Effects of Deletion of the Matrix Metalloproteinase 9 Gene on Development of Murine Thoracic Aortic Aneurysms

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Background—The matrix metalloproteinases (MMPs) contribute to cardiovascular remodeling, and MMPs, such as the gelatinases (MMP-9 and MMP-2), have been identified in thoracic aortic aneurysmal (TAA) tissue, but a cause-effect relationship has not been clearly established. Accordingly, this study examined TAA progression in mice devoid of the MMP-9 gene.

Methods and Results—The descending thoracic aortas of wild-type (WT) FVB (n = 17) and MMP-9 gene knockout (KO, n = 11) mice were exposed to 0.5 mol/L of CaCl₂ for 15 minutes with terminal studies performed at 4 weeks. Aortic lumen diameter was measured using video micrometry at baseline and at 4 weeks (TAA) followed by aortic tissue analysis. In WT mice, aortic diameter increased by 138 ± 5% at 4 weeks (P < 0.05), consistent with TAA formation. In the KO mice, aortic diameter increased from baseline by 120 ± 4% (P < 0.05) but was attenuated from WT TAA values (P < 0.05). Gelatin zymography performed on TAA segments confirmed the absence of MMP-9 in the KO mice but a >8-fold relative increase in the active form of MMP-2 compared with WT (P < 0.05). Despite this, MMP-2 activity was relatively increased (P < 0.05) and colocalized to smooth muscle cell actin in a differential pattern favoring medial distraction in the WT TAA compared with the KO TAA segments.

Conclusions—These results demonstrate that MMP-9 gene deletion attenuated TAA formation despite an increase in the zymographic levels of MMP-2. These unique findings suggest that an interaction between these 2 MMPs is necessary to facilitate TAA progression. (Circulation. 2005;112[suppl I]:I-242–I-248.)

Key Words: aneurysm ■ aorta ■ thorax ■ MMP-9 ■ MMP-2

Thoracic aortic aneurysm (TAA) disease is a serious condition with high mortality and morbidity rates.1 Aneurysm formation is a complicated, dynamic process involving both cellular and extracellular processes. Once initiated, chronic inflammation and pathologic remodeling of the vascular extracellular matrix (ECM) are principal features of TAA formation.2 A large body of literature has shown increased abundance of matrix metalloproteinases (MMPs), particularly of the gelatinase subset (MMP-2 and MMP-9), in developing aortic aneurysms.3–7

Substantial evidence implicates MMP-9 to play an important role in cardiovascular system remodeling. Specifically, MMP-9 gene deletion has been shown to modify left ventricular dilatation after myocardial infarction4 and aortic aneurysm formation.5,6 In addition, MMP-9 has many other nonproteolytic actions, which may affect the abundance and activity of other MMPs.9–13

Based on the potential importance of MMP-9, the present study was designed to test the central hypothesis that deletion of this specific MMP would modify the course of TAA formation. The first objective was to quantify whether changes in TAA formation would occur in mice devoid of the MMP-9 gene compared with wild-type (WT) mice.4,14 The second objective was to determine whether and to what degree a compensatory change would occur with respect to the other gelatinase, MMP-2, after TAA formation in the MMP-9 gene deletion construct.

Methods

Experimental Design

Animals used to create TAAs were 8- to 12-week-old adult WT (n = 17) and MMP-9 gene knockout (KO, n = 11, The Jackson Laboratory) FVB mice. For biochemical analysis, the above animals were compared with age-matched control-unoperated animals (WT, n = 14; KO, n = 8). No preference was given to animal gender. All of the animals were treated and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All of the studies were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee.

TAA Induction and Video Micrometry

The operative technique for TAA induction was based on aneurysm models reported previously6,16–18 and has been described in detail.

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previously. Briefly, the mouse was anesthetized (2% isoflurane), the descending thoracic aorta exposed via a left thoracotomy, and digital images of the aorta were obtained using a color CCD camera (KP D208; Hitachi Kokusai Electric Inc.) linked to a laptop computer with digital imaging software (WinTV2000; Hauppauge Computer Works, Inc.). Aortic diameter measurements were made via a digital video caliper (DMZR; Techni-Quip). A 3×1×10 mm sponge soaked in 0.5 mol/L of CaCl2 was placed on the distal half of the descending thoracic aorta under direct vision. After 15 minutes, the sponge was removed, the chest was irrigated liberally with normal saline, and the lung was reexpanded. The overall mortality rate was 35% because of pulmonary complications, and no cases of aortic rupture were observed.

We published results previously with this model using confocal microscopy on formalin-fixed specimens and documented a 25% increase in aortic diameter after 4 weeks. We believe that the in vivo aortic measurement used in the present study gives a more accurate representation of the true diameter changes within the nonpreserved, physiologically pressure-distended aorta over time.

At 4 weeks, the animals underwent thoracotomy, exposure of the descending thoracic aorta, and diameter measurement. The animals were euthanized with an intracardiac cadmium chloride (0.1 mmol/L) injection. The aorta was carefully harvested from its root to the iliac bifurcation and divided into ascending (ending at the left subclavian artery), descending thoracic (aneurysm segment, ending at the diaphragm), and abdominal portions.

### Gelatin Zymography

Substrate zymography was performed to assess the relative gelatinase (MMP-2 and MMP-9) content and has been described in detail previously. Briefly, harvested 4-week and unoperated control aortic segments were snap frozen. Processed aortic extracts (5 μg of total protein) were subjected to electrophoretic separation over a substrate containing gelatin (1 mg/mL type III gelatin, Sigma). All of the measurements were performed in duplicate, and the zymographic signals were analyzed using densitometric methods (Gel Pro Analyzer, Media Cybernetics) to obtain 2D integrated optical density values.

### MMP-2 Activity Assay

Endogenous MMP-2 activity was measured by an MMP-2 activity assay system (RPN 2631, Amersham Pharmacia Biotech) as described previously. Briefly, the proform and active form of MMP-2 in the aorta homogenate (25 μg of total protein) was allowed to bind to a monoclonal MMP-2 antibody (12 hour; 4°C), which was immobilized on a 96-well microtiter plate. After the wells were washed vigorously, an enzyme substrate solution and a chromogenic peptide substrate S-2444 were added. The reaction was allowed to proceed at 37°C for 6 hours, and the absorbance at 405 nm was recorded. MMP-2 proteolytic activity was determined from a colorimetric reaction (Multiskan MCC/340, 405 nm, Fisher Scientific). Actual MMP-2 activity was calibrated through the use-activated recombinant standard. Because the absorbance change is linear with respect to the square of incubation time, absorbencies for MMP-2 standards and aorta homogenate samples were converted to rates of change of MMP activity. The antisera used recognizes both proforms and active forms of MMP-2 and does not cross-react with other MMPs and tissue inhibitors of metalloproteinase (TIMPs).

MMP-2 activity was then expressed as a percentage of change from respective unoperated values.

### Smooth Muscle Actin/MMP-2 Colocalization

Longitudinal sections of the entire thoracic aorta were placed in histology freezing medium (Triangle Biomedical Sciences) and flash frozen. Full-thickness sections were then fixed in 3% paraformaldehyde, washed, and incubated with anti-α-smooth muscle actin (Sigma, A-2547, 1:500 dilution) and monoclonal antibody against MMP-2 (Chemicon AB809, 1:250 dilution) for 2 hours at room temperature. The specifically bound primary antisera was then conjugated with a corresponding fluorescent secondary antibody conjugated with a corresponding fluorescent secondary antibody only and IgG controls were negative.

### MMP-12 Immunoblotting

The abundance of MMP-12 was examined in aortic extracts by immunoblotting as described previously. Briefly, TAA extracts (10 μg of total protein) were loaded onto a 4% to 12% SDS-PAGE and subjected to electrophoretic separation. The separated proteins were then transferred to a nitrocellulose membrane. Membranes were probed using a polyclonal antibody corresponding to MMP-12 (0.2 μg/mL, AB19051, Chemicon). Membranes were then washed and incubated in horseradish peroxidase-conjugated antirabbit antibody (1:5000 dilution, Vector). After incubation with the secondary antibody, immunoreactive signals were detected by chemiluminescence (Western Lighting, Perkin Elmer). Prestained molecular weight markers (Invitrogen) were used to assess molecular weight and to ensure adequate protein separation and transfer. The relative abundance of MMP-12 in the aorta extracts was analyzed using densitometric methods, and values were expressed as a percentage of the MMP-12 abundance in unoperated aortic extracts. Secondary antibody-only controls were negative.

### Statistical Analysis

All of the statistical procedures were performed using the Statate statistical software package (Statata Corporation). MMP-2 activity assay results and aortic diameter results expressed as a percentage of baseline (BL) or unoperated values were compared using 2-tailed Student's t test analysis. Comparisons of aortic wall diameters and zymographic and immunoblotting analysis within and between experimental groups were made using ANOVA with post hoc Bonferroni corrections. Data are presented as mean±SEM, and values of P<0.05 were considered to be statistically significant.

### Results

#### TAA Induction and Aortic Diameter

Dilatation of the descending thoracic aorta (TAA) was observed over time in both murine phenotypes compared with BL measurements, with a significant increase in aortic diameter in the WT group compared with the KO animals (Figure 1).Expressed as a percentage of BL, the WT descending thoracic aorta diameter increased to 138±5% compared with a 120±4% increase observed in the KO
animals (P<0.05, WT versus KO). No gender-related differences in aneurysm size were observed.

Gelatin Zymography

A 92-kDa band corresponding to MMP-9 was seen in all of the WT aortas but was absent in the MMP-9 KO mice (Figure 2). Zymography for MMP-2 revealed bands at 72 and 66 kDa corresponding to the latent proform and the active form, respectively (Figure 2, bottom). Whereas the latent form of MMP-2 appeared to be increased in both phenotypes 4 weeks after CaCl₂ treatment, the active form of MMP-2 was most abundant in the KO descending thoracic aorta, corresponding to the aneurysm site. The quantitative results from the zymographic analysis for total MMP-2 is shown in Figure 3. The unoperated KO animals had significantly less zymographic MMP-2 levels than the WT unoperated animals. However, in contrast to the WT mice, a significant increase in total MMP-2 was seen at the aneurysm site of the KO animals compared with KO unoperated animals.

Figure 2. Zymographic analysis of the harvested aortic segments. Top: a 92-kDa band corresponding to MMP-9 was observed in all WT unoperated (Unop) and treated (TAA) aortas. No MMP-9 bands were seen in the MMP-9 KO mice. Bottom: representative MMP-2 zymographic bands at 72 and 66 kDa corresponding to the latent proform and the active form, respectively, are shown. There was an increase in the latent form of MMP-2 in both the WT and KO mice 4 weeks after CaCl₂ treatment. In addition, the active form of MMP-2 was most abundant in the KO descending thoracic aorta (Desc, circled), corresponding to the aneurysm site. Asc indicates ascending aorta; Abd, abdominal aorta.

Figure 3. Zymography for total MMP-2. The results are expressed as a percent change in optical density compared with the WT unoperated mice, which are shown on the left as 3 white bars at 100%. The descending thoracic aortic segment is labeled TAA as the site of CaCl₂ treatment. The unoperated MMP-9 KO animals had significantly less total MMP-2 than the WT unoperated animals. Total MMP-2 abundance was significantly increased in MMP-9 KO animals in the aneurysm site compared with KO unoperated animals. This effect was not seen in the WT mice. Asc indicates ascending aorta; Desc, descending thoracic aorta; Abd, abdominal aorta.

MMP-2 Activity Assay

Figure 4 summarizes the optical density values for the active (66 kDa) form of MMP-2 expressed relative to the segments for the unoperated animals from each respective group. In contrast to the WT mice, a trend was seen toward increased abundance of active MMP-2 in all of the aortic segments in the 4-week MMP-9 KO mice, with a substantial increase in the TAA site.

Figure 4. Optical density values for the active (66 kDa) form of MMP-2 expressed relative to the segments for the unoperated animals from each respective group. The descending thoracic aortic segment is labeled TAA as the site of CaCl₂ treatment. There is a trend toward increase in abundance of active MMP-2 in all of the aortic segments in the 4-week MMP-9 KO mice, with a significant increase in the aneurysm site, an effect not seen with the KO mice.
Smooth Muscle Actin/MMP-2 Colocalization
Representative photomicrographs of murine descending thoracic aortas using confocal microscopy and colocalization of smooth muscle actin and MMP-2 are shown in Figure 6. In control aortic sections, MMP-2 was localized to all regions of the aorta and was clearly visible in the medial layer. Staining indicative of smooth muscle cells (SMCs) or myofibroblasts was confined to the perimeter of the aorta. At 4 weeks after induction of a TAA, clear margination of smooth muscle actin staining occurred. Moreover, clear colocalization of MMP-2 and smooth muscle actin could be appreciated throughout the aneurysmal section in WT mice, especially in the media closest to the aortic lumen. However, in the KO mice, the distribution of α-smooth muscle actin-positive cells was reduced and more patchy in distribution, and colocalization of MMP-2 occurred in regions of the aneurysmal section more remote (ie, in the adventitia) from the media.

MMP-12 Immunoblotting
Figure 7 summarizes MMP-12 levels in the thoracic aorta segments. MMP-12 levels, expressed as a percentage of respective unoperated aortic segments, were relatively lower in the KO animals, with a significant decrease occurring in the KO TAA segment.

Discussion
Vascular ECM remodeling is critical for the development of aortic aneurysm disease, a process that involves the MMP enzyme system. Additional evolution in the understanding of MMPs may allow for the development of novel strategies to reduce, halt, or even reverse aneurysm progression. In addition, identification of the proteolytic and nonproteolytic effects of the specific MMP cassette responsible for adverse aortic ECM remodeling could allow for inhibition of isolated MMPs, thereby providing a more favorable side effect profile than that potentially associated with global MMP inhibition. With regard to specific MMP types, numerous reports have shown increased abundance and activity of the gelatinases (MMP-2 and MMP-9) in animal models of abdominal aortic aneurysms and in human aortic specimens taken at the time of surgery. This report adds several unique findings to this body of knowledge. First, MMP-9 gene deletion attenuated aortic dilatation in a murine model of TAAs. Second, this study demonstrated a decreased abundance of MMP-2 in the unoperated aorta of MMP-9-deficient animals. Third, there was a large increase in active MMP-2 in the aneurysm segment of the MMP-9 KO animals compared with the WTs. Despite this increase, the net MMP-2 activity within the KO TAA segment was reduced compared with the WT TAA segments. Fourth, confocal immunohistochemistry demonstrated colocalization of MMP-2 and smooth muscle actin in differential patterns within the WT and KO TAA segments. These results, in conjunction with decreased MMP-12

Figure 5. MMP-2 activity expressed relative to the segments for the unoperated animals from each respective group. The results show an increase in MMP-2 activity in the WT TAA segment.

Figure 6. Representative photomicrographs of murine descending thoracic aortas using confocal microscopy and colocalization of smooth muscle actin (red) and MMP-2 (yellow). Left: control aortic sections show localization of MMP-2 to all regions of the aorta and within the medial layer. Red staining indicative of SMCs was confined to the perimeter of the aorta. Middle: in the WT TAA segments, clear margination of smooth muscle actin staining occurred, and colocalization of MMP-2 (orange staining) was seen in a uniform distribution throughout the aneurysmal section in WT mice, especially in the media closest to the aortic lumen. Right: in mice devoid of MMP-9, the margination of α-smooth muscle actin-positive cells was reduced and more patchy in distribution, and colocalization of MMP-2 occurred in regions of the aneurysmal section more remote (ie, in the adventitia) from the media. AL indicates aortic lumen.
amounts observed in the KO TAAs, favor increased medial destruction and dilatation in the WT TAA.

**TAA Induction, MMP-9, and Aortic Diameter**

The CaCl₂ model used in this study is a modification of the methods originally used by Gertz et al. in the carotid artery and subsequently by Freestone et al., Chiou et al., and Longo et al. in the abdominal aorta. This model recapitulates the clinical situation where aortic aneurysmal dilatation is discovered and followed over time with serial imaging studies without referral for surgery because of insufficient size of the aneurysm to impose a significant rupture risk. Investigations using models such as that used in the present study could allow discovery of an active treatment strategy that could be applied to this patient population to reduce, arrest, or even reverse aneurysm progression.

Whereas application of this model in the murine abdominal aorta resulted in a diameter increase of up to 74%, a 38% increase in diameter was observed in the present study in the thoracic aorta. Several factors may account for this size difference. First, the thoracic aorta is inherently thicker than the abdominal aorta. Second, the intercostal vessels and adherent right pleura allow exposure of only a portion of the descending thoracic aortic surface. More circumferential exposure for CaCl₂ contact could account for a greater diameter increase in the abdominal aorta. Third, murine strain differences may cause subtle variations in aortic structure and biology resulting in a varied response to CaCl₂ treatment. Fourth, regional biological heterogeneity within and around the aorta may account for some of the diameter difference.

Gelatinase B (MMP-9) is one of the prominently expressed MMPs in aortic aneurysms. It is produced by infiltrating neutrophils and macrophages and is not present in large amounts in nondiseased aorta. MMP-9 has activity against insoluble elastin fibers and is the most abundant elastolytic protease produced by human aneurysm tissue. Clinically, the amount of MMP-9 in aneurysm tissue correlates positively with aneurysm diameter. Elevated circulating levels of MMP-9 regularly occur in the plasma of patients with aneurysms. Studies using mice with targeted MMP-9 gene deletion indicate that MMP-9 plays a critical role in abdominal aneurysmal degeneration.

It is not surprising, in the light of previous work, that a decrease in murine TAA size was observed with MMP-9 gene deletion. However, past reports have documented near complete abolishment of murine abdominal aneurysm production with MMP-9 gene deletion, in contrast to the present study where the aortic diameter increase was blunted with MMP-9 gene deletion but was still significantly greater than the unoperated state. This disparity may be accounted for by the differences in murine strain and regional aortic heterogeneity cited earlier. In addition, other proteolytic enzymes (such as the plasmin system) are likely activated within the aorta, which operates independently of MMP-9 activity and which could produce some dilatation. In addition, Lessner et al. demonstrated that MMP-9 gene deficiency reduced both vascular remodeling and sensitivity to macrophage infiltration within experimental atherosclerotic lesions in an apolipoprotein E KO mouse model. The present study supports these results, because reduced MMP-12 (macrophage elastase) was observed within the KO aneurysmal segment compared with unoperated control segments.

**Effect of MMP-9 Gene Deletion on MMP-2**

Gelatin zymographic analysis showed that MMP-9 gene deletion decreased total MMP-2 abundance in the entire
unoperated aorta compared with WT animals. However, CaCl₂ treatment resulted in a significant total MMP-2 increase in the aneurysmal segment compared with the WT aorta. In addition, the active MMP-2 fraction was greatly increased in the KO aneurysmal segment compared with the WT animals. Despite this, MMP-2 activity was increased within the WT aneurysm segment and colocalized with smooth muscle actin to the inner media of the aorta. These above results suggest multiple interactive effects of MMP-9 on MMP-2 at levels ranging from MMP-2 gene transcription to MMP-2 proteolytic activity. In addition to proteolysis, MMP-9 has as substrates a number of proinflammatory molecules and chemokines, including tumor necrosis factor α, transforming growth factor β, endothelin 1, and interleukin 1β and also processes the integrin-matrix interface. Each of these substrates can induce MMP transcription by the generation of transcription factors including activator protein 1, activator protein 2, and nuclear factor κB. All of these effects correlate with MMP-2 transcription, which is known to be induced by endothelin 1 and interleukin 1β. Therefore, the loss of the MMP-9 gene could significantly alter the extracellular and intracellular MMP transcription signaling milieu and explain the decrease in total MMP-2 abundance in the unoperated KO mouse aorta. However, CaCl₂ treatment may superimpose a proinflammatory state, which could additionally modify the extracellular environment in favor of increased MMP-2 gene transcription and, therefore, total MMP-2 abundance as seen in the present report. The exact nature of this shift from inhibition of MMP-2 transcription to activation is currently unknown and warrants additional study.

MMP-2 activation from its latent proform is a complex event involving binding to the endogenous MMP inhibitor/activator TIMP-2 and additional processing by MMP-14 (membrane-type MMP-1). Increased active MMP-2 observed in the present study may reflect a modification of this complex TIMP-2/membrane-type MMP-1/pro-MMP-2 interaction as a result of MMP-9 gene deletion, and/or it may represent an equilibrium shift secondary to increased availability of pro-MMP-2. However, despite this increase in overall MMP-2 amount, effective MMP-2 activity was reduced in the KO TAA segments. The reason for this observation is unknown but may reflect increased binding to MMP-2 by endogenous inhibitors as a result of upregulated synthesis or may represent a mass binding effect in the absence of MMP-9. Additional investigations are required to explore these possibilities.

Colocalization studies revealed differential distribution of MMP-2 within the aneurysm wall in a pattern that favors medial destruction in the WT mice. Recent studies by Johnson and Galis indicate that MMP-9 exerts a crucial role in SMC migration into areas of intimal hyperplasia and organization of local ECM collagen. These results are supported in the present study, where clear colocalization of SMC actin and MMP-2 within the inner media occurred in the WT but not the KO TAA segments. This suggests that MMP-9 may exert an additional influence on MMP-2 formation in that MMP-9 is required to allow SMC and/or myofibroblast migration (with subsequent MMP-2 secretion) into critical sites for medial destruction. Furthermore, cooperativity between these 2 enzymes is reflected in past work which has shown that MMP-2, acting as an interstitial collagenase, may cleave of the elastin triple helix into single α chains, which could be additionally degraded by MMP-9. The cooperative nature of the gelatinase subclass of MMPs is supported by work in an abdominal aortic aneurysm model by Longo et al.

**Study Limitations and Conclusions**

There are certain limitations associated with this report. This study was conducted using a murine system; therefore, extrapolation to human TAAs must be done with caution. In addition, TAA production was examined at 4 weeks after CaCl₂ treatment; thus, the changes associated with an additional temporal evolution of TAA formation remain unexplored. Furthermore, this report describes the changes in MMP-2 and MMP-12 associated with MMP-9 gene deletion. It remains unclear whether and to what degree MMP-9 gene deletion affects other MMPs or the endogenous inhibitors of the MMPs within the aorta or how TAA production is influenced by these changes.

In conclusion, the above results demonstrate attenuation of murine TAA production with MMP-9 gene deletion and suggest a complex cooperative effect between MMP-2 and MMP-9. These data hold clinical relevance in that they suggest that therapeutic strategies designed to limit thoracic aneurysm progression need not be targeted for both MMP-9 and MMP-2. In addition, MMP-2 may not be an appropriate candidate as a plasma marker to track aneurysm progression and prognosis.

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**References**

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