Comparison of Gadofluorine-M and Gd-DTPA for Noninvasive Staging of Atherosclerotic Plaque Stability Using MRI

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Background—Inflammation and neovascularization play critical roles in the stability of atherosclerotic plaques. Whole-body quantitative assessment of these plaque features may improve patient risk-stratification for life-threatening thromboembolic events and direct appropriate intervention. In this report, we determined the utility of the MR contrast agent gadofluorine-M (GdF) for staging plaque stability and compared this to the conventional agent Gd-DTPA.

Methods and Results—Five control and 7 atherosclerotic rabbits were sequentially imaged after administration of Gd-DTPA (0.2 mmol/kg) and GdF (0.1 mmol/kg) using a T1-weighted pulse sequence on a 3-T MRI scanner. Diseased aortic wall could be distinguished from normal wall based on wall-to-muscle contrast-to-noise values after GdF administration. RAM-11 (macrophages) and CD-31 (endothelial cells) immunostaining of MR-matched histological sections revealed that GdF accumulation was related to the degree of inflammation at the surface of plaques and the extent of core neovascularization. Importantly, an MR measure of GdF accumulation at both 1 and 24 hours after injection but not Gd-DTPA at peak enhancement was shown to correlate with a quantitative histological morphology index related to these 2 plaque features.

Conclusions—GdF-enhanced MRI of atherosclerotic plaques allows noninvasive quantitative information about plaque composition to be acquired at multiple time points after injection (within 1 and up to 24 hours after injection). This dramatically widens the imaging window for assessing plaque stability that is currently attainable with clinically approved MR agents, therefore opening the possibility of whole-body (including coronary) detection of unstable plaques in the future and potentially improved mitigation of cataclysmic cardiovascular events. (Circ Cardiovasc Imaging. 2009;2:226-234.)

Key Words: MRI • atherosclerosis • inflammation • angiogenesis • contrast media

Atherosclerosis is the underlying cause of life-threatening cardiovascular events such as myocardial infarction and represents the leading cause of morbidity and mortality. It is characterized by the formation of inflammatory plaques that occupy the intimal layer of arterial walls. Acute plaque rupture leads to the formation of thrombi that disrupt blood flow to downstream tissues. The current clinical standard for identifying atherosclerotic arteries is x-ray angiography, which assesses the degree of luminal narrowing. However, it is well recognized that plaque composition has more influence on their propensity to rupture than does the degree of luminal narrowing they cause. Hence, new imaging techniques reporting on plaque composition are urgently needed, which would improve stratification of patients at risk for future thromboembolic events and allow monitoring of antiatherosclerotic treatment.

Two classic morphological features of atherosclerotic plaque stability are the lipid-rich necrotic core and the collagen-rich fibrous cap. In addition, the number of activated macrophages that have infiltrated the cap directly influences plaque stability. Plaque stability is also influenced largely by the amount of intraplaque neovascularization. Neovascularization provides a new pathway for blood constituents to enter the plaque including macrophages, lipids, and red blood cells. Due to the roles that fibrocellular tissue composition and neovascularization have on plaque stability, an imaging technique that is capable of quantitatively evaluating these 2 components would be of great clinical value.
MRI is a leading candidate modality for staging plaque stability due to its ability to directly image the vessel wall at high resolutions. In particular, contrast-enhanced MRI using extracellular gadolinium (Gd)-based contrast agents has shown promise for highlighting specific plaque components, allowing determination of the thickness of the fibrous cap, the plaque’s fibrocapsular tissue composition, the size of the lipid core, and the degree of plaque neovascularization.14–17 Two drawbacks of these conventional agents for plaque imaging is the short time frame in which these plaque features can be assessed and the necessity to image single plaques over time during the agent’s rapid wash-in phase to assess neovascular volume.16 An agent that can widen this imaging window and report on the composition of multiple plaques in multiple vascular beds would be much more beneficial for diagnostic and therapeutic purposes.

One particular agent of interest that has been tested primarily in different animal models of atherosclerosis is gadofluorine-M (GdF). GdF first gained attention when it was shown to preferentially accumulate in diseased wall versus normal wall and allowed detection of plaques that were not detectable using noncontrast MRI.18 Further studies showed a correlation of the degree of GdF enhancement at 24 hours after injection with the amount of lipid present within plaques19 and the ability to track plaque progression.20 Finally, GdF has also been shown to have a strong affinity for fibrous (tenascin, proteoglycans, and collagen), not lipidic, components of plaques, suggesting that GdF-enhanced high-resolution MRI may allow better evaluation of plaque composition.21

The aims of this study were to understand the potential mechanisms by which GdF accumulates into plaques, relate these mechanisms to the ability of GdF-enhanced MRI to report on plaque stability, and compare the MR assessment of stability with GdF with a conventional Gd-based agent, Gd-DTPA. This highlighted advantages for the use of GdF for noninvasive assessment of plaque stability over conventional contrast agents, which may allow whole-body assessment of plaque vulnerability in the future.

Methods

Animal Model

A total of 14 male New Zealand White rabbits were used. Eight rabbits were fed 100 g/d of cholesterol (CH)-supplemented rabbit chow for 27 to 32 months, and the CH level was titrated between 0.125 and 0.25% (wt/wt) to promote aortic atherosclerotic plaque formation as previously described.22–24 Six age-matched rabbits were used as controls and fed normal chow. Twelve of the 14 rabbits were used in MRI experiments, whereas 1 control and 1 CH-fed rabbit were euthanized without contrast media injection. Animals were cared for in accordance with guidelines of the Canadian Council on Animal Care.

Contrast Media

Gadofluorine-M (Bayer Schering Pharma, Berlin, Germany) is an amphiphilic, macrocyclic, gadolinium-containing complex (1528 g/mol). It is a derivative of Gd-DOTA containing a perfluorocetyl side chain and a mannoside moiety. GdF has an r1 relaxivity of approximately 17.5 mmol/L−1 s−1 in blood (1.5 T and 37°C)25 and a blood half-life of approximately 10 hours in rabbits.26 Carbocyanine-labeled GdF (cc-GdF) is a formulation in which mannosamine has been replaced by carbocyanine (2% of total GdF content) is fluorescently tagged.21 Gd-DTPA (Magnevist, Bayer Schering Pharma, Berlin, Germany) has an r1 relaxivity of approximately 3.9 mmol/L−1 s−1 in human plasma (1.5 T and 37°C)26 and a plasma half-life of approximately 28 minutes.18

Magnetic Resonance Imaging

Anesthetized rabbits were imaged in the supine position using a clinical 3-T MRI scanner (GE Signa HD ×12, GE Healthcare, Waukesha, Wis) interfaced with a two-channel phased array surface RF coil.18 Each rabbit was scanned sequentially with Gd-DTPA and cc-GdF (or GdF in 2 control rabbits) using a standardized MRI protocol. Please see online supplemental (OS) text for technical specifications of anesthesia and MRI. Briefly, high-resolution (0.156×0.156×3 mm3) images were collected before and during the first 22 minutes after Gd-DTPA injection (0.2 mmol/kg) or before and at 1 and 24 hours after GdF administration (0.1 mmol/kg). A T1-weighted (T1w) quadruple inversion recovery fast-spin-echo (QIR-FSE) sequence developed previously for quantitative contrast-enhanced imaging was used.27

Histology

Animals were euthanized after imaging with an intravenous injection of ketamine (200 mg) and transcardially perfused under pressure with ~1.5 L of heparinized (1 IU/mL) Hank balanced salt solution. One control and 1 CH-fed rabbit without contrast agent injection were also euthanized. After the rabbits were killed, while the animal was in the supine position (similar to MRI), the imaged aortic segments (2, 3, and 4 cm superior to the celiac bifurcation) were carefully isolated, marked on the ventral surface with Evan blue dye for matching to MRI, dissected, and frozen in OCT. Fresh-frozen sections of each segment were collected and cc-GdF distribution within each section was imaged. Sections were then stained for various plaque components including collagen (picrosirius red), lipid (Oil Red O), macrophages (RAM-11 immunostaining), and endothelial cells (CD-31 immunostaining). Please see OS text for staining procedures. All microscopy was performed using a Zeiss Axiosplan 2ie microscope (Carl Zeiss Canada, Toronto, Ontario, Canada).

MR and Histological Image Analysis

MR images were analyzed with OsiriX DICOM reader (version 2.7.5, Geneva, Switzerland). The inner and outer vessel wall boundaries were traced to determine average and total wall signal intensity (SImuscle, wall, and SImuscle, total-wall, respectively). Regions of interest were placed in both the paraspinal muscle adjacent to the aorta and in a motion-free region outside the animal (air) to determine average muscle signal intensity (SMuscle) and the standard deviation of the noise signal (σNoise), respectively. Contrast-to-noise ratio (CNR) between the vessel wall and adjacent muscle (CNRwall=σnoise−σNoise) was calculated. Finally, the change in total wall SI (ΔSImuscle, total-wall) between various time points postcontrast and baseline values was calculated (ΔSImuscle, total-wall=SItotal-wall-postcontrast−SItotal-wall-baseline).

For histology, collagen-stained (picrosirius red) sections were analyzed for the area of loose connective tissue (LCT; ArealCT, Air) at the surface of the plaque (RAM-11 staining confirmed that these regions were also highly inflamed). Additionally, sections stained for endothelial cells (CD-31) were analyzed for the extent of neovascularization (NV) in the core (Arenv, Air). Arenv, Air was defined as the total area circumscribing neovascularized regions within the core (RAM-11 staining confirmed that these areas were also highly inflamed). From this, a histological morphology index (HMI) was calculated (HMI=Arenv, Air/ArelCT, Air) to give a quantitative measure of the combined extent of these 2 features of plaque instability.

Statistics

For comparisons of MR data between control and CH-fed animals in which images of the same plaque were collected at multiple time points per animal, a 2-way repeated-measures ANOVA was performed. For this analysis, measurements from multiple plaques in a single animal were averaged to generate a mean MR measurement per animal at each time point. For all other comparisons of data
either a 1-way ANOVA followed by a post hoc Tukey multiple comparison test or a 2-tailed $t$ test was performed. Univariate Pearson correlational analysis was performed between MRI values ($ΔS_{\text{total-wall}}$) and HMI, using data from individual plaques. Multivariate regression analysis was performed to assess the effects of both HMI and rabbit identity (multiple plaques imaged per rabbit) on MRI values ($ΔS_{\text{total-wall}}$). The nominal level of significance for all tests was $P<0.05$. Both GraphPad Prism 4.0a (GraphPad Software Inc, San Diego, Calif) and Statistical Analysis Software (SAS Institute Inc, Cary, NC) were used for statistical analysis.

**Results**

**GdF Accumulates More in Diseased Wall Versus Normal Wall and Improves the Contrast of Diseased Vessel Wall to Muscle 24 Hours After Administration**

To quantify the enhancement of normal and diseased vessel wall after either GdF or Gd-DTPA administration, we performed high-resolution (0.156 × 0.156 × 3 mm$^3$) black-blood imaging before contrast (baseline) and at various time points after contrast (first 22 minutes for Gd-DTPA; 1 and 24 hours for GdF) (Figure 1). As shown, blood nulling was successful both before and after administration of either contrast agent, allowing good delineation of inner wall boundaries at all time points. Qualitatively, diseased wall enhanced at both 1 hour and 24 hours after GdF; however, the patterns of enhancement appeared different between the 2 time points (Figure 1A). At 1 hour, a trilayered structure (bright inner surface, dark core, bright outer surface) was more apparent than at 24 hours. The more obvious bright inner surface at 1 hour versus 24 hours is probably caused by the gradual accumulation of GdF deeper within the plaque as time passes. Similar results showing deeper penetration of GdF at 6 hours compared with 0.5 and 2 hours after administration have been reported. The more obvious bright outer surface at 1 hour versus 24 hours may be explained by significant amounts of GdF circulating through neovessels present in both the media and core of the plaque at 1 hour, whereas at 24 hours GdF blood levels are significantly reduced (blood half-life of $\approx 10$ hours). As expected in diseased wall enhanced with Gd-DTPA, however, the enhancement appeared more homogenous within the vessel wall (Figure 1B). Surprisingly, normal wall also appeared to enhance at both 1 and 24 hours after GdF (Figure 1C), contrary to what has been previously reported. Enhancement of normal wall was also apparent after Gd-DTPA administration (Figure 1D).

To measure the ability of either GdF or Gd-DTPA to improve the conspicuity of diseased or normal wall from surrounding muscle, the wall-to-muscle contrast-to-noise ratio ($\text{CNR}_{\text{outer}}$) was determined (Figure 2). $\text{CNR}_{\text{outer}}$ values...
were significantly increased after GdF administration for normal wall at both 1 and 24 hours, whereas diseased wall conspicuity was significantly increased at 24 hours only (Figure 2A). Furthermore, diseased wall could be clearly distinguished from normal wall based on the highly positive CNR values at 24 hours after GdF administration versus the negative values for normal wall at all time points, consistent with previous findings at 48 hours. Similarly, Gd-DTPA administration improved the conspicuity of both normal and diseased wall shortly after administration (Figure 2B).

Next, the change in total SI ($\DeltaSI_{\text{total-wall}}$; reflecting total contrast agent accumulation) within vessel wall after contrast agent administration was determined (Figure 3). At 1 hour, a trend toward increased GdF accumulation was seen within diseased wall compared with normal wall (Figure 3A). At 24 hours, GdF accumulated within diseased wall significantly more than in normal wall (Figure 3A). Finally, a trend for greater GdF accumulation in normal wall at 1 versus 24 hours was seen, whereas this trend was not seen in diseased wall (Figure 3A), suggesting that GdF is retained better in diseased wall than normal wall. As expected for Gd-DTPA use, diseased wall accumulated more contrast agent than normal wall (Figure 3B).

**GdF Accumulates Primarily in Collagenous Regions Near the Surface of Atherosclerotic Plaques, but the Amount Is Influenced by the Plaque’s Fibrocellular Tissue Composition**

To detect the distribution of cc-GdF within diseased and normal wall, we first maximized our ability to detect cc-GdF fluorescence. Using normal and diseased vessel wall from animals not injected with contrast agent, we adjusted our microscope acquisition parameters to a point where autofluorescence from the aortic tissue was just nulled (Figure S1 in OS text). By doing this, cc-GdF was visualized in both diseased and normal vessel wall from animals injected with the agent 24 hours before killing (Figure S1), confirming our MR observations that both wall types enhanced using GdF. It was also apparent that the fluorescent signal was stronger in diseased wall, also confirming the MR results that diseased wall accumulates more GdF than normal wall (Figure 3A; significantly higher $\DeltaSI_{\text{total-wall}}$).

Similar to previous findings, imaging of cc-GdF revealed that within the majority of plaques (15 of 20 plaques), cc-GdF accumulation was restricted to the luminal surface of the plaque (Figure 4a). The highest density of GdF was present in regions composed of dense, organized collagen bundles, consistent with the concept that GdF binds to fibrotic plaque components (Figure 4B). However, interestingly, the penetration and amount of GdF in these regions appeared to be dictated by the degree of macrophage infiltrate present within the cap (Figure 5). Areas with minimal macrophages were often composed solely of dense, organized collagen bundles, and this appeared to limit the amount of GdF that penetrated into the plaque (Figure 5A). The presumed mechanism of this is limited plaque permeability and a high number of binding partners directly at the surface of the plaque. However, as the number of macrophages at the surface increased, the amount of GdF entering the plaque also increased, presumably due to increased permeability and decreased fibrotic binding partners directly at the surface (Figure 5B and 5C). Finally, whereas the majority of GdF was found to be associated with collagen, in contrast to previous findings that found paracellular GdF accumulation, it was also clearly apparent that a small pool of GdF accumulated within macrophage-derived foam cells present at the plaque surface (Figure S2).

**GdF Accumulates in Highly Inflamed, Lipid-Rich Cores With Significant Neovascularization**

Whereas in most plaques cc-GdF accumulation was restricted to the plaque surface, 5 of the 20 plaques showed evidence of deep accumulation within the core. Similar to the surface, core cc-GdF accumulation (Figure 6A) was found in areas rich in lipid-laden macrophages (Figure 6B and 6C) and with less collagenous material present (Figure 6D). However, for intravenously injected GdF to accumulate into these areas, we assumed an additional route (not directly from aorta lumen) of entry into the plaque core would need to be present. Hence we hypothesized that these areas would also be rich in intraplaque neovessels. As shown, this hypothesis held true as positive core CD-31 immunostaining was found only in the locations of those 5 plaques where core GdF was found (Figure 6E).
Change in Diseased Wall SI After GdF But Not Gd-DTPA Administration Correlates With a Histological Index of Morphological Features Related to Plaque Instability

As the amount of GdF accumulating within the plaque appeared to be determined by both the plaque’s fibrocellular tissue composition and the amount of neovascularization within the core, we correlated the $\Delta S_{I_{\text{total-wall}}}$ values obtained from the MR data to a histological measure of the additive extent of these 2 plaque features, referred to as the plaque’s HMI. Scatterplots of HMI values versus $\Delta S_{I_{\text{total-wall}}}$ values at 1 and 24 hours after GdF administration or 22 minutes after Gd-DTPA administration are shown in Figure 7, a and b, respectively. Univariate correlational analysis revealed significant positive correlations between HMI and $\Delta S_{I_{\text{total-wall}}}$ values at both 1 ($r$ value=0.8458, $P<0.001$) and 24 hours.

Figure 4. GdF accumulation appears restricted to the surface of the majority of plaques and accumulates in collagen rich regions. A, 15 of the 20 plaques attained 24 hours after GdF administration showed significant accumulation of GdF near the surface of plaques. Staining for collagen revealed that plaques were highly fibrotic in nature. B, Higher magnification images showed that areas of dense collagen bundles had the highest density of GdF accumulation (+), compared with areas with less collagen (*). White arrow in upper left panel represents fluorescence from Evan blue dye, not cc-GdF fluorescence (scale bars; low magnification=500 $\mu$m; high magnification=100 $\mu$m).

Figure 5. The degree of inflammation within the fibrous cap (fibrocellular tissue composition) influenced the accumulation of GdF within the plaque. A, Plaque caps with minimal macrophages present were composed mostly of dense, organized collagen bundles. These surfaces tended to have limited GdF accumulation. B and C, As the macrophage content increased within the cap, the collagenous network became more disorganized, presumably the plaque’s permeability was increased, and more GdF was present (scale bars=100 $\mu$m).
(r value=0.7962, P<0.0001) after GdF administration (Table). In contrast, $\Delta S_{I_{\text{total-wall}}}$ from a single time point during the peak enhancement phase after Gd-DTPA administration showed a weaker, nonsignificant correlation ($r$ value=0.5335, $P=0.0605$) (Figure 7B). Finally, multivariate regression analysis was performed to investigate any effects of rabbit identity on the variability in the MR data because multiple plaques were imaged per rabbit (Table). A significant effect ($P=0.02$) of rabbit identity was noted for MR data collected at 24 hours after GdF administration, but this accounted for only 14.6% of the variability (Table). No effect of rabbit identity was seen for the MR data collected at 1 hour after GdF administration or 22 minutes after Gd-DTPA administration.

**Discussion**

Noninvasive measures of atherosclerotic plaque composition in multiple vascular beds will dramatically improve the ability to identify patients at risk of life-threatening thromboembolic events and will aid in directing appropriate, patient-specific therapies. Gd-based MR contrast agents have been shown to be previously useful for aiding in determination of plaque composition. However, this typically is limited to 1 vascular bed (or even 1 MR slice) due to the rapid wash-in/out plaque kinetics of currently approved agents. In this study we sought to determine the ability of GdF, an agent with a long plaque half-life, to report on plaque composition at multiple time points in a rabbit model of atherosclerosis. We present evidence showing that GdF administration improved the conspicuity of diseased vessel wall to muscle 24 hours after administration and that GdF accumulated more in diseased wall than normal wall. Importantly, we found that total GdF accumulation within diseased wall as measured by $\Delta S_{I_{\text{total-wall}}}$ at both 1 and 24 hours positively correlated with a histological index of morphological features implicated in plaque instability, namely the plaque’s fibrocellular tissue composition and extent of intraplaque neovascularization. In contrast, accumulation within the same plaques...
during the peak enhancement phase using the conventional agent Gd-DTPA did not correlate with this measure. This suggests an advantage for using GdF over currently approved agents for quantifying these 2 plaque features, as this agent can be used within a short time period after administration (currently 1 hour) but also widens the imaging window (up to 24 hours), potentially allowing multiple vascular beds to be examined.

The 2 primary features of advanced atherosclerotic plaques are the formation of a lipid-rich necrotic core with an overlying fibrous cap. The composition of the cap and core is intrinsically linked to plaque stability. This includes the cap’s fibrocellular tissue composition and the degree of neovascularization in the core. Strong evidence suggests that plaques with a large number of macrophages at the surface and significant neovascularization are unstable and susceptible to rupture. Hence, an imaging agent that accumulates according to the plaque’s fibrocellular tissue composition and degree of neovascularization, such as GdF, would allow the staging of advanced plaque development and help identify plaques that are destabilizing at an accelerated rate.

As mentioned, Gd-based extracellular contrast agents have shown promise for identifying plaques with altered fibrocellular density and plaque neovascularization. Although these are significant advances in in vivo imaging of plaque composition, 1 potential drawback of using conventional agents is that the imaging window after injection of the agent is short (typically the first 20 minutes). This will limit the use of these agents for imaging multiple plaques in a single vascular bed or multiple vascular beds in a single patient (or animal). Since atherosclerosis is considered a systemic disease affecting multiple vascular beds, the use of an imaging agent capable of extending this imaging window would be of great benefit for clinical assessment of plaque vulnerability on a whole-body basis. GdF may be such an agent because it is retained in plaques for significant periods of time and its diffusion within the plaque is directly related to the plaque’s composition. Another added benefit of using GdF is its higher $r_1$ relaxivity compared with conventional extracellular agents (17.4 mmol/L $-1$s$^{-1}$ versus $\approx$4 mmol/L $-1$s$^{-1}$ at 37°C and 1.5 T, respectively) and therefore should improve detection of these plaque features at similar doses or may alternatively be used at lower doses.

Several groups have theorized about how GdF accumulates within plaques. One study showed that the amount of lipid within plaques correlated with the degree of enhancement of the plaques, and it was theorized that GdF binds to lipidic

| Table. Multivariate Regression Parameters for $S_{\text{total-wall}}$ Based on Models Including Rabbit Identity and HMI |

<table>
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<tr>
<th>Contrast (Time)</th>
<th>Independent Variable</th>
<th>$P$ Value</th>
<th>Partial $R^2$</th>
<th>Model $R^2$</th>
<th>Variability $r$ Value*</th>
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<tr>
<td>GdF Rabbit NS</td>
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<td>0.8458</td>
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<td>Gd-DTPA-Gd Rabbit NS</td>
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NS indicates not significant.

*Pearson correlation coefficient in univariate analysis of HMI to $S_{\text{total-wall}}$. 

Figure 7. Scatterplots show histological morphology index related to plaque inflammation and neovascularization versus the change in MR signal intensity ($\Delta S_{\text{total-wall}}$) within plaques after either GdF or Gd-DTPA administration. Regression lines are drawn. A, At both 1 (left) and 24 hours (right) after GdF administration, the $\Delta S_{\text{total-wall}}$ values obtained by MRI correlated well with the index of a plaque. B, In contrast, $\Delta S_{\text{total-wall}}$ values obtained at single time points within the peak enhancement phase after Gd-DTPA administration did not significantly correlate with this index.
components of plaques via interactions with its hydrophobic tail.\textsuperscript{19} This theory was tested in another study and it was shown that GdF enters plaques bound to albumin (hydrophobic interaction) and once in plaques it has a high affinity for other hydrophobic partners such as tenascin, proteoglycans, and collagen found in more fibrous, not lipidic, regions of plaques.\textsuperscript{21} These results suggest that GdF should accumulate in highly fibrotic regions of plaques. Clearly, a discrepancy exists between the MR imaging results of the first study and the biochemical analysis of GdF affinity in the second study. Our study provides new evidence that helps to resolve this discrepancy. We have shown that although GdF does preferentially bind to collagenous (fibrous) material within plaques, consistent with the Meding study,\textsuperscript{21} it also appears to accumulate more and penetrate deeper into plaques that contain a large number of lipid-rich macrophages (foam cells), consistent with the Sirol study.\textsuperscript{19}

Two results in this study were in striking contrast to previously published results using GdF in atherosclerosis models. First, we found evidence of macrophage uptake of GdF, whereas cc-GdF was previously found to paralocalize to macrophages within plaques.\textsuperscript{21} We believe this result is not unexpected for 3 reasons: (1) monocytes spontaneously phagocytose GdF in vitro\textsuperscript{20}; (2) GdF enhancement of atherosclerotic wall can last for up to 2 months after a single injection, which may be due to GdF being taken up by phagocytes\textsuperscript{20}; and (3) recent evidence has shown GdF colocalization with macrophages in optical nerves in rats with experimental autoimmune encephalomyelitis rats.\textsuperscript{31} Our second contrasting result to previous studies\textsuperscript{18,19} is the significant enhancement of normal vessel wall using GdF. However, we are confident in our results because we also confirmed the presence of GdF within normal wall using both epifluorescent and confocal (not shown) microscopy. We believe that the discrepancy between our finding and previous literature is partially because these previous studies used significantly lower resolution MR imaging protocols than ours, leading to partial voluming effects that probably limited their ability to detect the substantial accumulation of GdF in the very thin normal wall.

In summary, we provide novel evidence for the use of the MR contrast agent GdF for noninvasive staging of plaque stability in a rabbit model of atherosclerosis. This agent allowed the combined effects of multiple plaque features related to plaque stability to be assessed simultaneously and is capable of doing so at multiple time points after administration, lessening the imaging timing restrictions typically required with currently approved Gd-based agents. We believe this agent could be useful for testing of the efficacy of current (eg, statins) or novel therapeutics (eg, antiangiogenic or antiinflammatory) aimed at affecting plaque stability in individual animals (or patients) over time. Finally, this agent would also allow plaques responsible for thromboembolic events, so-called ruptured or “culprit” plaques, to be localized noninvasively before these life-threatening events occur and guide clinicians to make appropriate medical or surgical decisions that could save lives.

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Disclosures
Dr Misselwitz is an employee of the contrast media company Bayer Schering Pharma AG (Berlin, Germany) and has financial interest in the products under investigation or subject matter discussed in the article.

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References


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SUPPLEMENTAL MATERIAL

Supplemental Methods

Magnetic Resonance Imaging

For imaging, animals were sedated via an intramuscular injection of stock anesthetic (ketamine (23.4 mg/kg), xylazine (1.3 mg/kg) and glycopyrolate (0.02 mg/kg)) followed by intravenous administration of a 10-fold dilution (in saline) of this stock at a rate of ~3-12 ml/hr. Rabbits were scanned prior to (baseline) and up to 22 minutes following injection of 0.2 mmol/kg Gd-DTPA. After 3 days (to allow Gd-DTPA clearance) previously scanned rabbits were scanned again prior to and at 1 and 24 hours following injection of 0.1 mmol/kg of either GdF (3 control rabbits) or cc-GdF (all 7 CH-fed and 2 control rabbits). A standardized scanning protocol was used at each session. At first, a 2D time-of-flight (TOF) MR angiogram was acquired (repetition time (TR)/echo time (TE) = 25/5.1 ms; flip angle (FA)= 45º; bandwidth (BW) = ±15.63 kHz; field of view (FOV) = 8 cm; matrix = 256 x 256; thickness = 1.5 mm; excitations = 1; 50 slices; scan time = 5:28). Next, 3 axial slices were collected using the celiac bifurcation seen in the angiogram as an anatomical reference and prescribing axial images at 2, 3 and 4 cm superior to the bifurcation. These images were collected using a T1-weighted (T1w) quadruple inversion recovery fast-spin-echo (QIR-FSE) sequence (TR/TE = 800/21.77 ms; inversion time (TI) = 520 ms; echo train length (ETL) = 6; BW = ±11.9 kHz; FOV = 5 cm; matrix = 320 x 320; resolution = 0.156 x 0.156 x 3 mm³; excitations = 10; scan time = 21 minutes, ~7 min per slice acquired serially). QIR-FSE was developed previously for quantitative contrast-enhanced imaging of atherosclerotic vessels using Gd-based imaging agents. A chemically-selective fat-suppression pulse was used to
null the signal from periadventitial fat. A minimum of 3 days was allowed to pass after imaging with the first agent before imaging the same rabbit again with the second agent. Care was taken to ensure that the RF coil was positioned in the same portion of individual animals during all imaging sessions.

**Histology**

Immunostaining was performed on aortic sections using either a mouse anti-rabbit macrophage IgG1 (RAM-11; 0.0164 mg/ml; DakoCytomation, Mississauga, ON) or mouse anti-human CD-31 monoclonal IgG1 (clone JC/70A; 0.003 mg/ml; Abcam Inc., Cambridge, MA) primary antibody, followed by incubation with biotinylated horse anti-mouse IgG secondary antibody (0.006 mg/ml; Vector Laboratories, Burlington, ON), processed using an ABC-HRP complex solution for 30 minutes (Vector Laboratories, Burlington, ON), and visualized with 3’-diaminobenzidine (0.5 mg/ml; Sigma-Aldrich Canada, Oakville, ON; countered with hematoxylin for 1 minute).
Supplemental Figures

Figure S1

(a) 24 Hr Post cc-GdF
(b) No Agent

Diseased Wall

(c) Normal Wall

Figure S2

cc-GdF
Macrophages
Lipid
Figure Legends

**Figure S1:** Carbocyanine-labeled GdF (cc-GdF) was detected within aortic sections of both diseased and normal wall 24 hours after administration. Diseased (a and b) and normal (c and d) wall from animals either injected with cc-GdF or not injected with any contrast agent. cc-GdF fluorescence was detected in both diseased and normal aortic sections from animals injected with the agent, confirming our MR results. Qualitatively, it was apparent that more cc-GdF accumulated in diseased wall than normal wall. White arrows represent fluorescence from Evan’s blue dye injected on ventral surface of the wall, not cc-GdF fluorescence (scale bar = 500 µm).

**Figure S2:** While the majority of cc-GdF accumulated in collagen-rich regions, a small pool of GdF was found within macrophages and macrophage-derived foam cells. Often at the surface of plaques cc-GdF fluorescence was visualized within cells (white arrows). cc-GdF imaged sections were then stained for macrophages (RAM-11) and adjacent sections stained for lipid (Oil Red O) and it was found that this fluorescence was actually within macrophages that were often engorged with lipid (black arrows; scale bar = 100 µm).

Supplemental References
