Stromelysin-1 (Matrix Metalloproteinase-3) and Tissue Inhibitor of Metalloproteinase-3 Are Overexpressed in the Wall of Abdominal Aortic Aneurysms

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Background—Atherosclerosis is implicated in the pathogenesis of abdominal aortic aneurysm (AAA) but more often causes aortic occlusive disease (AOD). The matrix metalloproteinases (MMPs) degrade extracellular matrix and may play a central role in the pathogenesis of AAA. The aim of this study was to examine differences in the patterns of MMP and MMP inhibitor expression between AAA and AOD.

Methods and Results—The expression of mRNA for 14 MMPs and 4 tissue inhibitors of metalloproteinases (TIMPs) was estimated in samples of aortic wall from 8 patients with AAA and 8 with AOD using the reverse-transcriptase polymerase chain reaction with a synthetic multicompetitor standard. AAA wall expressed significantly more stromelysin-1 (MMP-3) (mean log10 ratio [copy enzyme cDNA/copy GAPDH cDNA], 1.9; range, 3.3 to 0.7) than the AOD wall (mean, 4; range, 5.7 to 2.4), P<0.005. TIMP-3 expression was significantly higher in AAA (mean, 1.7; range, 2.9 to 1.0) than AOD (mean, 3.6; range, 5.7 to 1.8), P<0.01. Expression of 8 other MMPs (1, 2, 7, 9, 11, 12, 14, and 17) was detected and was similar in AAA and AOD. Expression of the remaining 5 MMPs (8, 10, 13, 15, and 16) was not detected in any of the samples.

Conclusions—Both AAA and AOD walls express similar levels of a wide range of MMPs, including cell membrane–bound MT-MMPs. Stromelysin-1 (MMP-3) and TIMP-3 were, however, over expressed in the AAA samples and may be involved aneurysm pathogenesis. Stromelysin-1 could provide a target for pharmacological inhibition. (Circulation. 2002;105:477-482.)

Key Words: aneurysm ■ aorta ■ arteriosclerosis ■ metalloproteinases ■ polymerase chain reaction

Abdominal aortic aneurysm rupture is responsible for >1% of deaths in the United Kingdom each year.1 Atherosclerosis is a feature of the common form of abdominal aortic aneurysm and is thought to play an important role in the development of the disease.2 Atherosclerosis is, however, more commonly associated with narrowing of the aortic lumen.

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Aneurysm expansion is characterized by inflammation, loss of elastin, and increased turnover of collagen in the aortic wall.3-5 The inflammatory cell infiltrate seen in the tunica media and adventitia of abdominal aortic aneurysms contains B- and T-lymphocytes, plasma cells, and macrophages.8 The matrix metalloproteinases (MMPs) are a group of enzymes produced by a wide range of cell types, including the fibroblasts, smooth muscle cells, and inflammatory cells found in aneurysm wall. The MMPs can degrade the components of extracellular matrix, including elastin and collagen, and have been implicated in the proteolysis that accompanies aneurysm expansion.

There is already evidence that the concentration of some of the MMPs, particularly gelatinase A (also known as MMP-2 and 72-kDa gelatinase) and gelatinase B (also known as MMP-9 and 92-kDa gelatinase), is elevated in aneurysm wall.7 The gelatinases are relatively easy to extract from tissues and to assay their activity. However, other soluble MMPs bind strongly to extracellular matrix or cell membranes and are difficult to extract and to assay enzyme activity. These include 2 elastases produced by macrophages, matrilysin (MMP-7) and macrophage metalloelastase (MME or MMP-12). Fibroblasts, smooth muscle cells, and macrophages can also express MMPs that are bound to the cell membrane. These membrane-type metalloproteinases (MT-MMPs) not only degrade extracellular matrix but are also key activators of other MMPs.8 To date, there have been conflicting reports on the expression of matrilysin and macrophage metalloelastase in abdominal aortic aneurysm9-10 and no...
reports of MT-MMP expression in the disease. It is possible that differences in the activity of one or more of the MMPs determines whether an atherosclerotic aorta dilates orstenoses.

Many aneurysms are detected incidentally or by screening when they are small and the low risk of rupture does not justify the risks of surgery.11–13 There is no proven effective treatment for these small aneurysms, and repeated scans are used to monitor the expansion of the aneurysm until the diameter of the aneurysm is >5.5 cm. At this stage, the risk of rupture is greater than the risk of operation, and surgery is indicated. The development of endovascular techniques of aneurysm repair promises a reduction in the morbidity and mortality associated with aneurysm repair, although continued expansion of the neck of the aneurysm after the procedure can lead to detachment and failure of the device. An effective pharmacological treatment that could slow or halt this expansion might avoid surgery in small aneurysms unsuitable for repair and might also be expected to reduce late complications after endovascular aneurysm repair. If specific metalloproteinases can be identified as responsible for proteolysis in abdominal aortic aneurysms, then inhibition of these enzymes may prove effective in slowing or even preventing aneurysm expansion.14

The aim of this study was to use semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) to determine whether there was differential expression of 14 of the known MMPs and the 4 natural tissue inhibitors of MMPs (TIMPs) in human abdominal aortic aneurysm wall compared with control tissue taken from stenosing atheromatous aortic wall.

Methods

Patients

Protease expression was measured in 8 patients undergoing elective repair of abdominal aortic aneurysms (AAAs) (ages 67 to 79 years, all male) and 8 patients (ages 58 to 81 years, 6 male and 2 female) undergoing aortic bypass surgery (aortoiliac or aortofemoral grafts) for aortic occlusive disease (AOD). All patients had their operations at St Thomas’ Hospital, London, UK, between January 1997 and June 1999. Full-thickness samples of diseased aorta were taken from the anterior aspect of the infrarenal aorta at operation and immediately diced and frozen in liquid nitrogen to minimize degradation of mRNA by endogenous ribonucleases. These aortic samples are taken as a routine part of the operation and are usually discarded.

RNA Extraction

Tissues (100 mg) were pulverized while still frozen using a reciprocating ball and cup Microdismembrator (Braun). The total RNA was extracted by homogenizing the frozen pulverized tissue in 2 mL of the Trizol (Life Technologies).15 The integrity of the RNA in each sample was confirmed by running each extract on an agarose formaldehyde gel and comparing intensities of the 18S and 28S bands.

Reverse Transcription

Approximately 1 μg of the total RNA from each sample was reverse transcribed to form complementary deoxyribonucleic acid (cDNA) using a cycle kit (Invitrogen). The reaction was primed with oligo (dT) (Invitrogen), complementary to the poly (A) tail of mRNA.

Primers and Synthetic Multicompetitor cDNA Standards and PCR

Primers developed and supplied by British Biotech were selected to amplify ~220-bp fragments of GAPDH (as a housekeeping gene) and 14 MMP and 4 TIMP genes in cellular cDNA. The 20-bp primers were designed to span ~1 exon to distinguish amplicons from genomic DNA and had a 50% G/C content to minimize bias from the length of the cDNA.16 Two synthetic multicompetitor cDNA standards, pQH1 and pQH6 (British Biotech, Figure 1; EMBL accession numbers AJ315845 and AJ315846, respectively) were used for PCR quantification. The standards had oligonucleotide sequences complementary to the primer pairs for each MMP or TIMP are separated by ~300 bp.

Polymerase Chain Reaction

The PCR was carried out in a total volume of 50 μL. The reaction mix contained 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9.0 at 25°C), 1.5 mmol/L MgCl2, 0.1% Triton X-100, 200 μmol/L of each dNTP, 0.5 μmol/L of both forward and reverse primers, and 1 U Taq DNA polymerase (Promega). This mixture was placed in a thermal cycler (Techne Genius) for 35 cycles of 95°C for 15 seconds, 57°C for 220 bp in length and 72°C for 60 seconds. Aliquots of 10 μL of each amplified sample cDNA, together with a DNA ladder, were electrophoresed at 200 V on a precast 6% polyacrylamide TBE gel (Novex) and stained with ethidium bromide. Bands of ~220 bp in length corresponded to amplified DNA specific for the MMP or TIMP genes (Figure 2).

Quantitative Polymerase Chain Reaction

Serial 3-fold dilutions of the pQH1 and pQH6 standards starting at 120 pg were prepared and added to the PCR mixture (as above) with a constant amount of sample cDNA in each tube. The PCR was then run after the addition of 1 μCi 32P α-dCTP 3000 Ci/mmol (Amer-
sham) and the sample and standard cDNA separated by gel electrophoresis (conditions as above).

Incorporation of the radiolabeled nucleotide was estimated by excising the sample (~220 bp) and standard DNA bands (~300 bp), placing each in 4 mL of scintillation fluid and measuring the counts per minute (cpm) in a scintillation counter. The counts were corrected for relative dCTP concentration in the sample and standard cDNAs. The ratio of the standard to sample corrected counts were plotted against the dilution of the standard on a log-log scale, and a regression curve was generated. The value at which the concentration of sample cDNA equaled that of the standard DNA was calculated and divided by the expression of the housekeeping (ie, constantly expressed) GAPDH gene. All values were expressed as log_{10} (copy enzyme cDNA/copy GAPDH cDNA). A typical quantitative PCR analysis is shown in Figure 3.

The threshold for accurate quantification was \(10^{-6}\) copies per copy of GAPDH. If a MMP or TIMP was detected but not accurately quantifiable, it was given an arbitrary value of \(10^{-6}\), representing the approximate lowest detectable level of quantification. Statistical

Figure 2. Example of a quantitative PCR amplification. A constant amount of cDNA, prepared from a sample of abdominal aortic aneurysm, has been amplified in the presence of serial dilutions of the synthetic standard pQH1 with GAPDH or 92-kDa gelatinase primer pairs. Lane 8 is a 100-bp DNA ladder (L); standard DNA is at ~300 bp, and sample DNA is at 220 bp.

Figure 3. Typical plot showing relative expression of 1 MMP, 3 TIMPs, and the GAPDH housekeeping gene in a sample of abdominal aortic aneurysm. Quantities of sample and standard cDNA are equivalent where the regression line crosses y=1. This value is then expressed as a proportion of the GAPDH value.

Figure 4. Expression of MMPs and their TIMP inhibitors in samples of abdominal aortic aneurysm (AAA, solid symbols) and aortic occlusive disease (AOD, open symbols). Expression is shown as the ratio of (copy enzyme cDNA/copy GAPDH cDNA) and plotted on a logarithmic scale.
significance was calculated with two-sample t testing, assuming unequal variances.

**Results**

Initial studies using PCR alone showed that 9 MMPs and 3 TIMPs were expressed in 4 aortic wall samples taken from patients with AAA and AOD (Table 1).

Significantly higher levels of expression of stromelysin-1 (MMP-3) and TIMP-3 were found in the aneurysm samples than in the occlusive samples. For stromelysin-1, mRNA was detected in all 8 AAA and 5 of 8 AOD samples, giving mean AAA of 5.7 to 1.9 (range, 3.6 to 1.7) and mean AOD of 3.59 to 0.01, $P=0.0083$.

Stromelysin-3 showed a tendency toward greater expression in AAA than AOD, but this did not reach statistical significance. The mRNA was detected in 6 of 8 AAA and 5 of 8 AOD samples, with a mean expression in AAA of −3.1 to 0.7 and mean AOD of −4.0 (range, −5.7 to −2.4), $P<0.3$. There was no significant difference seen in the expression of other MMPs or TIMPs in between AAA and AOD wall.

The mean expression of the MMPs and TIMPs is summarized in Table 2. Statistical significance is indicated by the $P$ value and $t$ statistic from two-sample $t$ testing, assuming unequal variances.

**Discussion**

The extracellular matrix, particularly the heavily collagenous tunica adventitia, of the aortic wall provides a strong structural safety net that resists aortic expansion in a healthy individual. These structural proteins must be degraded for aneurysm expansion to occur. Aneurysm expansion is an active process and involves inflammation, proteolysis, and protein deposition. The MMP family of enzymes has been implicated in this degradation of the aortic wall. The MMPs are also intimately involved with development and rupture of atherosclerotic plaques. It is possible that differences in the activity of the MMPs may determine whether an atherosclerotic aorta develops aneurysmal or stenosing disease.

The MMPs are a large family of Zn$^{2+}$- and Ca$^{2+}$-dependent enzymes found in the extracellular matrix in both soluble and cell membrane–bound forms. Each MMP has substrate specificities for several different components of extracellular matrix, including different phenotypes of collagen and elastin, the major structural proteins of arterial wall. The production of MMPs is regulated at the transcriptional level, and MMP activity is additionally regulated by proteolytic cleavage of inactive proenzymes to active forms and inhibition by their specific inhibitors, the TIMPs.

Extraction and activity assay of some MMPs is relatively easy, and most previous studies have focused on these enzymes. Gelatinases A and B (also known as 72-kD gelatinase/MMP-2 and 92-kD gelatinase/MMP-9, respectively) in particular have been studied much more extensively than other MMPs with substrate gel zymography and enzyme-linked immunosorbent assay. Interpretation of

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>AAA</th>
<th>AOD</th>
<th>$P$</th>
<th>$t$</th>
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<tbody>
<tr>
<td>Gelatinase A (MMP-2)</td>
<td>−3.22</td>
<td>−1.57</td>
<td>0.36</td>
<td>−0.99</td>
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<tr>
<td>Gelatinase B (MMP-9)</td>
<td>−1.12</td>
<td>−0.85</td>
<td>0.7</td>
<td>0.4</td>
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<tr>
<td>Interstitial collagenase (MMP-1)</td>
<td>−2.81</td>
<td>−2.93</td>
<td>0.89</td>
<td>0.14</td>
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<tr>
<td>Stromelysin-1 (MMP-3)</td>
<td>−1.92</td>
<td>−4.03</td>
<td>0.0047</td>
<td>3.63</td>
</tr>
<tr>
<td>Stromelysin-3 (MMP-11)</td>
<td>−3.12</td>
<td>−4.01</td>
<td>0.29</td>
<td>1.1</td>
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<td>MT1-MMP (MMP-14)</td>
<td>−2.9</td>
<td>−2.3</td>
<td>0.31</td>
<td>−1.1</td>
</tr>
<tr>
<td>MT4-MMP (MMP-17)</td>
<td>−4.21</td>
<td>−4.3</td>
<td>0.92</td>
<td>0.11</td>
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<tr>
<td>Matrixin-1 (MMP-7)</td>
<td>−2.29</td>
<td>−2.09</td>
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<td>−0.29</td>
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<td>MME (MMP-12)</td>
<td>−2.36</td>
<td>−1.86</td>
<td>0.46</td>
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<td>TIMP-1</td>
<td>0.52</td>
<td>0.35</td>
<td>0.44</td>
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<tr>
<td>TIMP-2</td>
<td>−1.45</td>
<td>−1.57</td>
<td>0.63</td>
<td>0.49</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>−1.71</td>
<td>−3.59</td>
<td>0.0083</td>
<td>3.28</td>
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</table>

Values are expressed as the mean log$_{10}$ (copy enzyme cDNA/copy GAPDH cDNA), with $P$ and $t$ statistics.
these zymograms is, however, complicated by the presence of several inactive and partially and fully activated forms of each enzyme, although all exhibit proteolytic activity on a zymogram gel. Furthermore, the TIMP inhibitors are separated during the electrophoresis, and no estimation of their effect is possible. The expression and activity of other MMPs in aneurysmal disease is even more poorly understood because of difficulties in extracting proteases strongly bound to connective tissues and in assaying enzyme activities. The semiquantitative RT-PCR technique we used in this study allows the estimation of the expression of all these MMPs and their inhibitors. The technique is highly sensitive, and strict internal standards are necessary to avoid erroneous results. The standards used in this study allowed compensation for interassay variation as well as quantification. The RT-PCR reaction, however, only gives an estimate of mRNA production for each of the enzymes and inhibitors, because it only reflects transcriptional regulation.

This study has demonstrated that the mRNA for 9 different MMPs and 3 TIMPs are expressed in both abdominal aortic aneurysm and atherosclerotic aortic occlusive disease. Expression did not vary greatly between the two disease states for 8 of these metalloproteinases, including gelatinases A and B, interstitial collagenase, matrilysin, macrophage metalloelastase, and stromelysin-3. Two of the 4 membrane-bound MT-MMPs were detected with similar expression of MT1-MMP and minimal expression of MT4-MMP in both the aneurysmal and occlusive aortic samples.

Stromelysin-1 (MMP-3) had a highly significant 40-fold increase in mean expression in the aneurysm samples over the occlusive aortic samples. This increased expression of stromelysin-1 in the aneurysm samples was matched by a 200-fold increase in one of the tissue inhibitors of metalloproteinases, TIMP-3. There was, however, no significant difference in the expression of the other inhibitors, TIMP-1 and TIMP-2, in aneurysmal wall compared with occlusive aortic samples.

The results of this study suggest that upregulation of stromelysin-1 (MMP-3) and TIMP-3 expression may play a significant role in the expansion of an atherosclerotic aorta to form an aneurysm. The great increase in the expression of TIMP-3 in the aneurysm samples implies that there is a significant amount of protease inhibition in abdominal aortic aneurysm. Like other TIMPs, TIMP-3 is an inhibitor of many MMPs, including the gelatinases and their proenzymes, the stromelysins, matrilysin, and macrophage metalloelastase. However, unlike the other TIMPs, TIMP-3 is strongly bound to extracellular matrix, perhaps reflecting an involvement in the cellular regulation of activity of the matrix metalloproteinases.

It is difficult to estimate whether the upregulation of TIMP-3 expression seen in this study is sufficient to inhibit any increased activity resulting from increased expression of stromelysin-1. Some assay techniques, such as substrate gel zymography, remove the TIMPs during electrophoresis and do not measure the effect of the inhibitors on tissue MMP activity. Other techniques, such as soluble enzyme activity assays, can estimate TIMP inhibition but rely on efficient extraction of soluble enzyme and inhibitor from tissue samples. But extraction of insoluble enzymes and inhibitors that are strongly bound to extracellular matrix, such as stromelysin-1 (MMP-3) and TIMP-3, is notoriously difficult and has effectively prevented accurate estimation of enzyme-inhibitor activities by soluble bioassays.

The similar levels of expression of the gelatinases, interstitial collagenase, stromelysin-3, matrilysin, macrophage metalloelastase, and MT1-MMP seen in both the aneurysmal and occlusive samples imply that it is not a specific upregulation of mRNA expression of any of these enzymes that determines whether an atherosclerotic aorta weakens to form an aneurysm. However, the activity of these MMPs may be upregulated at a posttranscriptional level by activation of their latent proenzymes by the protease activators stromelysin-1 (MMP-3) and plasmin.

Stromelysin-1 (MMP-3) digests collagen and several other extracellular matrix proteins, which are important in maintaining the structural integrity of the aortic wall. It also plays a central role in the pericellular MMP activation cascade by cleaving other MMP proenzymes, such as interstitial collagenase, matrilysin, and gelatinase B, to their active forms. Immunohistochemical studies have previously localized the expression of stromelysin-1 in abdominal aortic aneurysm to macrophages within the aortic wall, additionally supporting the idea that aneurysm expansion is a chronic inflammatory process.

It seems plausible that excessive production of stromelysin-1 in an atherosclerotic aorta weakens the aortic wall and causes additional proteolysis by activating other latent, constitutively expressed metalloproteinases. The cause of this increased stromelysin-1 expression in certain individuals prone to aneurysm formation is not known, but a combination of genetic and environmental factors may be responsible. This is supported by a recent Finnish study suggesting that a polymorphism (5A MMP3 allele) in the promoter sequence for stromelysin-1 may be a genetic risk factor for developing abdominal aortic aneurysm. Pharmacological inhibition of proteolysis holds promise as a means of slowing or preventing additional expansion of aneurysms. Stromelysin-1 may prove to be a better therapeutic target than the gelatinases.

Acknowledgments

T. Carrel is a Research Fellow of the Royal College of Surgeons of England.

References


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