Effects of tissue inhibitor of metalloproteinase 2 deficiency on aneurysm formation

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Objective: Matrix metalloproteinase (MMP)-2 has been shown to play a pivotal role in aortic aneurysm formation. Its activation requires formation of a trimolecular complex of MMP-2, tissue inhibitor of metalloproteinase-2 (TIMP-2), and membrane type 1 (MT1)-MMP, which is attached to the cell surface. At higher concentrations, TIMP-2 becomes an inhibitor of MMP-2. Thus, TIMP-2 could both augment and inhibit matrix degradation. This study was undertaken to define the net effect of TIMP-2 on matrix destruction and aneurysm formation.

Methods: The abdominal aortas of wild-type and TIMP-2–deficient (TIMP-2−/−) mice were exposed to 0.25 mol/L CaCl2 or 0.9% NaCl for 15 minutes after laparotomy. Aortic diameters were measured before treatment and 6 weeks after aneurysm induction. In addition, aortic tissues were studied for MMP-2 activation by zymography, and matrix structure was studied by connective tissue staining.

Results: The aortic diameter increased in both wild-type and TIMP-2−/− mice. The increase in the TIMP-2−/− mice was significantly smaller after CaCl2 treatment (51% ± 5%) compared with the diameter of wild-type mice (67% ± 4%). Connective staining of aortic sections from the CaCl2-treated mice revealed disruption and fragmentation of the medial elastic lamellae in both wild-type and TIMP-2−/− mice. Zymographic analysis showed that active MMP-2 levels were decreased in TIMP-2−/− aortas compared with wild-type mice.

Conclusions: Targeted deletion of TIMP-2 results in attenuation of aneurysm development. Despite its name as an inhibitor of MMPs, TIMP-2 promotes aortic enlargement in vivo, presumably through its role as a cofactor in the activation of MMP-2.

Clinical Relevance: Abdominal aortic aneurysmal (AAA) disease is a potentially fatal disorder that screening studies have detected in 2% to 9% of the general population. Medical therapy designed to inhibit the progression of small aneurysms includes control of hypertension and smoking cessation; neither of these measures is of proven benefit. Effective and directed medical treatments for small AAs await elucidation of key etiologic factors. Understanding precisely which molecules mediate AAA development, and blocking the activity of these molecules, could lead to important new therapies. Through our research, we have found that tissue inhibitor of metalloproteinase (TIMP)-2 has a role in this process in an experimental model of aortic aneurysms. We believe that TIMP-2 promotes aortic enlargement in vivo by activating matrix metalloproteinase 2.

Abdominal aortic aneurysms (AAA) represent a complicated and dynamic remodeling process in which the aorta enlarges to a point at which rupture may occur. The structural matrix proteins, collagen and elastin, are durable and relatively resistant to degradation by all but a few specific enzymes. Studies of human AAA tissues and animal models of AAA consistently show increased production of matrix metalloproteinases (MMPs), a family of proteases capable of efficiently degrading matrix macromolecules. In addition, aneurysm tissue exhibits altered tissue inhibitor/MMP ratios, in association with disruption of the orderly lamellar structure of the aorta.

To date, the MMP family is composed of 24 members classified into 5 major subfamilies: collagenases, gelatinases, elastase, stromelysins, and membrane-type MMPs (MT-MMPs) (Table 1). MMPs are typically synthesized as latent zymogens and require proteolytic cleavage to achieve their catalytic activity. This posttranslational modification is one of the key steps in modulating local matrix destruction by MMPs. The extracellular activation of MMP-2, one of the MMPs essential for aneurysm formation, occurs through the formation of a trimolecular complex formed on the cell surface. MMP expression and activity are controlled by the endogenous physiological tissue inhibitors of MMPs (TIMPs). It is believed that disruption of the MMP/TIMP balance is responsible for the excessive elastic degradation seen in AAA. The TIMP family currently includes four different members (TIMP-1 to TIMP-4). TIMP-1 is a known specific inhibitor of MMP-1 and MMP-9. Deletion of the TIMP-1 gene results in increased and continued progression of aneurysm formation in a mouse aortic aneurysm model. At higher levels, TIMP-2 selectively inhibits MMP-2 gelatinolytic activity in vitro. At low concentrations, TIMP-2 augments pro–MMP-2 activation by the formation of a trimolecular complex formed of...
Methods

Mice. The homozygous TIMP-2 gene knockout (TIMP-2/−) mice were obtained from Cornell University, New York, NY (Dr Paul D. Soloway). These mice were back-crossed seven times into a C57BL/6 background such that they shared 99.22% genetic similarity with C57BL/6. The homozygote TIMP-2 gene knockout mice breed normally, and all were bred from the homozygous mice obtained from and genotyped by Dr Soloway. The phenotype was verified in our laboratory by using reverse zymography, which confirmed the absence of TIMP-2 (Fig 1). The appropriate control mice, the standard C57BL/6 mice, were purchased from The Jackson Laboratory (Bar Harbor, Me). All experiments were performed in accordance with the guidelines of the University of Nebraska Medical Center Animal Care Committee for the use and care of laboratory animals. All mice were maintained in a pathogen-free animal facility.

Aneurysm induction model. TIMP-2−/− mice and their controls (aged 8 weeks) underwent surgery as described previously. Briefly, the mice were anesthetized with intraperitoneal 2,2,2-tribromoethanol (Avertin; Sigma, St Louis, Mo) at 200 mg/kg before undergoing laparotomy. The abdominal aorta between the renal arteries and bifurcation of the iliac arteries was isolated from the surrounding retroperitoneal structures. The diameter of the aorta was measured in triplicate midway between the renal artery origin and iliac artery bifurcation. After baseline measurements, 0.25 mol/L CaCl2 was applied to the external surface of the aorta for 15 minutes. The aorta was then rinsed with 0.9% sterile saline, and the incision was closed. NaCl (0.9%) was substituted for CaCl2 in sham control mice. Six weeks later, the mice underwent laparotomy and dissection. Measurements were repeated at the same location in the mid infrarenal aorta. Typically, there was diffuse, homogeneous dilatation of the infrarenal aorta in wild-type mice and, to a lesser extent, in the knockout mice. The aorta was collected for zymographic analysis of MMP proteins. The aorta was perfusion-fixed with 10% neutral buffered formalin for histologic studies.

Histologic analysis. After perfusion-fixation, abdominal aortic tissues were embedded in paraffin and cut into 4-μm sections. Standard Masson trichrome staining was performed. The slides were examined and photographed with light microscopy (Kodak, Tokyo, Japan; magnification, ×20).

Gelatin zymography. Aortic proteins were extracted as previously described. The protein concentration for aortic proteins was standardized with Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif). Gelatin zymography was performed as described previously by Longo et al with 0.8% gelatin in a 10% sodium dodecylsulfate-polyacrylamide gel. The molecular sizes of gelatinolytic activities were determined by using protein standards (Bio-Rad).

Reverse zymography. Aortic proteins were separated by electrophoresis on 17% sodium dodecylsulfate-polyacrylamide gel copolymerized with 0.8% gelatin and 0.16 μg/mL MMP-2. After electrophoresis, the sodium dodecylsulfate was removed from the gel by washing in 2.5% Triton X-100 for 2 hours. The gels were incubated at 37°C overnight in development buffer (50 mmol/L Tris-HCl [pH 7.5], 5 mmol/L CaCl2, 1 μmol/L ZnCl2, and 3 mmol/L NaNO3), stained with 0.5% Coomassie brilliant blue G-250 for 20 minutes, and destained in gel-destaining buffer (40% methanol and 10% glacial acetic acid) until the background was clear.

Reverse transcription-polymerase chain reaction. Aortas from wild-type mice were collected at 6 weeks after treatment with 0.9% NaCl or 0.25 mmol/L CaCl2. Total RNA was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA), and TIMP-2 expression was analyzed by reverse transcription-polymerase chain reaction. First-strand complementary DNAs were synthesized by reverse transcription by using the first-strand synthesis kit (Invitrogen) primed with oligo(dT)20. The polymerase chain reaction was performed by using Platinum Taq DNA polymerase (Invitrogen) and primers specific for TIMP-2. Polymerase chain reaction products were analyzed by using Table I. Matrix metalloproteinases

<table>
<thead>
<tr>
<th>MMP family</th>
<th>Members</th>
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<tbody>
<tr>
<td>Gelatinase</td>
<td>MMP-2, MMP-9</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>MMP-3</td>
</tr>
<tr>
<td>Elastase</td>
<td>MMP-12</td>
</tr>
<tr>
<td>Collagenase</td>
<td>MMP-1, MMP-13</td>
</tr>
<tr>
<td>Membrane type</td>
<td>MT-MMP (1-4)</td>
</tr>
</tbody>
</table>

MMP, Matrix metalloproteinase; MT, membrane type.
MMP-2 in vitro. In addition, messenger RNA levels of TIMP-2 are required for efficient activation of pro-MMP-2. To test this hypothesis, TIMP-2 deficiency would decrease MMP-2 activation and inhibit aneurysm formation in a murine AAA model. Alternatively, deficiency of TIMP-2 could lead to promotion of AAA if the primary effect, in vivo, is inhibition of MMP-2 activity. To test this hypothesis, TIMP-2−/− mice and wild-type control mice of the same background were tested for their response to CaCl2 aneurysm induction. Six weeks after periaortic application of CaCl2, wild-type control mice showed a 67% ± 4% increase in aortic diameter after CaCl2 treatment. The aortic diameter of the TIMP-2−/− mice also increased significantly (51% ± 3%). The final aortic diameter in the TIMP-2−/− mice was, however, less than the final diameter of the wild-type controls (P = .0159; Table II). There was no change in the aortic diameter of the sham mice (NaCl substituted for CaCl2). If the primary physiologic role of TIMP-2 were MMP inhibition, one would expect augmentation of the aneurysm in its absence. In contrast, we observed moderate inhibition in the absence of TIMP-2, showing that overall, it promotes aneurysm formation.

Histologic changes. Compared with mice treated with NaCl, in which the aorta appeared normal (Fig 3, a and b), connective staining of aortic sections from the CaCl2-treated wild-type and TIMP-2−/− mice revealed a similar degree of disruption and fragmentation of the medial elastic lamellae (Fig 3, c and d). There were no discernible differences on trichrome staining between wild-type and TIMP-2−/− mice.

MMP-2 activation. The processing of MMP-2 in mouse aortas was examined by gelatin zymography (Fig 4). Processed MMP-2 was identified not only in the aortic extracts of wild-type mice, but also in the extracts of TIMP-2−/− mice. This is consistent with the observations of Morrison et al,19 who have demonstrated some MMP-2 processing through pathways independent of TIMP-2. That TIMP-2 has a primary role in MMP-2 activation was shown by the significant decrease in levels of processed MMP-2 from TIMP-2−/− mice compared with wild-type mice (Fig 4, a; P = .0183). These findings suggest that the attenuated aortic dilatation seen in the TIMP-2−/− deficient mice is attributable to the lesser degree of MMP-2 activation (Table II).

RESULTS

Aortic diameter change. Increased expression of MMP-2 has been observed in AAA tissue.16,17 Transgenic mice with no MMP-2 expression are resistant to elastin degradation and the formation of AAA.2 There is evidence that TIMP-2 is required for efficient activation of pro-MMP-2 in vitro.18 In addition, messenger RNA levels of TIMP-2 were significantly increased in aneurysmal CaCl2-treated aorta relative to non-aneurysmal NaCl-treated controls (Fig 2). Therefore, we hypothesized that TIMP-2 deficiency would decrease MMP-2 activation and inhibit aneurysm formation in a murine AAA model. Alternatively, deficiency of TIMP-2 could lead to promotion of AAA if the primary effect, in vivo, is inhibition of MMP-2 activity.

![Graph showing relative levels of TIMP-2 mRNA in CaCl2-treated aorta](image)

Fig 2. Expression of tissue inhibitor of metalloproteinase (TIMP)-2. Six weeks after 0.9% NaCl or 0.25 mol/L CaCl2 treatment, wild-type mouse aortas were harvested. Total RNA from each aorta was extracted, and TIMP-2 messenger RNA (mRNA) levels were examined by reverse transcription-polymerase chain reaction. Alpha actin was used as the internal standard. The gel is representative of three separate experiments. The TIMP-2 expression is normalized to α-actin expression. The relative levels of TIMP-2 mRNA in CaCl2-treated aorta to NaCl-treated aorta are expressed in the bar graph.
thought to play a role in weakening the aortic wall. TIMPs are ubiquitously produced in the extracellular aortic milieu, and unstimulated smooth muscle cells express high levels of TIMP-1 and TIMP-2 messenger RNA and protein. TIMP-1 and TIMP-2 have been extensively studied in the aorta. TIMP-1 can inhibit MMP-1, -9, and -3 activity. A number of studies have found an increase in TIMP-1 levels in AAA. Overexpression of TIMP-1 in one model of AAA inhibited aneurysm formation and led to the accumulation of matrix proteins. Further studies using TIMP-1 gene knockout mice showed that deletion of TIMP-1 augments aneurysm formation.

We have demonstrated in a previous study that MMP-2 is required for aneurysm formation in the CaCl\textsubscript{2} murine aneurysm model. Aneurysm formation and matrix degradation are completely abrogated by the targeted deletion of MMP-2. TIMP-2 is a 22-kd protein that has a curious dual role in regulating MMP-2. At low concentrations, TIMP-2 forms a ternary complex with MT1-MMP and pro–MMP-2 on the cell surface. Modeling studies suggest that this results in binding of an additional pro–MMP-2 molecule. An adjacent TIMP-free active MT1-MMP molecule then activates the pro–MMP-2 to MMP-2. At significantly higher concentrations, TIMP-2 blocks MT1-MMP-mediated degradation of TIMP-2.

Table II. Changes in aortic diameter in C57BL/6 WT controls and C57BL/6 TIMP-2\textsuperscript{-/-} mice after treatment with NaCl or CaCl\textsubscript{2}

<table>
<thead>
<tr>
<th>Variable</th>
<th>NaCl</th>
<th>CaCl\textsubscript{2}</th>
<th>NaCl</th>
<th>CaCl\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{n}</td>
<td>11</td>
<td>11</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>Pretreatment (\mu m)</td>
<td>555 ± 8.3</td>
<td>533 ± 8.3</td>
<td>510 ± 14.3</td>
<td>502 ± 5.4</td>
</tr>
<tr>
<td>Posttreatment (\mu m)</td>
<td>551 ± 7.7</td>
<td>887 ± 14.8*</td>
<td>547 ± 21.1</td>
<td>757 ± 14.7*†</td>
</tr>
<tr>
<td>% Increase</td>
<td>3.4</td>
<td>67.3</td>
<td>7.2</td>
<td>51.1</td>
</tr>
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</table>

WT, Wild type; TIMP, tissue inhibitor of metalloproteinase.

Aortic diameters were measured before NaCl or CaCl\textsubscript{2} application (pretreatment) and before death (posttreatment). Measurements of aortic diameter are expressed as mean ± SE. The percentage increase represents the percentage change compared with the pretreatment diameter.

\*\(P < .01\), paired Student \(t\) test, comparing the pretreatment value with the final diameter.

†\(P < .05\), ANOVA, final diameter of CaCl\textsubscript{2} TIMP-2\textsuperscript{-/-} vs CaCl\textsubscript{2} treated WT control mice.

Fig 3. Histologic changes in mouse aorta by trichrome staining. One example of NaCl or CaCl\textsubscript{2} treatment of C57BL/6 wild-type (WT) controls (a and c) and C57BL/6 tissue inhibitor of metalloproteinase (TIMP)\textsuperscript{-/-} (b and d) mice is shown. The staining is representative of three to five samples with similar results. The matrix damage is profound in the CaCl\textsubscript{2}-treated WT mice. There is also significant matrix destruction in the TIMP-2\textsuperscript{-/-} mice.
In this study, the role of TIMP-2 in AAA was studied by using TIMP-2−/− mice. In the absence of TIMP-2, mice developed significant aortic enlargement in response to CaCl₂ treatment. The aneurysms, however, were significantly smaller than those that developed in the corresponding wild-type controls. This is in contrast to studies using the elastase model of aneurysms with TIMP-1−/− mice. The TIMP-1−/− mice developed larger aneurysms than the wild-type control mice, thus confirming that TIMP-1 acts primarily as an inhibitor of MMPs. To evaluate the mechanism of the effects of TIMP-2, gelatin zymography was used to assess the proportion of latent and processed MMP-2. This showed a decrease in the processed or active form of MMP-2 in TIMP-2−/− mice. The decrease in active MMP-2 is consistent with the known participation of the TIMP-2 molecule in the activation of MMP-2. There is some MMP-2 activation in TIMP-2−/− mice, thus illustrating that alternative, TIMP-2−/− independent, pathways for MMP-2 activation are operative. This finding is consistent with the observations of Morrison et al., who observed that MMP-2 could be activated through an alternative pathway involving MT2-MMP. It is also possible in knockout mice that there may be compensatory changes, such as the upregulation of other MT-MMPs, that could affect the phenotype of the mouse.

This study was conducted to determine the net effect (promotion vs inhibition of matrix destruction) of TIMP-2 in vivo in a model of AAA. If the primary effect of TIMP-2 were similar to the MMP inhibitory effects of TIMP-1, we would have observed larger aneurysms in CaCl₂-exposed TIMP-2−/− mice compared with wild-type control mice. Instead, we observed that the dilatation and matrix damage of CaCl₂-treated TIMP-2−/− mice were significantly less than those of CaCl₂-treated wild-type mice. That a small proportion of MMP-2 in the TIMP-2−/− mice is processed to the active form confirms that alternative TIMP-2–independent pathways were operative in vivo. Even though there was only one fourth as much active MMP-2 in the CaCl₂-treated TIMP-2−/− mice, there was significant dilatation and matrix damage, thus indicating the efficiency of processed MMP-2 against structural matrix macromolecules of the aorta. In vitro studies have shown that TIMP-2 can act as both a promoter and an inhibitor of matrix destruction in a concentration-dependent manner through activation of MMP-2 or inhibition of active MMP-2. In this study, using a murine model of aortic aneurysm, we showed that, in vivo, TIMP-2 acts primarily to promote rather than inhibit matrix degradation. This effect seems to be mediated through the processing of latent MMP-2 to its active form.

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AUTHOR CONTRIBUTIONS

Conception and design: WX, BTB
Analysis and interpretation: WX, BTB
Writing the article: WX, BTB
Critical revision of the article: WX, RK, JM, BTB
Final approval of the article: WX, RK, JM, BTB
Data collection: WX
Provision of materials, patients, or resources: WX, RK, JM
Statistical expertise: WX, BTB
Obtaining funding: BTB
Literature search: WX, JM
Administrative, technical, or logistic support: WX, RK

REFERENCES


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