Serial Studies of Mouse Atherosclerosis by In Vivo Magnetic Resonance Imaging Detect Lesion Regression After Correction of Dyslipidemia


Objective—We determined the effects of sustained normcholesterolemia on advanced mouse atherosclerosis and whether changes in plaque size and composition can be detected noninvasively by MRI.

Methods and Results—Aortic arch segments containing advanced lesions from apolipoprotein E–deficient (apoE−/−) mice (total cholesterol 1281±97 mg/dL) were transplanted into syngeneic wild-type (WT; 111±11 mg/dL) or apoE−/− (702±74 mg/dL) recipient mice on chow diet. Mice underwent serial MRI at 3, 5, 7, and 9 weeks after transplantation. Compared with 3 weeks, correction of dyslipidemia in WT recipient mice resulted in a monotonic decrease (regression) in arterial wall volume, whereas in apoE−/− recipient mice, further plaque progression was noted (P<0.05). MRI and histological measurements were closely correlated (R=0.937). The lesional content of macrophages decreased >90% (P<0.001), and smooth muscle cells increased in the WT recipient mice. In vivo T1−, T2−, and proton density–weighted images of the mouse thoracic aorta differentiated intraplaque lipid and collagen.

Conclusions—Plaque changes can be noninvasively monitored by serial in vivo MRI of a mouse regression model. Our ability to image the thoracic aorta and perform in vivo plaque characterization will further enhance atherosclerosis studies. (Arterioscler Thromb Vasc Biol. 2004;24:1714-1719.)

Key Words: atherosclerosis ■ imaging ■ lipoproteins ■ remodeling ■ transplantation
arch as donor tissue is particularly advantageous because (1) lesions form relatively faster and in a predictable manner in this location, and (2) the preservation of the curvature of the implanted aortic arch provides for a clearer landmark for MRI.

We and others have shown that MRI can noninvasively detect atherosclerotic plaques in living mice. To our knowledge, the ability to measure plaque regression and differentiate plaque components in mice has not been demonstrated. Differentiation among lipid, fibrous, and cellular components in atherosclerotic lesions is desirable because, in humans, susceptibility to rupture is not predicted by plaque size alone, but rather by composition. To date, in vivo MRI characterization of plaque has been reported in humans and in rabbits. In mice, however, the size of arteries is extremely small (diameter <1 mm), and the presence of cardiac, respiratory, or blood flow–induced artifacts make in vivo vascular imaging a challenge.

In this study, aortic arches containing advanced atherosclerotic lesions were transplanted into WT or apoE recipient mice, and the graft lesion burden was quantified in individual mice by serial in vivo MRI at 3, 5, 7, and 9 weeks later. Correction of dyslipidemia resulted in a monotonic decrease in arterial lesion burden and correlated well with histological measurements. The normalization of plasma lipoprotein levels was associated with depletion of macrophage foam cells and an increased content of smooth muscle cells (SMCs), characteristics of human lesions associated with increased plaque stability. In addition, we performed multicontrast imaging of the mouse thoracic aorta, overcoming technical difficulties associated with cardiac and respiratory motion artifacts, to characterize the lipid and collagenous components of mouse lesions in vivo.

Methods

Methods are available online at http://atvb.ahajournals.org.

Results

Plasma Lipid Levels and Lipoprotein Characteristics

The plasma cholesterol level of the baseline group after 10 months on the high-cholesterol Western diet (see Figure 1 for study design) was 1281 ± 97 mg/dL (all data reexpressed as mean ± SEM). Nine weeks after arterial transplantation, the mean plasma cholesterol level of transplant recipient mice maintained on a chow diet in the apoE−/− recipient mice was 702 ± 74 and in the WT recipient mice was 111 ± 11 (P < 0.0001). The high-density lipoprotein cholesterol (HDL-C) levels in WT recipient mice (60 ± 2.7) were significantly increased compared with both the baseline (30 ± 2.7) and the apoE−/− recipient (28 ± 2.5) groups. Fast protein liquid fractionation of pooled plasma revealed that the cholesterol was distributed primarily in the HDL fraction in the WT recipient mice and in the very low–density lipoprotein (VLDL) and intermediate-density lipoprotein/LDL fractions in the apoE−/− recipient group (data not shown).

Serial In Vivo MRI Detects the Progression and Regression of Mouse Atherosclerosis

The baseline group was analyzed by histopathology. Transplant recipient mice were imaged serially at 3, 5, 7, and 9 weeks after implantation of an aortic arch graft containing complex atherosclerotic lesions (Figure 1). The images obtained exhibited no substantial motion artifacts. The slice thickness (0.5 mm) and sequence parameters in the present study were the best compromise between an acceptable signal-to-noise ratio and the acquisition time. Wall tracings of MR images by 2 independent observers showed no statistical difference between the measurements. Coefficient of variance between the data from 2 observers measuring the same MR images ranged from 9.0% to 12%. Morphometric analysis of the serial in vivo MRI-acquired images (Figure 2A) revealed...
that, compared with 3 weeks after transplant, the arterial wall volume decreased in the WT recipient (regression) group at 5, 7, and 9 weeks (−11.6% ± 2.7, P<0.01; −20.6% ± 3.8, P<0.01; and −29.6% ± 4.9, P<0.01, respectively) and increased in the apoE−/− recipient (progression) group (+13.6% ± 3.2, P<0.05; +22.6% ± 5.0, P<0.01; and +27.7% ± 8.2, P=0.01, respectively). Furthermore, the mean arterial wall volumes of mice in the regression group were significantly less than the corresponding MR measurements in the progression group at 5 weeks (26.1 ± 1.9 mm³ versus 37.3 ± 1.5 mm³, P=0.001), 7 weeks (23.3 ± 1.7 mm³ versus 37.9 ± 2.1 mm³, P<0.001), and 9 weeks (20.9 ± 2.2 mm³ versus 39.2 ± 2.4 mm³, P<0.0001). Representative MR images from the progression (top) and regression (bottom) recipient groups 9 weeks after transplantation are shown in Figure 2B.

**Histological Analysis**

Representative combined Masson’s elastin (CME)–stained cross-sections of the arch segment are shown in Figure 3A. Compared with the baseline group, and in agreement with the MRI results, lesion size increased in the progression group and decreased in the regression group (P<0.001) at 9 weeks after aortic arch transplantation. Quantitative analysis of arterial wall volume (Figure 3B) of the entire graft confirmed the visual impression in Figure 3A.

**Lesion Burden by MRI Is Closely Correlated With Histopathology**

The arterial wall volume measurements obtained by MRI and histopathology are graphed against each other (Figure 3C).

The visual impression of a positive relationship between these techniques was confirmed by correlation analysis (for the complete data set, r=0.94; for the progression aortae, r=0.70; and for the regression aortae, r=0.60). Consistent with this was the outcome of the Bland–Altman analysis, a test designed to examine the relationship between 2 different measurement techniques, which showed that the paired in vivo MRI and ex vivo histological measurements of arterial wall volume strongly agreed.

**Correction of Dyslipidemia Decreased Macrophage and Increased SMC Content of Preexisting Advanced Atherosclerotic Lesions**

In addition to determining the effects of normalized plasma cholesterol levels on the size of preestablished lesions, we also determined the changes in cellular composition. Histological sections obtained from mice from each group were stained with the macrophage marker CD68 or the SMC marker α-actin, and the areas of staining were determined by digital image analysis. In the regression group, there was a notable absence of macrophages in lesions (mean area of 0.0045±0.001 mm²) and was in sharp contrast to the baseline (0.051±0.010; P<0.001) and the progression (0.053±0.015; P<0.001) groups (Figure 4A and 4B). Similarly, as would be predicted from the reduction of lesional foam cell content, in a separate study using frozen sections (to perform oil red O staining), the lipid content of lesions in the regression group as early as 1 week after transplantation was significantly reduced (P<0.001 versus baseline).

Because plaque-stabilizing changes in human lesions are thought to include reduced macrophage and increased SMC content, we also determined the effect of a normalization in...
plasma cholesterol levels on lesional SMC content by immunostaining for α-actin. Compared with the baseline and the progression groups, there was a marked increase in α-actin positive SMC in the regression group (Figure 4A), with staining predominantly in the fibrous cap region. Interestingly, lesions from animals of the progression group had diminished α-actin staining, although the lesions that appeared by light microscopy to be cells lining the outer periphery of the plaque (arrowhead in Figure 4A).

In Vivo Multicontrast MRI Can Noninvasively Characterize Plaque Composition in the Mouse Ascending Aorta

Plaque characterization has been previously reported for rabbit and pig experimental models, in ex vivo specimens, and in human carotid arteries and aortas in vivo. However, the small diameter of mouse arteries has made such efforts in mouse models of atherosclerosis a challenge, efforts which, to our knowledge, have not been previously successful.

ApoE−/− mice were fed Western diet for 32 weeks, and the ascending aorta was imaged. Multicontrast images (T1-weighted [T1W], T2-weighted [T2W], and proton density-weighted [PDW]) were acquired. Localization of the correct plane angulation for the multislice experiments was planned from coronal and sagittal scout images. The in-plane resolution was 94 × 94 μm², which was sufficient for visualization of the outer lining of the arterial wall and lumen circumference.

The results are summarized in Figure 5. Morphologically, the lesions in the MR images corresponded closely to histopathological cross-sections taken from the same location. We were able to differentiate the lipid and fibrous tissue components within the atherosclerotic lesions. Collagen and lipid content were confirmed by picrosirius red and oil red O staining, respectively. Picrosirius red staining is thought to be specific for collagen. When used with polarized light, collagen is stained yellow by picrosirius red (Figure 5F); corresponding bright areas are clearly visible on the T1W and PDW images. Areas of hyperintense signal on T1W images and of hypointense signal on PDW and T2W images correlated well in size and appearance with the lipid-rich regions on the oil red O positively-stained areas (red staining; Figure 5E). A combined parametric (colored) MR image was obtained by superimposing images in Figure 5A through 5C with red, green, and blue channels assigned to each image, respectively, as described. The resulting parametric image has a spatial distribution according to the type of tissue present in the specimen, with lipid areas appearing red (T1W contribution) and collagen as green (PDW contribution).

Discussion

The present study used a novel mouse aortic-arch transplantation model to investigate by noninvasive in vivo MRI, for the first time, the effects of a sustained correction of dyslipidemia on the serial regression of mouse atherosclerotic plaque. Compared with the baseline, normalization of plasma lipoprotein levels results in a monotonic reduction of lesion burden, whereas under continued dyslipidemia in apoE−/− recipient mice, the arterial lesion burden is further increased. MRI measurements were closely correlated with histopathology. Intimal remodeling was evident by the decrease in foam cells and the increase in SMCs that are associated with plaque stabilization in human lesions. Another important feature of the present studies is that respiratory and cardiac motion-related artifacts were overcome so that multicontrast MRI (T1W, T2W, and PDW) could be performed in living mice. With this imaging approach, the lipid and collagenous components of plaque in the mouse aortic arch were inferred, with merged composites closely matching the corresponding histology.

Genetically-manipulated mice have become widely used models of human atherosclerosis, and transgenic and knock-out technologies have been widely used to test the in vivo role of individual genes in the disease process. Current practice frequently requires the use of large numbers of mice, euthanized at multiple time points, to determine the degree of atherosclerosis. Serial in vivo MRI is likely to reduce the number of animals required to demonstrate statistical significance because each mouse serves as its own control.

With aortic transplantation, the plasma lipoprotein environment of a lesion at any stage of development can be rapidly altered, and the change can be sustained indefinitely. Although regression of atherosclerosis has been demonstrated in nonhuman primates, those studies mostly involved dietary manipulations over several months to years, and the degree of quantitative regression was generally modest. In contrast, regression induced by correction of dyslipidemia in our transplantation model was rapid, and the extent of regression was marked.

We previously reported that transplantation of a segment of the descending thoracic aorta containing advanced lesions into apoE−/− mice overexpressing (human) apoAI (resulting in selective elevation of plasma HDL-C) was associated with decreases in lesional macrophages and increases in SMC content
similar to the present findings, but surprisingly, and in contrast to this study, regression of lesion size was not achieved. Another group reported similar results after injecting apoE−/− mice with apoAI-milano.28 These observations suggest that increasing HDL-C alone partially reverses plaque pathology, but that normalization of non−HDL-C is also needed for more complete reversal.

As noted above, the changes in the lesions in the regression group acquired the appearance of what in human samples would be considered a stable plaque; namely, that the contents of foam cells and SMCs were decreased and increased, respectively (for a review, see).29 Similar changes were also observed in previous studies in rabbits where plasma cholesterol was lowered by dietary means30 or with statins.31 The loss of macrophages, as we have observed, bears a striking resemblance to the findings in classic studies in nonhuman primates.32–38 As summarized by Small,39 when diet-sensitive monkeys, such as Macaca fascicularis, were fed a high-cholesterol diet for 18 months to induce lesions then switched to low-cholesterol feeding, serial changes in the lesions also demonstrated a loss in foam cells. These similarities indicate that the regression of atherosclerotic lesions may be achieved by processes that are common among a number of mammalian species, adding support to the relevance of our findings to humans.

Although lesions in the progression group contained cellular material in the "fibrous cap" region, α-actin staining in these cells was notably absent (Figure 4A, arrowhead). We recently reported that primary mouse SMCs loaded in vitro with cholesterol downregulate their expression of α-actin as well as other SMC-specific markers and gain a "macrophage-like" phenotype.36 Whether the decreased α-actin expression is associated with the same "transdifferentiation" phenomenon observed in vitro or whether the changes are associated with SMC proliferation or migration37 will require further investigation.

Sites of predilection and of early development of atherosclerosis in the apoE−/− model include the aortic root and the lesser curvature of the aortic arch and its branches.38 The ascending aorta, however, presents a technical challenge for MRI because of cardiac and respiratory motion of the heart and thorax. In our study, stable cardiac and respiratory gating was essential to ensure the quality of the in vivo images. With a typical heart rate of 400 bpm, accurate triggering was crucial to imaging the native aortic segment. Combined respiratory and cardiac triggering has rarely been used in atherosclerosis studies,39 and in those studies, rats were not breathing freely, but were mechanically ventilated, an invasive intervention that complicates and slows down the preparation of animals for imaging. We previously imaged the abdominal aorta13 of mice; however, we reported substantial respiratory motion artifacts in the MR images close to the diaphragm, and thus excluded the thoracic aorta from the study. We recently reported imaging the aortic root, but those measurements were limited to ex vivo analysis.26 In the present study, we have shown that diet-induced spontaneous lesion development can be studied if the appropriate imaging method is used to avoid the respiratory and cardiac motion.

We31 and others have noted that MR measurements of arterial wall parameters are exaggerated compared with those obtained by histopathology, an effect likely caused by tissue shrinkage during the fixation and paraffin-embedding process or to anatomic misalignment of MR and histopathology sections. To circumvent this latter difficulty, we have used an alternative strategy by calculating the total lesion volume (ie, burden) in the atherosclerotic graft by obtaining serial in vivo MR images and ex vivo histological sections throughout the entire aortic arch.

The present results demonstrate, for the first time, that in vivo MRI can noninvasively detect fibrous and lipid components of aortic atherosclerosis in apoE−/− mice. As established in previous reports,19,41 the lipid areas have a high T2W signal and low signal in T1W and PDW images, and are very distinctive from the fibrous areas that have a high signal on T1W and PDW images. In some sections, where the fibrous component appeared diffuse by histopathology (Figure 5), the MR image will tend to overestimate this component because of the volume averaging with adjacent voxels. Nonetheless, despite these limitations, we have shown that we are able to detect these components, although it is clear that repeated studies as well as further improvements in resolution will be necessary to fully define the sensitivity and specificity of the measurement of atherosclerotic plaque composition by MRI. In addition, the development of new, targeted MRI contrast agents may further aid in defining atherosclerotic plaque components.42

In summary, we have shown that the normalization of lipoprotein levels reduces the size and foam cell content of complex atherosclerotic plaques, and that MRI in vivo can accurately and noninvasively detect changes in lesion size and composition. This ability of in vivo MRI to serially quantify lesion regression and differentiate intraplaque composition should provide a powerful assessment tool in future studies of interventions intended to reverse preexisting disease in mouse models of atherosclerosis.

Acknowledgments

The authors thank Sabina Omerhodzic for her technical expertise. E.T. was supported by a National Institutes of Health (NIH) Training Grant in Molecular and Cellular Cardiology (HL-07824). These studies were supported by NIH Grants HL-61814 and HL-70524 (to E.A.F.) and the Howard Hughes Medical Institute Biomedical Research Support Program Grant (to Z.A.F.).

References


Serial Studies of Mouse Atherosclerosis by In Vivo Magnetic Resonance Imaging Detect Lesion Regression After Correction of Dyslipidemia

Arterioscler Thromb Vasc Biol. 2004;24:1714-1719; originally published online July 15, 2004; doi: 10.1161/01.ATV.0000139313.69015.1c
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/24/9/1714

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2004/09/10/24.9.1714.DC1

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

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

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Methods for Trogan et al.

Animals.

All animal experiments followed approved protocols by the Animal Care and Use Committee. ApoE-deficient and WT mice were purchased from Jackson Laboratories or generated from mating colonies. Mice were maintained on a light/dark, 12/12-hour cycle at 22 °C, and received food and water ad libitum.

Study Design and Aortic Transplant (see Figure 1).

To accelerate development of complex lesions in the aortic arch, apoE-deficient donor mice were fed a Western diet (WD; containing 21% fat wt/wt, 0.15% cholesterol) for 40 weeks. Some animals (n = 5) were sacrificed immediately after WD feeding and served as the Baseline group. The remaining animals were used for syngeneic aortic arch transplantations (n = 12) into the infra-renal abdominal aorta of either WT or apoE-deficient mice (28 – 36 weeks of age) maintained on a regular chow diet. The aortic arch transplant was performed as previously described.

In vivo MRI.

The graft segments of WT (n = 6) and apoE-deficient (n = 6) mice were imaged by MR at 3, 5, 7, and 9 weeks after transplantation. In vivo MR imaging was performed with a Bruker 9.4 Tesla, 89 mm-bore system operating at a proton frequency of 400MHz. A gradient insert (ID, 75mm) was capable of generating a maximum of 50 gauss/cm. Mice were anesthetized with continuous inhaled isofluorane and were placed in a 30mm (inner diameter) transmit/receive birdcage coil. Constant body temperature of 37°C was
maintained using a thermocouple/heater system. The aortic arch was identified in an approximately coronal section on a localizing sequence. Contiguous, 0.5 mm-thick transverse slices spanning the entire graft were acquired (94 \times 94 \, \mu m^2 \text{ in plane resolution}) using fat suppressed spin-echo sequence. Repetition and echo times for the proton density-weighted (PDW) images were 2000 ms and 9 ms, respectively. The total imaging time was \sim 34 \text{ minutes/mouse}.

For the purpose of imaging the native aortic arch and for determination of lesional composition, multicontrast-weighted MR images (T_1 (TR/TE = 500/9 \text{ ms}), PDW (2000/9), and T_2W (2000/25)) of the ascending aorta of apoE-deficient mice (fed western diet for 32 weeks) were acquired on a 9.4T Bruker imaging system. The imaging sequence used was a combined respiratory and cardiac triggered spin echo. Cardiac gating (on R wave) was performed using three subcutaneous silver electrodes connected to a PhysioGuard SM785 NMR triggering unit (Bruker). To avoid gradient activity interfering with the ECG waveform, the wires were double insulated in the magnet bore and grounded. A 60-Hz filter was inserted in the triggering line to reduce frequency leakage (zipper artifact on MR images). A respiratory sensor was placed on the abdomen to monitor the depth and frequency of respiration. The output of the ECG trigger is accepted or rejected based on the respiratory cycle as determined by the respiratory trigger. For a specified period of time (trigger window), after detection of the respiratory trigger, all ECG triggers received were rejected to avoid breathing artifact in the images. The sequence was single-sliced (0.5mm), matrix size = 256^2, and field of view (FOV) = 22 \times 22 \text{ mm}. The RGB composite was produced by combining T_1-weighted, PDW, and T_2-weighted greyscale images as red, green, and blue channels, respectively.
Identification of lipid and collagen was based on the different signal intensities in each of the different MR images (T₁W, PDW, and T₂W) and confirmed by oil-red-O² and picrosirius red³ histological stains.

**Lipoprotein measurements.**

Total plasma cholesterol and HDL-C were measured at time of sacrifice using a colorimetric assay (Sigma). High density lipoproteins (d< 1.063) were isolated by serial ultracentrifugation. For FPLC size fractionation, pooled plasma (obtained at time of sacrifice) was separated on 2 in-series Superose 6 columns, and the cholesterol content in the eluted fractions was measured.

**Histology.**

After completion of the last imaging time point (9 weeks after transplantation), mice were exsanguinated under general anesthesia (ketamine/xylazine) by perfusion-fixation with PBS followed by 4% paraformaldehyde at physiological pressure. The aortic arch graft was removed en bloc, transferred to 4% paraformaldehyde, decalcified in 10% formic acid, and re-immersed in 4% paraformaldehyde for at least 24 hours. For the Baseline group, the aortic arch was dissected, and processed in 4% paraformaldehyde for 24 hours. The specimens were dehydrated, paraffin embedded, and serial sections were obtained from the entire graft (5-µm thickness, 40-µm intervals). Sections were stained with combined Masson elastin stain (CME). For immunohistochemistry, macrophage-specific marker CD68 and smooth muscle cell-specific marker α-actin were used as previously described ⁴.
Lipid content was assessed by Oil red-O staining followed by counterstaining with Mayer’s Hematoxylin. For identification of collagen, sections were stained with picrosirius red (0.1% Sirius red in saturated picric acid solution for 30 min.), dehydrated through increasing concentrations of ethanol, mounted, and examined by polarizing light microscopy.

Morphometric Analysis.

Images were digitized, and computer-aided morphometry was performed by an operator blinded to the origin of the samples using Image Pro Plus Version 3.0 software (Media Cybernetics, Silver Spring, MD). Arterial wall volume was derived from measurements of wall area of 10 –15 equally spaced sections (500 µm intervals) spanning the entire graft. For MR images, MRI Analyze 3.1 software (Biomedical Imaging Resource, Mayo Foundation, Rochester, MN) was used to manually trace inner and outer wall boundaries of the entire transplanted graft. Two independent observers traced the wall areas, and the average values were used for subsequent analysis. The wall volume was computed as the product of the difference between lumen and the outer wall area of all slices.

Statistical Analysis.

Changes in lesion size were statistically assessed by a two-tailed paired t test and simple regression analysis (GraphPad Prism, GraphPad Software Inc., San Diego, CA). Other parameters (mean arterial wall volume, and total and HDL cholesterol levels) were analyzed by unpaired two-tailed t tests. A P value ≤ 0.05 was considered significant.
addition, to assess data agreement, Bland-Altman and correlation analysis were performed.
References for Trogan et al.


