In Vivo Serial Assessment of Aortic Aneurysm Formation in Apolipoprotein E–Deficient Mice via MRI

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Background—Hyperlipidemic mice administered angiotensin II have been used for the study of abdominal aortic aneurysms (AAAs). The purpose of this study was to examine the use of MRI for studying AAA development and for examining the effects of pharmacological intervention on AAA development in the apolipoprotein E–deficient mouse.

Methods and Results—Suprarenal aortic aneurysms were generated in apolipoprotein E–deficient mice administered angiotensin II (1000 ng/kg per min) for up to 28 days. In vivo MRI was performed serially (once weekly) to assess AAA development and rupture. Comparison of AAA size as measured by in vivo and ex vivo MRI resulted in excellent agreement ($r=0.96$, $P<0.0001$). In addition, MRI correlated with histology-derived AAA area assessment (in vivo versus histology: $r=0.84$, $P<0.0001$; ex vivo versus histology: $r=0.89$, $P<0.0001$). In a separate study, angiotensin II–administered apolipoprotein E–deficient mice were treated with doxycycline (broad-based matrix metalloproteinase inhibitor; 30 mg/kg per day for 28 days). MRI was able to noninvasively assess a reduced rate of AAA development (46% versus 71%, $P<0.05$), a decreased AAA area (2.56 versus 4.02 mm$^2$, $P<0.01$), and decreased incidence of rupture (43% versus 100%) in treated versus control animals. Inhibition of aorta matrix metalloproteinase 2/9 activity was observed in the treated animals.

Conclusions—These results demonstrate the use of MRI to noninvasively and temporally assess AAA development on pharmacological intervention in this preclinical cardiovascular disease model. *(Circ Cardiovasc Imaging. 2008; 1:220-226.)*

Key Words: abdominal aortic aneurysms ■ imaging ■ MRI ■ doxycycline ■ MMP

Abdominal aortic aneurysms (AAAs) are generally characterized by dilation of the aorta wall and occur as a result of degradation of the collagen and elastin in the medial wall.1–5 However, when a rupture of the media occurs and blood clots at the media-adventitia interface, this is known as a false aneurysm or pseudoaneurysm.6,7 To further understand the pathology and underlying mechanism behind the onset and progression of human aneurysm development, several different animal models have been described.1,8–15 Recently, Daugherty et al reported that aortic aneurysms could be reliably generated in hyperlipidemic mice administered angiotensin II (Ang II; Sigma-Aldrich, St Louis, Mo) via osmotic minipumps. The resulting aneurysm development is primarily located in the suprarenal aortic region covering ≈1 cm and has a gross morphology consistent with a pseudoaneurysm, with 2 distinct regions: a remodeled adventitia that results in compression of the aortic lumen and a rupture of the medial vascular wall, which allows blood to flow into the remodeled adventitial space.8

The characterization of aneurysms in preclinical mouse models has been performed mostly through gross morphometry of the harvested aorta8,16 or histological evaluation of sectioned aortas.1,3,8 Although these methods provide morphological and immunohistochemical information, they are terminal measurements and, therefore, limit their use for studying the development and progression of AAAs. For this reason, ultrasound imaging has been used to assess AAA morphometry in mouse in vivo,17,18 but detailed morphological changes are difficult to determine because of limited sensitivity and/or poor signal to noise. MRI has been used to monitor aortic wall dimensions for studying atherosclerosis19,20 and the progression of aortic dilation at the sinus level in Marfan syndrome mice.21 Spontaneous aortic aneurysms in aged apolipoprotein E–deficient (apoE$^{-/-}$) mice have been visualized via MRI.22 However, this earlier aneurysm study was performed at low magnetic field (2 T), did not use an intervention to drive AAA development (such as Ang II), and did not use bright-blood contrast to visualize blood flowing into the aneurysms. To date, there have been no reports of imaging studies examining pharmacological intervention on AAA development in mice.
In the current work, we use high magnetic field (9.4 T) MRI to follow the temporal development of AAAs in vivo. High-field MRI provides the means to track the progression of aneurysm development at greater contrast sensitivity and signal to noise than ultrasound. In addition, the imaging was optimized to visualize blood flow for the detection of aortic medial wall rupture, an additional measure of aneurysm severity. Following validation of the MRI technique, a pharmacological intervention study was performed using doxycycline, a broad spectrum matrix metalloproteinase (MMP) inhibitor, which was previously demonstrated to inhibit AAA development.4 The current study, to the authors’ knowledge, is the first use of bright-blood MRI to study in vivo AAA temporal development and pharmacological intervention on this development in an Ang II–infused mouse model.

Methods

Animals and Study Design

Male apoe-/- mice (age, 28 weeks; weight, 28 to 35 g) were obtained from Taconic Labs (Hudson, NY), housed in a clean barrier, and administered a standard laboratory diet. Osmotic minipumps (Alzet, Cupertino, Calif) delivering 1000 ng/kg per min of Ang II for up to 28 days were implanted subcutaneously after acquisition of baseline images. All procedures were approved by the Animal Care and Use Committee at GlaxoSmithKline (King of acquisition of baseline images. All procedures were approved by the Animal Care and Use Committee at GlaxoSmithKline (King of proximaal to the right renal branch. After sections were deparaffinized, rehydrated, and placed in 0.05 mol/L Tris buffer (pH=7.6), they were stained on a DAKO autostainer (DAKO Corporation, Carpinteria, Calif) using reagents from the catalyzed signal amplification system provided by DAKO. Serial sections were stained using a Masson trichrome (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). A total of 19 sections contained a measurable aneurysm.

MMP Activity

The thoracic aorta was used to assess MMP activity as this region of aorta is activated by Ang II but did not contain aneurysms and therefore could be controlled for variability associated with AAA sections.24 Aortas were extracted for MMP quantitation by grinding in liquid nitrogen and followed by incubation for 18 hours at 4°C in 0.5% Triton X-100 (Sigma-Aldrich) in PBS containing 0.01% sodium azide (1.3 wt/vol) on a bench shaker. After extraction was complete, the samples were centrifuged at 14 000 rpm (10 minutes, 4°C) and the supernatants were collected. Protein concentration for each sample was determined with the BioRad DC protein assay kit (BioRad Laboratories, Hercules, Calif).

MMP enzyme activity was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) zymography as described previously.25 Enzyme activity of MMP-9 and MMP-2 was measured using a Masson trichrome (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). A total of 19 sections contained a measurable aneurysm.

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Image Analysis

MRI image analysis was performed using Analyze AVW software (AnalyzeDirect, Leneka, Kans) and histological image analysis was performed using Image-Pro-Plus software (Media Cybernetics, Bethesda, Md). In the MRI images, the lumen and remodeled vessel
wall of the AAA were traced separately for area measurements. In histological sections, the AAA was traced using the medial wall of the aorta and the collagen capsule separating the aneurysm from the adventitia as boundaries. Cross-sectional area measurement of all image slices through the aneurysm (defined as remodeling of vessel wall area >50% of lumen area) was performed to obtain the section with the largest aneurysm area. This single section of aneurysm was then analyzed serially throughout the study. For comparison between in vivo and ex vivo MRI images, area measurements (using the same procedure as the in vivo measurements) were made in both sets of data on 3 slices per aneurysm. MRI and histology sections were coregistered based on anatomic landmarks and distance from the right renal branch.

Analytic Analysis
End-of-study plasma doxycycline concentration was measured using a standard protein precipitation, and API-4000 LC/MS/MS (Applied Biosystems, Calif) and plasma lipid were measured using an Olympus AU640 chemical analyzer (Olympus America Inc, Melville, NY).

Statistical Analysis
All statistics (log-rank for survival and AAA development, Student t test for area measurement, correlations for AAA area and lumen comparisons) were performed using either the Prism (GraphPad, San Diego, Calif) software package for MRI or Microsoft Excel (Microsoft, Redmond, Wash) for zymography. P<0.05 was used for significance.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
Assessment of AAA Development
Imaging of the mice was performed weekly as the initiation of AAA development was variable, but generally ranged from 1 to 4 weeks. Figure 1A illustrates an example of a MR image in the true-coronal plane through an aorta at baseline, whereas Figure 1B shows the aorta after development of an AAA. A slight stenosis of the aortic lumen may be observed in Figure 1B approximately where the plane (Figure 1D; 1 mm thick) intersects the aorta. Also observable is an area of blood flow immediately adjacent to the aortic lumen, where the plane (Figure 1E) intersects the aorta, indicating a break in the aorta medial wall. The location of this aneurysm is 1 mm superior to the right renal branch. The corresponding transverse cross-sections of the abdominal aorta from Figures 1A and 1B are shown in Figures 1C through 1E. At baseline (Figure 1C), the abdominal aorta appears concentric with no aneurysm present. After 1 week of Ang II infusion, an AAA has developed in the same area of the aorta (Figure 1D) and the lumen is stenosed because of the presence of an aneurysm. The aneurysm appears dark compared with the bright signal from the blood within the lumen of the aorta, indicating vessel remodeling with no existing aorta medial wall rupture. In a different section of the same AAA (Figure 1E) there is bright signal indicating the presence of a rupture of the aorta medial wall allowing blood to flow into the aneurysm.

Ex Vivo Validation of In Vivo MRI
AAA Detection
To assess the accuracy of in vivo, MRI-derived morphometric measurement of the aneurysm, ex vivo imaging was performed on the abdominal aortas obtained immediately following the final in vivo imaging session. Similar qualitative features of the aneurysm were observed in both in vivo and ex vivo images. Figure 2A represents a typical transverse image obtained in the mouse with the obvious feature of the osmotic minipump present subcutaneously. The AAA may be observed as noted. Figure 2B represents the region of interest of the AAA, and Figure 2C represents the coregistered ex vivo image of the AAA. Similar structures can be observed in both images, in greater detail than simply a gross remodelling of the adventitia. The original lumen of the aorta (Figures 2B and 2C, i) is prominent and there appears to be blood flow into the remodelled adventitia in the in vivo image and an open dilation of the adventitial matrix in the ex vivo image. Within the remodelled aorta, the ex vivo image shows structure (Figure 2C, ii, iii) that can also be observed in vivo (Figure 2B, ii, iii).

A coregistered trichrome stained section of the same AAA is shown in Figure 2D. Qualitative comparison between both histology and MRI images revealed a similar remodeled
region within the AAA. Collagen, represented by blue stain in
the histology (Figure 2D, iii), corresponds to darker areas in
the MRI images (Figure 2C, iii), whereas areas of thrombus
(red for red blood cells and pink for fibrin, Figure 2D, ii)
formation results in a lighter appearance (Figure 2C, ii).

In vivo and ex vivo cross-sectional areas of AAA and
aortic lumen as determined by image analysis strongly
correlated ($r=0.96$, $P<0.0001$, Figure 3A, and $r=0.86$,
$P<0.0001$, Figure 3B, respectively). Statistically significant
cross-sectional area correlations between histology and both
in vivo MRI ($r=0.83$, $P<0.0001$, Figure 3C) and ex vivo
MRI ($r=0.88$, $P<0.0001$, Figure 3D) were observed. These
measures validate the accuracy of using MRI for quantitating
in vivo aneurysm formation and progression in the mouse. In
addition, the mean AAA area measured by ex vivo MRI was
not different from that measured by in vivo MRI or histology
(2.99±0.22 versus 2.87±0.22 versus 2.16±0.23 mm$^2$,
respectively).

**Effect of Doxycycline on AAA Formation**

After ≈1 week of Ang II administration, study animals began
to die or become moribund (Figure 4A). The percentage of
animals that died or became moribund over the course of the
study was 47% versus 33% (vehicle versus doxycycline,
$P=NS$). Plasma cholesterol, low-density lipoprotein, and
triglycerides were higher in the doxycycline versus vehicle
group ($P<0.05$), with no difference in high-density lipopro-
ten. Plasma concentration of doxycycline at the end of the
study was 282±33 ng/mL.

There was a greater incidence of aneurysm formation in the
vehicle versus doxycycline group following each weekly

**Figure 2.** Comparison of in vivo MRI, ex vivo MRI, and histology of AAA. A, In vivo transverse MRI image of AAA in an apoE$^{-/-}$ mouse. The presence of the osmotic minipump is apparent, and the aneurysm is highlighted by the white box with a zoomed-in view shown in B. In vivo (B) and ex vivo (C) transverse MRI images of the same segment of an AAA are shown. Similar features, such as aortic lumen (i) and remodeled adventitia (ii, iii) with an area of flowing blood, are present in both images. D, Masson trichrome staining of the same section is shown (magnification, ×2). Darker areas in the ex vivo MRI image (C, iii) correspond to areas stained for collagen (blue; D, iii), whereas lighter areas (C, ii) correspond to areas stained for red blood cells and fibrin (red and pink; D, ii).

**Figure 3.** Correlation between MRI and histological measurements of AAA. Significant correlations in measured AAA area (A) and lumen area (B) were observed between in vivo and ex vivo MRI ($r=0.96$, $P=0.0001$ and $r=0.86$, $P<0.0001$, respectively). In 8 of the aortas, area measurements from MRI and histology were compared. Correlation in measured AAA area was observed between in vivo MRI and histology (C; $r=0.83$, $P<0.0001$) and between ex vivo MRI and histology (D; $r=0.88$, $P<0.0001$).
After 4 weeks of Ang II infusion, not only were there fewer aneurysms detected by MRI and necropsy (47% versus 71% in doxycycline versus vehicle group, \( P < 0.05 \)), but the rate of AAA development was retarded in the doxycycline group (Figure 4B). On examination of only the first 2 weeks of data, a significant difference in time-to-aneurysm formation using MRI only (\( P < 0.05 \)) and MRI and necropsy combined (\( P < 0.01 \)) was observed.

Cross-sectional area of AAA measured from transverse image slices obtained at the same location were analyzed throughout the duration of the study. Weekly AAA areas of aneurysms detected in the end of study survivors for both groups are shown in Figure 5A. Aneurysm development was rapid on onset, but also exhibited continuous growth throughout the duration of the experiment. Doxycycline treatment resulted in a reduction of aneurysm size (\( P < 0.05 \) at 2- and 4-week time points). The aorta lumen area changes due to remodeling of the vessel wall were also measured throughout the study. The doxycycline group had reduced lumen stenosis during the first 3 weeks of the study, after which the lumen size was reduced to a level similar to vehicle (Figure 5B).

In addition to onset of aneurysm and aneurysm growth, MRI also allowed for the detection of blood flow within the aneurysm (Figure 1E), indicating the presence of a rupture of the aorta medial wall. Over the course of the study, the number of aneurysms for which MRI was able to detect a rupture was greater in the vehicle group (100%) versus doxycycline (43%).

**Effect of Doxycycline on MMP Inhibition**

To confirm treatment with doxycycline resulted in reduced MMP activity, gel zymography was performed on thoracic aortas from both groups at end of study. Thoracic aorta was chosen to eliminate any bias because of the presence of macrophage-rich AAA tissue. After 4 weeks of treatment, the doxycycline group had lower levels of MMP2 (\(2\times 38\%\), \( P < 0.05 \)), pro-MMP2 (\(2\times 31\%\), \( P < 0.05 \)), and pro-MMP9 (\(2\times 21\%\), \( P < 0.05 \)) activity, consistent with the reduced development of AAAs detected by MRI in this group.

**Discussion**

To understand and characterize the etiology of aortic aneurysm development, animal models that can consistently generate AAAs in a predictable manner have been developed.1-8 One such model is the Ang II administered, hyperlipidemic apoE\(^{-/-}\) mouse, which reliably, and as indicated by the present in vivo MRI results, rapidly generates AAAs. To accurately characterize aneurysm development using traditional measurement of aorta diameter on necropsy would require a large number of animals to generate enough power at each time point in the development timeframe. To mitigate this issue, in vivo imaging technologies would be of obvious use. One such technology is ultrasound imaging, which has
been used to track the development of AAA formation in mouse in vivo, but unfortunately suffers from limited contrast or signal-to-noise sensitivity and as such limits the ability to provide a detailed assessment of the remodeled adventitia. In the present study, MRI was shown to provide delineation between the original lumen and the remodeled adventitia with or without an aorta medial rupture. Although ultrasound can monitor luminal dilation, there have been no reports in mice using ultrasound techniques to distinguish between the lumen and the so called pseudoaneurysm. MRI also allows for reliable replication of imaging slice placement based on physiological landmarks without operator dependency, which may be problematic for serial ultrasound use. The accuracy of AAA area determination by in vivo MRI was clearly demonstrated by the high degree of correlation with measurements made using ex vivo MRI and histology. To ensure the comparison was accurate and valid, animals were euthanized and vessels were fixed immediately following AAA detection in vivo.

In addition to visualization of adventitial remodeling, in vivo MRI reveals some degree of functional information about the aortic lumen. By using short MR pulse repetition times, excitation of fresh nuclear spins brought in by flowing blood results in higher MRI signal than in surrounding tissue. This bright-blood contrast allows for the effects of AAA development on the aortic lumen to be examined in vivo. Dilation of the lumen due to aorta medial rupture is a characteristic of AAAs in this model. Although the exact location of medial wall rupture is difficult to define by in vivo MRI given the 1-mm slice thickness in the present study, the technique provides for the visualization of blood flow into the dilated lumen. Although bright-blood contrast was implemented this study, the use of black-blood contrast where the vessel lumen appears dark compared with the surrounding tissue, would be of particular interest for examining the vessel wall as this technique would provide superior contrast in comparison with bright-blood imaging. However, in the present study, the interest was in luminal visualization and the effect of blood flow through medial breaks into the adventitial space rather than obtaining a detailed visualization of the remodeled aortic wall. In addition, black-blood imaging typically requires longer scanning times to produce optimal contrast, especially if physiological triggering is used. Because of the throughput required for preclinical serial imaging pharmacology studies, rapid scan times are beneficial. Full appreciation of the advantages and disadvantages of these 2 imaging methods in this animal model requires further investigation.

A second characteristic of the AAA described by Daugherty was remodeling of the media-adventitia interface. MRI provided adequate contrast to distinguish between the blood-filled luminal dilation and remodeled adventitia. The formation of a thrombus and degraded collagen within the adventitial space resulted in varying degrees of stenosis in the aorta immediately adjacent to the remodeled adventitia that could be monitored by MRI. Barisione et al reported luminal dilation in aortas with AAAs detected by ultrasound; however, ultrasound lacks the contrast sensitivity of MRI and may not be able to distinguish between the compressed lumen and the remodeled wall. We also detected an absence of extravasation of blood into the adventitial space in some animals. Similar aneurysms have been reported previously in apoE−/− mice fed a high fat diet. In these AAAs evidence of the medial rupture typically reported in this model was absent at the time of the initial detection of the aneurysm and throughout the subsequent weekly scans.

The in vivo images often yielded distinct structures within the remodeled adventitia. These structures were also found in the ex vivo images in the same segment of the aorta. Trichrome staining revealed differences in ex vivo MR contrast, which corresponded to either collagen (dark in the MR images) or thrombus (light in the MR images). The composition of aneurysms determined by histology was similar to that found in previous studies. Variations in MR contrast within the aneurysm could yield valuable information on AAA development beyond changes in gross morphology and blood flow. Changes in composition of the remodeled adventitia and the presence and size of thrombus in relation to the presence or absence of an aorta medial rupture reflects pathological changes in the early development of AAAs that could potentially be followed by repeated scanning during the early stage of aneurysm development. Further, the parameters used in this study were optimized to emphasize blood flow. Variations in the weighting of image contrast and the use of black-blood imaging techniques could be further refined to allow greater emphasis on specific features of AAAs.

The use of this technique for pharmacological studies was clearly demonstrated by treating mice with the broad spectrum MMP inhibitor, doxycycline. Protection from doxycycline occurs most likely as a result of MMP-2 and MMP-9 inhibition both of which have been closely linked to AAA development. Over the 4 weeks of Ang II infusion, treatment effects of AAA development (P < 0.05) and size could be detected (P < 0.05). However, there were significant differences in time-to-aneurysm formation (P < 0.01) and aneurysm size (P < 0.05) after only 2 weeks. Because of the increased risk for mortality in this disease model, the longer animals are administered Ang II, the greater the chance group size differences could confound results. In our study, there was slight decrease in mortality, albeit nonsignificant, in the treatment versus vehicle group (33% versus 47%, respectively). In previous studies published by Daugherty et al, a range of mortality has been reported from as low as 10% up to 40%. One inherent limitation in this model is that not every animal given Ang II will develop an AAA during the 28-day dosing period. In an earlier study by Manning et al using a similar protocol, after 4 weeks of Ang II infusion, 86% of the vehicle mice versus 35% of the doxycycline treated mice generated AAAs. In the current study, 71% of the vehicle and 46% of the treated mice produced AAAs. A potential explanation for the differences between the 2 studies may be in the age (28 weeks in the present study versus 8 weeks) and/or strain (apoE−/− in the present study versus low-density lipoprotein−/−) of the mouse model used. A detailed comparison of AAA development between the 2 strains of mice has not been performed.
In summary, MRIs potential for noninvasively and temporally assessing AAA development and drug efficacy in this preclinical cardiovascular disease model was presented. MR images yielded multiple end points including morphometry and composition, whereas bright-blood contrast allowed the visualization of blood flow into the remodeled adventitia as a consequence of a rupture of the media. The ability to evaluate AAAs in vivo provides a mechanism for refining drug studies in this model. By obtaining serial data on AAA progression, future optimization of study design with proper statistical power while adjusting for incidence of mortality may be performed. In addition, AAA development may be tracked serially in the same animals resulting in the use of fewer animals to assess AAA modulation. The use of MRI to refine drug studies in the preclinical discovery phase may provide valuable input for clinical advancement decision making using a technology that is clinically translatable.

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