenous administration of 0.03 mmol/kg gadofosveset (Bayer Schering Pharma AG, Berlin, Germany). Additionally, control and 12-week HFD-fed ApoE−/− mice (n=3 per group) were imaged 50 minutes after intravenous administration of a covalently bound albumin-gadolinium contrast agent (4 mL/kg; Galbumin, BioPal, Worcester, MA). Anesthesia was induced with 5% isoflurane and maintained with 1% to 2% isoflurane during the MRI experiments.

After a 3-dimensional gradient recalled echo scout scan, contrast-enhanced angiography images were acquired for visualization of the aortic arch and the brachiocephalic and carotid arteries, with a field of view (FOV)=30×30×8 mm, matrix=200×200, in-plane resolution=0.15×0.15 mm (reconstructed=0.10×0.10 mm), slice thickness=0.5 mm, repetition time/echo time=15/6.1 ms, and flip angle=40°. The maximum intensity projection images were used to plan the subsequent delayed-enhancement (DE) and T1 mapping scans. A 2-dimensional Look-Locker sequence planned perpendicular to the ascending aorta was used to determine the optimal inversion time for blood signal nulling. Acquisition parameters were as follows: FOV=30×30 mm, matrix=80×80, in-plane resolution=0.38×0.38 mm, slice thickness=2 mm, repetition time/echo time=19/8.6 ms, repetition time between subsequent inversion recovery pulses=1000 ms, and flip angle=10°. An inversion recovery 3-dimensional fast gradient echo sequence was acquired 30 minutes after injection and was used for DE MRI and visualization of contrast uptake. Imaging parameters were as follows: FOV=30×30×8 mm, matrix=304×304, in-plane resolution=0.1×0.1 mm, measured slice thickness=0.5 mm, slices=32, repetition time/echo time=28/8 ms, repetition time between subsequent inversion recovery pulses=1000 ms, and flip angle=30°. T1 mapping was performed with the use of a sequence that employs 2 nonselective inversion pulses with inversion times ranging from 20 to 2000 ms, followed by 8 segmented readouts for 8 individual images. The 2 imaging trains resulted in a set of 16 images per slice with increasing inversion times. For T1 mapping, the acquisition parameters were as follows: FOV=36×22×8 mm, matrix=192×102, in-plane resolution=0.18×0.22 mm, measured slice thickness=0.5 mm, slices=16, repetition time/echo time=9.64/9.4 ms, flip angle=10°. T1 values were computed on a pixel-by-pixel basis with the use of in-house Matlab software.

Vessel wall area was calculated by manually segmenting the visually enhanced region of the vessel wall as seen on the DE MRI images with the use of OsiriX (OsiriX Foundation, Geneva, Switzerland). To ensure that the segmented area encompassed the vessel wall, the DE MRI images were coregistered and fused with the MR angiography images.

In Vivo MRI Protocol at 7 T

WT mice were fed a normal Chow diet, whereas ApoE−/− mice were fed a HFD for 12 weeks. A subgroup of ApoE−/− mice was treated with statins as described above. Cardiovascular MR images (n=6 per group) were acquired before and 20 to 30 minutes after intraperitoneal injection of acetylcholine (50 μL from a stock solution of 10 mg/mL; 16.6 mg/kg) to test the vasomotor responses of the vessels. Cardiovascular MR was performed on a horizontal MR scanner (Varian Inc, Palo Alto, CA) with mice positioned in the prone position.
position. The gradient coil had an inner diameter of 12 cm, a gradient strength of 1000 mT/m, and a rise time of 120 μs. A quadrature transmit/receive coil (RAPID Biomedical GmbH, Würzburg, Germany) with an internal diameter of 39 mm was used. Anesthesia was achieved as described above, and body temperature was maintained at 37°C with the use of a warm air fan (SA Instruments, Stony Brook, NY). ECG gating was achieved via 2 metallic needles placed subcutaneously in the front paws, and a pressure transducer was placed on the abdomen for respiratory gating (SA Instruments). Temporally resolved dynamic short-axis images of the brachiocephalic and carotid arteries were acquired with a cine gradient echo sequence. For the brachiocephalic artery, both ECG triggering and respiratory gating were used, whereas for the carotid arteries, only ECG gating was used to compensate for motion artifacts. Imaging parameters included the following: repetition time=RR interval/number of frames (typically ~10 ms), echo time=1 ms, FOV=25×25 mm, matrix size=192×192, in-plane resolution=0.13×0.13 mm, slice thickness=1 mm, flip angle=40°, averages=3, slices=1, 1 k-space line per frame, 8 to 12 frames per cardiac cycle depending on the length of the RR interval, and scan time=8 to 0.5 minutes. Heart rates varied between 500 and 600 bpm. Respiration rate varied between 80 and 50 cycles per minute. Images were analyzed with the use of semi-automated in-house software programmed in C++. Pixels encompassing the blood pool were clustered on the basis of their signal intensity, and the vessel wall borders of the end-diastolic and end-systolic phases were defined.

**Plasma Analysis**

Blood was collected by cardiac puncture in citrate-coated tubes, and plasma was separated by centrifugation at 1500 rpm for 15 minutes. Quantification of albumin levels was performed with the use of a mouse albumin enzyme-linked immunosorbent assay kit (Benthyl Laboratories Inc, Cambridge, UK).

**Histology**

**Tissue Harvesting**

For all histological procedures, mice were anesthetized with isoflurane and perfused through the left ventricle with either physiological saline (for electron microscopic studies) or 10% formaldehyde (for Evans blue staining and immunohistochemistry) for 10 minutes. Finally, mice were cuffed with a ketamine/xylazine overdose.

**Evans Blue Staining**

Vascular permeability was assessed by visualizing and quantifying the leakage of EBD into the vascular wall. EBD was extracted by incubation in formamide for 24 hours at 60°C, and the absorbance was measured at 620 nm. Standard curves for pure EBD were used to calculate the total amount of dye in the tissue (n=3 per group).

**Immunohistochemistry**

The brachiocephalic artery together with the right subclavian artery, the aortic arch, and the carotid arteries were removed en block and immediately transferred in tissue freezing medium for cryosectioning. Three individual animals were analyzed at each time point. Transverse sections (5 μm thick) were collected at 4 locations along the brachiocephalic artery starting from the left subclavian artery to the aortic arch at every 200-μm interval. Three sections were analyzed at each location, and a total of 12 sections per animals were included in the analysis. The aortic arch and the subclavian artery were used as internal landmarks for coregistration between histological sections and MR images. The left carotid artery was used as a plaque-free segment. Immunohistochemistry was performed with the avenin-biotin-peroxidase method (Vector Laboratories, Burlingame, CA). Anti-mouse goat polyclonal antibody for albumin (1:50, Abcam, Cambridge, MA) and rat monoclonal antibody for CD31 (1:50, BD Pharmingen, Oxford, UK) were used. Immunopositive areas were analyzed on digital images by computerized planimetry with the use of ImageJ (National Institutes of Health). The immunopositive area was segmented on the images and expressed as a percentage of the total vessel wall area.

**Transmission Electron Microscopy**

Brachiocephalic and left carotid arteries (n=2 per group) were pinned down and fixed in 2% gluteraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) for 2 hours, washed with sodium phosphate buffer for 2 hours, and postfixed in 1% OsO4 for 2 hours. Each brachiocephalic artery was divided into 3 segments starting from the subclavian bifurcation to the aortic arch and was processed for histological analysis. Subsequently, 3 to 4 sections were examined from each block. Therefore, 9 to 12 sections were analyzed per animal. Samples were dehydrated through a graded series of ethanol and embedded in epoxy resin. Semithin sections (0.2 μm) were stained with toluidine blue for light microscopy examinations and were used to guide sampling for transmission electron microscopy studies. Thin sections (0.09 μm) were collected on 150-mesh copper grids and double stained with uranyl acetate and lead citrate for electron microscopy examinations (H7650, Hitachi, Tokyo, Japan). Tight junction width was measured on electron microscopy sections cut perpendicular to the long axis of the vessel wall with the use of ImageJ (National Institutes of Health). Nine to 12 measurements were performed in each sample, and the data points were averaged for each animal group.

**Electron Probe X-Ray Microanalysis**

Brachiocephalic arteries were prepared by cryofixation under liquid nitrogen (n=2 at 12 weeks of HFD). Cryosections were cut at −120°C, transferred to Pioloform-coated nickel grids, and freeze-dried overnight. The sections were coated with a thin layer of carbon and viewed and analyzed in an FEI Tecnai 12 electron microscope equipped with an EDAX energy-dispersive x-ray spectroscopy detector. Mapping was achieved with EDAX software (EDAX).

**Inductively Coupled Plasma Mass Spectrometry**

Gadolinium concentrations were determined by inductively coupled plasma. The aortic arch with the brachiocephalic artery was collected and snap-frozen in liquid nitrogen (n=3 per group). Before inductively coupled plasma mass spectrometry, the samples were digested in 300 μL 70% nitric acid overnight at room temperature, followed by dilution in 1 mL of deionized water.

**Western Blot**

Brachiocephalic arteries were washed in ice-cold phosphate-buffered saline, cleaned of adhering tissue, and stored at ~80°C (n=3 per group). Before immunoblotting, the samples were weighed and solubilized in 0.5 mL lysis containing 8 mL L thiourea 2 mol/L, Tris-HCl 0.05 mol/L, dithiothreitol 0.075 mol/L, 2% sodium dodecyl sulfate, and 0.05% bromophenol blue, pH 6.8. Samples were boiled for 10 minutes and centrifuged for 15 minutes at 14,000g at 4°C to remove insoluble materials. Protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad, No. 23227), and 10 μg total protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and subjected to immunoblotting. Primary antibodies for albumin (1:5000; Abcam No. 19194) and GAPDH (1:1000; Cell Signaling No. 2118) were detected by anti-goat and anti-rabbit secondary antibodies, respectively, linked to horseradish peroxidase. Specific protein bands were detected by enhanced chemiluminescence.

**Statistical Analysis**

The Statistical Package for the Social Sciences 18.0 (SPSS Inc, Chicago, IL) was used. Two-group comparisons of continuous variables were performed with a Student t test (unpaired, 2-tailed) or a Mann-Whitney nonparametric test after the variables were ranked. In cases of >2 groups, statistical comparisons were performed by 1-way ANOVA followed by the Bonferroni post hoc test. A trend analysis for control and for 4, 8, and 12 weeks of a HFD was
performed with a Jonckheere trend test. The data are presented as mean±SD. P values <0.05 were used to define statistical significance.

**Results**

**Pharmacokinetics of Gadofosveset in ApoE−/− Mice Fed a HFD for 12 Weeks**

The pharmacokinetics of gadofosveset in mice was investigated to determine the optimum imaging time point for vessel wall imaging. The relaxation rate (R₁ = 1/T₁) of the brachiocephalic artery and blood peaked at 30 minutes after injection of gadofosveset and returned to baseline at 4.5 and 8 hours, respectively (Figure 1). The maximum difference in R₁ between blood and vessel wall occurred at 30 minutes. Thus, DE MRI was performed 30 minutes after injection.

**Gadofosveset Uptake Correlates With Endothelial Permeability and Atherosclerosis Progression**

On postcontrast DE MRI, a greater enhancement of the brachiocephalic artery of ApoE−/− mice exposed to a HFD was observed compared with WT mice (Figure 2A1 through 2A2). Pravastatin-treated ApoE−/− mice showed decreased uptake compared with untreated ApoE−/− mice (Figure 2E1 and 2E2). Interestingly, vessel wall enhancement was also observed in WT mice treated with RVV compared with untreated WT mice (Figure 2F1 and 2F2). Similar findings were observed on R₁ maps that were used to quantify uptake of gadofosveset within the vessel wall (Figure 2A3 through 2A4). Consistently, EBD staining (Figure 2B1 and 2B2) showed a localized and increased uptake of the dye during atherosclerosis progression. Furthermore, extravasation of gadofosveset resulted in a time-dependent increase of the vessel wall area (Figure II in the online-only Data Supplement).

Figure 3 illustrates a quantitative assessment of the findings described in Figure 2. The contrast-enhanced area of the vessel wall (Figure 3A), EBD uptake (Figure 3B), and R₁ (Figure 3C) gradually increased with progression of atherosclerosis in ApoE−/− mice and after RVV treatment in plaque-free WT mice. Conversely, these measurements decreased in the pravastatin-treated group and in WT animals. There was a trend for a linear increase of the contrast-enhanced vessel wall area (P<0.001), EBD (P<0.001), and R₁ (P<0.001) when animals were kept on a HFD for a longer duration. Correlation between the vessel wall R₁ and gadolinium concentration (Figure 3D), as determined by inductively coupled plasma mass spectrometry, revealed a relaxivity r₁=17.95 mmol/L per second, which is closer to that reported for the bound fraction of gadofosveset.

The association of gadofosveset and EBD uptake with disease progression is illustrated in Figure III in the online-only Data Supplement. Our data show that there is a good correlation between the plaque area calculated on the DE MR images and the plaque area calculated histologically, suggesting that the extent of vessel wall enhancement reflects the atherosclerotic burden. Furthermore, there was a good correlation between the DE MRI area and the vessel wall concentration of gadofosveset as measured by inductively coupled plasma, the R₁ relaxation rate, and the concentration of EBD.

**Hypercholesterolemia and RVV Damage of ECs and Their Tight Junctions**

To understand the mechanism of gadofosveset uptake, the fine structure of the ECs and their tight junctions was studied by electron microscopy. Normal ECs from WT mice were attached to the basal membrane and appeared flattened and elongated, with a prominent glycoalyx on the luminal side and smooth contours (Figure 4A). Conversely, in HFD-fed ApoE−/− mice, the ECs appeared cuboidal and swollen with increased lysosomal-like inclusions and mitochondria (Figure 4B), cytoplasmic extensions on the luminal side, and vacuoles occupying large areas of the cytoplasm (Figure 4C). In more advanced stages, dead ECs were detached from the basal membrane, lacked a nucleus, and had an electron translucent cytoplasm (Figure 4D). Denuded regions and exposure of the subendothelial space were also observed (Figure 4E). Interestingly, statin treatment appeared to preserve the endothelial lining and restore or delay the effects of hyperlipidemia on the morphology of the endothelium. Extremely elongated ECs (Figure 4F) and ECs that exhibited increased thickness, luminal projections (microvilli), and pseudopods (Figure 4G) were observed. RVV-treated mice exhibited signs of EC necrosis with condensed nucleus, clear perinuclear cytoplasm, and desquamation (Figure 4H). Dying ECs and vascular denudation were also observed (Figure 4I). Examination of the endothelial junctions revealed widening of the cell-cell junctions with atherosclerosis progression and RVV treatment. In WT mice, adjacent ECs had considerable overlap and narrow junctions (22±4 nm) (Figure 4J). Conversely,
“open” junctions were observed in ApoE−/− mice (57±8, 1600±45, 2400±52 nm at 4, 8, 12 weeks of HFD, respectively) (Figure 4K). Interestingly, statin-treated animals showed preservation of the EC overlap and narrower junctions (65±5 nm) (Figure 4L) compared with the untreated animals. Despite the absence of atherosclerosis, RVV injection damaged the endothelial junctions that appeared open (620±31 nm) (Figure 4L).

The changes in the tight junction width observed in different animal groups are summarized in Figure 4N.

**Figure 2.** Uptake of gadofosveset correlates with endothelial permeability and plaque progression. A1 through F1 and A2 through F2. Cross-sectional delayed-enhancement magnetic resonance imaging (DE-MRI) and DE-MRI fused with magnetic resonance angiography images of the brachiocephalic artery. Apolipoprotein E–deficient mice on a high-fat diet (HFD) show a gradual increase of vessel wall enhancement corresponding to plaque progression, whereas statin-treated apolipoprotein E–deficient mice show less enhancement. Wild-type mice injected with Russell’s viper venom also show increase enhancement compared with noninjected WT mice. A3 through F3. Corresponding relaxation rate (R1) maps quantify the amount of gadofosveset within the vessel wall. Intense yellow signal indicates increased gadofosveset concentration. A4 through F4. In situ Evans blue staining shows increased endothelial leakage in regions of DE-MRI with the use of gadofosveset. n=8 per group for the MRI experiments and n=3 per group for the Evans blue dye staining.

**Vessel Wall and Plasma Albumin Measurements**

Immunopositive areas of albumin were observed in the brachiocephalic artery of WT mice. The distribution across the vessel wall showed low intensities in the subendothelial space, lower intensities in the media, and high intensities in the adventitia (Figure 5A and 5B). In ApoE−/− mice, albumin was present within the plaque, whereas the intensities and extent of the immunopositive areas in the other regions of the vessel wall were increased compared with the WT arteries.
The percent immunopositive area for albumin observed in different groups is illustrated in Figure 5E. Vessel wall albumin significantly increased in ApoE−/− mice after 8 and 12 weeks of HFD (48.93 ± 3.38% and 56.30 ± 9.18%, respectively) compared with mice fed a HFD for a shorter duration and WT mice (30.52 ± 3.56%). Vessel wall albumin was also increased in RVV-treated WT mice (50.98 ± 4.65%) compared with untreated WT animals (30.52 ± 3.56%), indicating increased vascular leakage. Conversely, vessel wall albumin was decreased in statin-treated ApoE−/− mice (34.0 ± 2.82%) compared with the untreated mice fed a HFD for 12 weeks. There was an increasing trend of the percent albumin (P < 0.01) when animals were kept on a HFD for a longer duration.

Similar findings were observed when the amount of vessel wall albumin was measured by Western blotting (Figure 5E). Vessel wall albumin significantly increased in ApoE−/− mice after 8 and 12 weeks of HFD (48.93 ± 3.38% and 56.30 ± 9.18%, respectively) compared with mice fed a HFD for a shorter duration and WT mice (30.52 ± 3.56%). Vessel wall albumin was also increased in RVV-treated WT mice (50.98 ± 4.65%) compared with untreated WT animals (30.52 ± 3.56%), indicating increased vascular leakage. Conversely, vessel wall albumin was decreased in statin-treated ApoE−/− mice (34.0 ± 2.82%) compared with the untreated mice fed a HFD for 12 weeks. There was an increasing trend of the percent albumin (P < 0.01) when animals were kept on a HFD for a longer duration.

In Vivo MRI Assessment of Endothelial Function
To investigate whether the observed morphological alterations of the ECs were accompanied by changes in their function, the endothelial vasodilating properties were assessed by comparing the end-diastolic vessel diameter before and after administration of acetylcholine. The vasculature and end-systolic and end-diastolic images of a WT mouse are illustrated in Figure 6A through 6C. The brachiocephalic artery of WT mice showed the expected vasodilation in response to acetylcholine (32.45 ± 12.35%), whereas the atherosclerotic brachiocephalic artery of HFD-fed ApoE−/− mice showed vasoconstriction (−22.22 ± 7.95%) (Figure 6D). Quantitative changes in vessel wall diameter observed in different animal groups are summarized in Figure 6E. The vasodilating changes in response to acetylcholine were also measured in the left carotid artery (LCA). In WT mice, the LCA showed vasodilation (18.18 ± 8.9%). However, the LCA of ApoE−/− mice showed less vasodilation (4.0 ± 15.5%) compared with WT mice despite the absence of atherosclerosis. Electron microscopic studies showed that initial morphological changes occurred in the ECs of the LCA in ApoE−/− mice (Figure IV in the online-only Data Supple-
ment) compared with WT mice, suggesting that endothelial morphological and functional changes may precede the presence of atherosclerosis. Moreover, statin-treated ApoE−/− mice showed less vasoconstriction in the brachiocephalic artery (\(8.12\%\) compared with untreated mice, suggesting that statins may improve EC function. Conversely, WT mice injected with RVV showed more vasoconstriction (\(10.37\%\) for the brachiocephalic artery and \(7.11\%\) for the LCA), verifying that morphological changes in the ECs (Figure 5H and 5I) are accompanied by alteration of their function even in the absence of atherosclerosis.

**Higher Dose of Gadofosveset Increases the Gadolinium Concentration in the Vessel Wall Without Proportional Increase of the Relaxation Rate (R1)**

To decipher the contribution of the bound and free fractions of gadofosveset to the signal enhancement of the vessel wall, the tissue gadolinium concentration and the corresponding R1 were compared between the clinical dose (0.03 mmol/kg, 0.5 mmol/L in blood, \(\approx 75\%_{\text{bound}}: 25\%_{\text{free}}\)) and a higher dose of gadofosveset (0.84 mmol/kg, 12.5 mmol/L in blood, \(\approx 50\%_{\text{bound}}: 50\%_{\text{free}}\)). Increasing the free fraction of gadofosveset in blood resulted in a statistically significant increase in the gadolinium concentration measured in the brachiocephalic artery with a more prominent increase in ApoE−/− mice (control: 13.34 ± 0.8–217 ± 34.3 μmol/L; 12 weeks of HFD: 189.5 ± 4.96–2796 ± 34.3 μmol/L; statin-treated: 34.12 ± 0.66–580 ± 16.83 μmol/L) (Figure 7A). However, the increase in R1 was not proportional to that of the gadolinium concentration (control: 2.20±0.3–2.89±0.25 s⁻¹; 12 weeks of HFD: 4.15±0.65–5.93±2.25 s⁻¹; statin-treated: 3.15±0.47–3.72±0.92 s⁻¹) (Figure 7B). This observation was better demonstrated when the fold increase in gadolinium concentration and the corresponding R1 were compared (Figure 7C). Although there was a 13- to 15-fold increase in gadofosveset concentration in the vessel wall, the R1 increased only up to 2-fold (Figure 7C).
Imaging With a Covalently Bound Albumin-Gadolinium Agent Shows Higher Uptake In Diseased Compared With Nondiseased Vessel Wall

To further understand the mechanism underlying the uptake of gadofosveset and vessel wall enhancement, we performed imaging experiments using a covalently bound albumin-gadolinium agent (Galbumin) to ensure the presence of only the albumin-bound fraction of the contrast agent and the absence of the free fraction in plaque (Figure 8). These data showed a higher vessel wall enhancement as seen on DE MRI (Figure 8A through 8F), a higher R₁ relaxation rate (Figure 8G), and higher gadolinium concentration as measured by inductively coupled plasma mass spectrometry in the diseased compared with the control vessel wall (Figure 8H).

Discussion

Although it has long been known that endothelial dysfunction is the primary event in atherogenesis, noninvasive assessment of endothelial permeability has not yet been established in vivo. In the present study, we assessed EC permeability and function associated with atherosclerosis in ApoE⁻/⁻ mice by...
in vivo MRI. We found that signal enhancement, after injection of gadofosveset, correlated with atherosclerosis progression in the brachiocephalic artery (BCA) and carotid arteries in a wild-type (WT) mouse. Conversely, WT mice and statin-treated ApoE–/– mice showed less uptake of gadofosveset. The uptake of gadofosveset was similar, both regionally and quantitatively, to that of EBD. Several studies have validated the use of EBD in assessing EC permeability in normal and injured vessel walls.37,38 Consistent with previous investigations, we found a range of morphological changes of ECs and widening of cell-cell junctions, but we did not observe CD31-positive neovessels39,40 in ApoE–/– mice fed a HFD for up to 12 weeks. Thus, endothelial damage alone appears sufficient to lead to gadofosveset uptake in the vessel of this animal model. In addition, we showed the ability of MRI to directly assess endothelial function by measuring the endothelial-dependent vasomotor responses to acetylcholine. We found that atherosclerotic vessels in ApoE–/– mice showed vasoconstriction, whereas nondiseased arteries in WT mice showed vasodilation. Conversely, statin-treated ApoE–/– mice showed improved endothelial function.

Previous studies suggested that extravasation of gadofosveset in experimental32 and human atherosclerosis33 occurs through leaky microvessels. However, in these studies, a detailed analysis of the endothelium was not performed. Recently, it was shown that gadofosveset enhancement also occurred in injured swine coronary arteries that lack neovessels, indicating that mechanical endothelial damage alone can cause extravasation of gadofosveset.34 Using transmission electron microscopy, we found that disease progression was accompanied by EC death, vascular denudation, and widening of the tight junctions. Collectively, those changes could increase vascular permeability to blood constituents and gadofosveset, leading to plaque progression and contrast enhancement, respectively. The changes we observed in endothelial morphology involved a change to a cuboidal shape, the presence of cytoplasmic extensions, cytoplasmic vacuolation, thinning of the glycocalyx, and an increased number of lysosome-like inclusions. The glycocalyx is important for regulating permeability, filtration, and cell adhesion, whereas cytoplasmic vacuolation represents an adaptive response, presumably for “damage limitation.”42 Vacuoles grow in number and size, and up to a certain threshold cells continue to function normally. We also found more severe endothelial damage characterized by dead ECs with an electron translucent cytoplasm, lack of nu-
cleus, detachment from the basal membrane, and open junctions with disease progression. Similar changes have been observed in other animal models and in human atherosclerosis.4,7,9,10

Consistent with previous reports, treatment with pravastatin attenuated endothelial damage in ApoE<sup>−/−</sup> mice, leading to a decreased uptake of gadofosveset and EBD, and improved the response to acetylcholine. Although statins reduce cardiovascular events by lowering plasma cholesterol levels, there is increasing evidence that they exert cholesterol-independent or pleiotropic vascular effects, including improvement of endothelial function,43,44 by increasing the bioavailability of nitric oxide, stimulating reendothelialization, and reducing EC death.45

The presence of an albumin-bound and a free fraction of gadofosveset in blood, both of which are small enough to leak through breaks in the endothelial junctions, raised the question of which fraction of gadofosveset generated the observed MRI signal. In our study, the combination of a T1 mapping sequence and inductively coupled plasma mass spectrometry showed that the relaxivity of gadolinium in the vessel wall (r<sub>1</sub> bound ≈ 17 mmol/L per second) was closer to that reported for the albumin-bound fraction (r<sub>1</sub> bound ≈ 25 mmol/L per second versus r<sub>1</sub> free ≈ 6.6 mmol/L per second),36 suggesting that gadofosveset primarily exists in the vessel wall in a bound form. The use of a higher dose of gadofosveset, which increased the free fraction from 20% to 50%, resulted in a significant fold increase (13–15 times) in gadolinium concent-

**Figure 7.** In vivo magnetic resonance imaging using a higher dose of gadofosveset compared with the clinical dose. A, Administration of a higher dose of gadofosveset significantly increased the gadolinium concentration in the brachiocephalic artery. B, The relaxation rate (R<sub>1</sub>) was increased, but the increase was not always significant. C, The fold change in gadolinium concentration and R<sub>1</sub> revealed that increasing the circulating levels of free gadofosveset results in a higher total concentration in the vessel wall, but it does not proportionally increase the R<sub>1</sub>. n=5 per group. ICP-MS indicates inductively coupled plasma mass spectrometry; HFD, high-fat diet.

**Figure 8.** In vivo magnetic resonance imaging using a covalently bound albumin-gadolinium agent (Galbumin) shows higher uptake in diseased compared with nondiseased vessel wall. A and B and D and E, Delayed-enhancement magnetic resonance imaging (DE-MRI) and fused magnetic resonance angiography and DE-MRI images show a higher uptake of Galbumin in the brachiocephalic artery of diseased compared with control mice. C and F, Corresponding vessel wall relaxation rate (R<sub>1</sub>) maps. G, Quantification of the vessel wall R<sub>1</sub>, n=3 per group. HFD indicates high-fat diet. H, Quantification of gadolinium concentration as measured by inductively coupled plasma mass spectrometry (ICP-MS).
tration in the vessel wall compared with the clinical dose for both control and diseased animals. However, the $R_1$ of the tissue was increased only by 1.2- to 1.6-fold despite the increased intravessel wall albumin measured in the brachio-cephalic artery of HFD-fed ApoE$^{-/-}$ mice. Three possible mechanisms may lead to gadofosveset uptake in the vessel wall: (1) unbound gadofosveset moves into the vessel wall; (2) unbound gadofosveset moves into the vessel wall and binds to intraplaque albumin; and (3) bound gadofosveset moves across disrupted endothelium into the vessel wall. Our experiments using the low and high doses of gadofosveset suggest that all 3 mechanisms of uptake are possible. The small change in vessel wall $R_1$, despite the significantly higher increase of gadolinium concentration, observed in the dose escalation experiment cannot exclude the possibility that unbound gadofosveset moves into the vessel wall and binds to intraplaque albumin. It may, however, suggest a saturation of the intraplaque albumin pool at the lower dose so that the higher dose has little additional effect on vessel wall $R_1$. Moreover, when the higher dose of gadofosveset was used, the vessel wall relaxivity $r_1$ decreased to $\approx 2.2$ mmol/L per second, further suggesting that the excess unbound gadolinium present in the vessel wall does not significantly contribute to the DE effect. Interestingly, the use of a contrast agent in which albumin is covalently and irreversibly bound to gadolinium and that lacks the free/unbound fraction showed higher uptake in the diseased compared with control vessel walls and further reinforced the hypothesis that albumin leakage in the vessel wall can reflect endothelial permeability and be an indirect measure for gap junction width. Collectively, these data suggest that gadofosveset, used at the clinical dose, might be a suitable contrast agent for the noninvasive assessment of endothelial permeability because albumin\(^46\) and albumin-bound dyes\(^37,38\) have been commonly used in permeability studies.

Although contrast enhancement with the use of nonspecific compounds, which rapidly extravasate into the extravascular space, has improved the detection of atherosclerosis, contrast uptake may be related to multiple factors including increased endothelial permeability,\(^34\) increased distribution volume (tissue necrosis, fibrosis, inflammation), and neovascularization.\(^17-21\) Although applicable in a clinical setting, current contrast agents provide only indirect and limited information on pathological processes in the vessel wall and yield limited signal strength. Interestingly, a previous study performed in our laboratory\(^26\) showed little uptake of gadolinium-DTPA (Magnevist, Bayer Pharma AG, Berlin, Germany) in the brachiocephalic artery of the HFD-fed ApoE$^{-/-}$ mouse model. Little to no visually apparent enhancement in the normal or atherosclerotic vessel wall, low $R_1$ values, and low gadolinium concentrations were observed for all time points after commencement of the HFD and for different time intervals after injection of the contrast agent. For these reasons, the experiments in which a conventional small gadolinium chelate was used were not repeated in our present study. These findings also suggest that in this mouse model, gadolinium-DTPA and the unbound fraction of gadofosveset do not behave similarly, probably because unbound gadofosveset may bind to intraplaque albumin, whereas gadolinium-DTPA does not. The reason why mouse plaques do not show enhancement with gadolinium-DTPA remains unknown, and further investigations are needed to elucidate the underlying differences between murine atherosclerosis compared with other species, which could account for such an effect.

Clinical assessment of endothelial function provides important prognostic information for future cardiovascular events.\(^47,48\) However, direct clinical assessment of endothelial function has been achieved mainly with the use of invasive methods, including angiography\(^11\) and intracoronary Doppler ultrasound,\(^49\) whereas noninvasive assessment with the use of venous occlusion plethysmography and high-resolution ultrasound\(^50\) is limited to the study of peripheral arteries. Only recently has MRI been used to assess coronary endothelial vasomotion in subjects with coronary artery disease.\(^27-29\)

In the present study, we showed that endothelial damage was accompanied by an impaired endothelial-dependent vasodilation in response to acetylcholine. Both the atherosclerotic ApoE$^{-/-}$ mice and WT animals injected with RVV showed vasoconstriction compared with uninjected WT animals that showed vasodilation. The vasomotor response of the endothelium was improved in statin-treated ApoE$^{-/-}$ mice, suggesting a beneficial role of statins on endothelial function.

**Conclusions**

We demonstrate the feasibility of contrast-enhanced MRI with the use of gadofosveset to noninvasively visualize and quantify endothelial permeability and the assessment of endothelial function as measured by cine MRI. This novel approach may provide new insights on the roles of ECs in atherosclerosis and allow detection of early lesions and monitoring of interventions that improve endothelial permeability and/or function and reduce cardiovascular risk. Because gadofosveset is a clinically approved contrast agent, it may allow translation of our findings to patients with cardiovascular disease.

**Acknowledgments**

We thank Dr Alexandros Tratsos for his expertise with molecular biology techniques and useful comments during the preparation of this manuscript.

**Sources of Funding**

This work was funded by British Heart Foundation grant PG/10/044/28343 (to Dr Botnar).

**Disclosures**

None.

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CLINICAL PERSPECTIVE

Despite the systemic and multifactorial nature of atherosclerosis, lesion development is focal and occurs at particular regions of the vasculature including the branches, the inner curvature, and the outer wall of bifurcations, where focal hemodynamic factors exert major damage on the vascular endothelium. Although several imaging modalities are now available for imaging of atherosclerosis, most of the work has focused on identifying anatomic changes associated with disease progression and risk, which have shown low predictive value. Conversely, the combination of imaging structural and functional properties of the vessel wall may allow investigation of the manner in which the extent of local artery disease is related to the degree of local abnormal endothelial function. Therefore, noninvasive physiological imaging of endothelial permeability and function may offer a comprehensive methodology for monitoring focal atherosclerotic progression in both early and later stages, as well as plaque instability, and testing the effectiveness of treatment interventions targeting vascular endothelium integrity and function.
Noninvasive Magnetic Resonance Imaging Evaluation of Endothelial Permeability in Murine Atherosclerosis Using an Albumin-Binding Contrast Agent
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_Circulation_. 2012;126:707-719; originally published online June 29, 2012;
doi: 10.1161/CIRCULATIONAHA.112.092098
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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SUPPLEMENTAL MATERIAL

Supplemental Methods

- Wild-type mice on normal chow
- Native (n=4) followed by contrast-enhanced MRI of control mice (n=8) and mice treated with Viper venom (n=8)
- Collect tissue for histology, ICP-MS, immunoblotting
- apoE−/− mice on high fat diet
- apoE−/− mice on high fat diet and statin treatment (pravastatin 40mg/kg/day)
- WT, WT treated with RVV, ApoE−/− on high-fat diet and ApoE−/− treated with statin
- Native (n=4) followed by contrast-enhanced MRI of apoE−/− mice (n=8)
- Collect tissue for histology, ICP-MS, immunoblotting
- Assessment of endothelial-dependent vasodilation in response to acetylcholine (n=6 per group)

Note: Each animal was imaged only one time

Figure 1. Experimental timeline. WT mice were imaged before and 48h after treatment with RVV (administered twice in 24h). ApoE−/− mice were imaged at 4, 8 and 12 weeks post commencement of the HFD. Statin treated ApoE−/− mice were scanned after 12 weeks of HFD. Images were acquired before and 30min after injection of gadofosveset (0.03mmmol/kg). After the MRI tissues were harvested for *ex vivo* studies. RVV: Russell’s’ viper venom, HFD: high-fat diet.
Figure 2. Bright-blood *in vivo* MRI of the time-depended changes in the vessel wall area after injection of gadofosveset. There is a time-depended increase in the vessel wall area suggestive of extravasation of the contrast agent. This effect was most prominent in the ApoE\(^{-}\) mice fed a high-fat diet for 12 weeks and WT mice treated with Russell’s viper venom suggesting that extravasation of gadofosveset occurs in regions with increased endothelial damage and leakage. N=8 per group.
Figure 3. The association of gadofosveset and EBD uptake with disease progression. A, The area calculated on the contrast-enhanced MR images correlates well with the plaque area calculated on the histological section. B-D, The vessel wall gadolinium concentration, the vessel wall relaxation rate (C), and the concentration of Evan’s blue dye (D) correlate with the area as calculated on the contrast-enhanced MR images. N=8 per group for the MRI experiments, N=3 per group for histology, ICP-MS and EBD.
Figure 4. Morphological changes of the endothelium and uptake of gadofosveset precede plaque development in the left carotid artery. A and F, Toluidine blue staining shows the absence of atherosclerosis. B and G, C and H, Low and high magnification images of CD31 stained vessels. Endothelial cells (brown staining) appear flat and elongated in WT (C) and cuboidal in the ApoE−/− mice (H). D and E, Transmission electron microscopy of a flat and elongated endothelial cell from a WT mouse. I and J, Transmission electron microscopy of the endothelium reveals initial morphological changes in the ApoE−/− mouse despite the absence of atherosclerosis. The cells contain luminal protrusions (I) and large cytoplasmic vacuoles (J). K, The R₁ of the left carotid artery also changes and follows the same pattern as that observed for the brachiocephalic artery. Importantly, the R₁ of the left carotid artery was always lower compared to that of the brachiocephalic artery indicating that gadofosveset uptake
correlated with disease severity. N=8 per group for the MRI experiments, N=3 per group for TEM, and N=3 for immunohistochemistry.