Activity of Matrix Metalloproteinase-2 and -9 in Abdominal Aortic Aneurysms. Relation to Size and Rupture

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Objectives: to investigate the activity of matrix metalloproteinase (MMP)-2 and -9 in asymptomatic abdominal aortic aneurysms (aAAAs) and ruptured abdominal aortic aneurysms (rAAAs).

Design: cross-sectional study.

Materials and methods: MMP-2 and MMP-9 activity was estimated in biopsies from the anterior wall of 60 AAAs using gelatin zymography. There were 20 medium-sized (diameter 5–7 cm) aAAAs, 20 large (>7 cm) aAAAs and 20 rAAAs. MMP activity was quantified using a laser densitometer and expressed as arbitrary units (au).

Results: mean (SEM) MMP-9 activity was significantly lower in large aAAAs (1190 au ± 247) than in rAAAs (2647 au ± 498, p<0.05). There was no difference in MMP-2 activity.

Conclusion: High MMP-9 activity in the AAA wall is associated with rupture.

Key Words: Matrix metalloproteinases; Abdominal aortic aneurysm.

Introduction

The development of abdominal aortic aneurysms (AAAs) is characterised by inflammation, degradation and remodelling of the aortic wall. The predominant proteins of the aortic wall are collagen and elastin, which provide the structural integrity and the main mechanical properties of the normal aorta.1 Histological studies demonstrate significantly altered architecture of the aortic wall in aneurysms, with disruption and fragmentation of elastin fibres and disordered collagen deposition. Elastin degradation is believed to be a primary event in aneurysm formation, and the elastolytic activity in the aneurysm wall is increased compared to normal aorta.5 Regardless of aneurysm size, elastase activity has been found significantly higher in ruptured than in non-ruptured aneurysms.5 Gelatinase A (MMP-2) and gelatinase B (MMP-9) are involved in aneurysm disease5–7 and both are capable of degrading the same matrix substrates,8 among these elastin.9,10 MMP-9 appears to be the predominant proteinase in AAAs in terms of mRNA expression, and the ratio of MMP/tissue inhibitor of metalloproteinases is increased in the AAAs compared to the normal aorta.11 MMP-9 is produced in mononuclear cells in the AAA wall.12 Different MMPs may be active at various times during aneurysm formation, as the expression of MMP-9 mRNA was found to be significantly higher in asymptomatic AAAs (aAAAs) 5–6.9 cm in diameter, compared to smaller and larger AAAs.14 However, aAAA is a heterogeneous group, containing aneurysms in different stages of development, not only in relation to size but also to the extent of matrix destruction. Hypothetically, larger aAAAs comprise a selected group of aneurysms, which retrospectively had a low risk of rupture and ruptured AAAs (rAAAs) represents aneurysms which had a high risk. By analysing these two groups of AAAs, it may be possible to reveal factors related to rupture.

The aim of this study was to investigate the activity matrix metalloproteinase (MMP)-2 and -9 in aAAAs and rAAAs using gelatin zymography. Activity in large (diameter ≥7 cm) aAAAs and rAAAs were of special interest, to investigate whether MMP activity may be related to AAA rupture.

Materials and Methods

Sixty patients undergoing infrarenal AAA repair at the Department of Surgery, Umeå University Hospital,
Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Medium-sized aAAAs (n = 20)</th>
<th>Large aAAAs (n = 20)</th>
<th>rAAAs (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>69</td>
<td>67</td>
<td>68</td>
</tr>
<tr>
<td>(median-range)</td>
<td>(52–80)</td>
<td>(57–84)</td>
<td>(56–81)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>15/5</td>
<td>15/5</td>
<td>18/2</td>
</tr>
<tr>
<td>Current smoking (%)</td>
<td>40</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Diagn. of hypertension (%)</td>
<td>55</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>AAA diameter (cm)</td>
<td>5.6 ± 0.1</td>
<td>8.0 ± 0.3</td>
<td>6.7 ± 0.3</td>
</tr>
</tbody>
</table>

rAAAs: ruptured abdominal aortic aneurysms. aAAAs: asymptomatic abdominal aortic aneurysms. Medium-sized: (5<7 cm). Large: (≥7 cm).

were studied (Table 1). Retrospective information on age, gender, and diagnosis of chronic obstructive pulmonary disease or hypertension was obtained. The maximal cross-sectional diameter of the AAA was measured by ultrasound and/or computed tomography scanning, carried out less than 6 weeks before operation. In two cases of aneurysm rupture no preoperative diameter measures were available and the diameter of the aneurysms was estimated preoperatively. Because of the known measure error in measuring aneurysm diameter, the diameter measures were corrected to the nearest 0.5 cm. There were 20 medium-sized (diameter 5–6.5 cm) aAAAs, 20 large (≥7 cm) aAAAs and 20 rAAAs. Our definition of medium-sized and large aneurysms is in accordance with the definition used by other authors.

The study was approved by the Scientific Ethical Committee, Umeå University Hospital and reported to the Central Control of Registers. All patients gave informed consent.

Specimen sampling

Full thickness specimens were collected from the central part of the anterior wall of the AAA. The specimens were cleaned and dissected and stored at −70°C.

Extraction of matrix metalloproteinases

Full thickness AAA tissue (approximately 2 g of wet weight) was rinsed twice with 10 volumes of 10 mM Tris (pH 7.5), 5 mM EDTA, 0.15 M NaCl and 2 mM phenylmethylsulfonylfluoride (PMSF) for 15 min on magnetic stirrer. The specimens were crushed to powder under liquid nitrogen and extracted three times by vigorous shaking with 10 volumes of 10 mM Tris (pH 7.5), 5 mM EDTA, 0.2 M NaCl and 2 mM PMSF for 12 h. In some experiments during the extraction procedure, a protease inhibitor Nα-p-Tosyl-l-lysine Chloromethyl Ketone (TLCK) was added. However, the inclusion of TLCK did not affect expression of MMP-2 or MMP-9 activity or the ratio of pro-enzyme to active enzyme. In the third extract of 0.2 M NaCl we found only traces of protein and negligible expressions of MMP-2 and -9. The first two 0.2 M NaCl extracts were combined, centrifuged at 20,000 g of 30 min, and filtered millipore 0.2 µm. Clear protein solutions were used for zymography. The concentration of protein in each homogenate was measured (mg/ml).

Gelatin zymography

Gelatin zymography was performed on a 10% polyacrylamide gel slab (6 cm long, 0.75 mm thickness) containing 0.3–0.6 mg/ml gelatin under Laemmli sodium dodecyl sulphate polyacrylamide gel electrophoresis conditions. The 0.2 M NaCl protein solutions were mixed 3:1 (vol/vol) with 4 times Laemmli sample solution. The samples were kept for 1 h at room temperature and used for zymography. Equivalent volumes (20, 10 or 5 µl) of each sample were loaded onto the gel. Electrophoresis was carried out at 4°C for 2.5 h keeping the voltage constant at 140 V. Following electrophoresis the gel was washed twice for 30 min each, in 100 ml of 2.5% Triton X-100, then rinsed three times with water and once with 0.05 M Tris buffer.
Abdominal Aortic Aneurysms and Matrix Metalloproteinase

Table 2. Activity of pro- and active enzyme of MMP-2 and MMP-9 in zymographic gels. Total (pro- plus active enzyme) and the ratio total MMP9/total MMP-2 (MMP-9/MMP-2) is calculated. The mean concentrations of protein in extractions from groups are shown. The protein concentration in each sample was used to adjust the total MMP-2 and total MMP-9 expression.

<table>
<thead>
<tr>
<th></th>
<th>Medium-sized aAAAs</th>
<th>Large aAAAs</th>
<th>rAAAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-MMP-2 (au)</td>
<td>529 ± 69</td>
<td>485 ± 65</td>
<td>596 ± 91</td>
</tr>
<tr>
<td>Act-MMP-2 (au)</td>
<td>283 ± 43</td>
<td>302 ± 50</td>
<td>266 ± 54</td>
</tr>
<tr>
<td>Pro-MMP-9 (au)</td>
<td>1118 ± 147</td>
<td>750 ± 87</td>
<td>1196 ± 133</td>
</tr>
<tr>
<td>Act-MMP-9 (au)</td>
<td>492 ± 107</td>
<td>310 ± 56</td>
<td>411 ± 64</td>
</tr>
<tr>
<td>Total MMP-2 (au)</td>
<td>812 ± 103</td>
<td>787 ± 108</td>
<td>863 ± 137</td>
</tr>
<tr>
<td>Total MMP-9 (au)</td>
<td>1610 ± 231</td>
<td>1060 ± 138</td>
<td>1607 ± 187</td>
</tr>
<tr>
<td>Total MMP-2 (au)</td>
<td>2.25 ± 0.29</td>
<td>1.47 ± 0.12*</td>
<td>2.46 ± 0.34</td>
</tr>
<tr>
<td>Protein extr. (mg/ml)</td>
<td>0.98 ± 0.10</td>
<td>1.38 ± 0.17</td>
<td>0.86 ± 0.11</td>
</tr>
<tr>
<td>Total MMP-2 per mg protein extracted (au/mg)</td>
<td>1092 ± 205</td>
<td>851 ± 214</td>
<td>1222 ± 200</td>
</tr>
<tr>
<td>Total MMP-9 per mg protein extracted (au/mg)</td>
<td>1907 ± 324</td>
<td>1190 ± 247*</td>
<td>2647 ± 498</td>
</tr>
</tbody>
</table>

(MMP: matrix metalloproteinases. Au: arbitrary units.)

(*p<0.05 vs large aAAAs). Values are expressed as mean ± standard error of mean. rAAAs: ruptured abdominal aortic aneurysms. aAAAs: asymptomatic abdominal aortic aneurysms. Medium-sized: (5<7 cm). Large: (≥7 cm).

Table 3. Multiple regression analysis. Aneurysm diameter is the only variable, which is negatively correlated to protein adjusted total MMP-9 activity in patients with aAAAs. No variable was correlated to protein adjusted total MMP-2 activity.

<table>
<thead>
<tr>
<th>aAAA</th>
<th>MMP-2 Beta</th>
<th>p-value</th>
<th>MMP-9 Beta</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−0.019</td>
<td>0.91</td>
<td>0.04</td>
<td>0.81</td>
</tr>
<tr>
<td>Diagnosis of hypertension</td>
<td>0.271</td>
<td>0.11</td>
<td>0.25</td>
<td>0.11</td>
</tr>
<tr>
<td>Current smoking</td>
<td>0.091</td>
<td>0.60</td>
<td>0.24</td>
<td>0.13</td>
</tr>
<tr>
<td>AAA diameter</td>
<td>−0.216</td>
<td>0.18</td>
<td>−0.31</td>
<td>0.04*</td>
</tr>
</tbody>
</table>

(*p<0.05). aAAAs: asymptomatic abdominal aortic aneurysms.

(pH 7.5), containing 0.005 M CaCl₂ and 0.001 M ZnCl₂ in 0.01 M acetic acid. Then the gel was incubated in the later buffer for 18 h at 37°C, after which it was stained with Coomassie Brilliant Blue G-250 (Fig. 1). The bands detected on the zymograms correspond to pro- and active MMP-9 (92 and 84 kDa) and pro- and active MMP-2 (72 and 62 kDa), respectively. The identity of these bands were confirmed using conditioned media of phorbol 12-myristate 13-acetate activated HL-cells, known to express MMP-2 and MMP-9 under these conditions, molecular markers and recombinant MMP-2 and MMP-9. The gelatinolytic activities were quantified by densitometry using a 2020 Ultrascan Laser Densitometer (LKB, Sweden). The amount of protein in each lane has previously been determined to be in the linear range for densitometric quantification. Each sample tested was electrophoresed twice and each lane on the zymogram was scanned triplicate. The relative activity of MMP-2 and MMP-9 (pro- and active enzyme) were expressed in arbitrary units (au).

**Statistical Methods**

The results were reported as mean ± standard error of mean (SEM) and compared by analysis of variance ANOVA and Tukey’s HSD to determine differences between groups. A multiple regression analysis and Spearman’s rank correlation coefficient were used to...
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Discussion

The character of aortic wall appears to differ markedly between different AAAs\(^{13,14,19,20}\) and may be related to the risk of rupture. Investigating rAAAs and large aAAAs may discover factors promoting rupture.

When biopsying the aneurysm wall it is difficult to be sure if the sample is representative; local differences in MMP activity are possible. We therefore chose to harvest biopsies in a standardized way, from the central part of the anterior wall of all aneurysms.

There are also problems using tissue homogenates, which may not represent the situation \textit{in vivo}. Proteases may be activated or deactivated, and not all the MMPs may be extracted. The use of urea may be necessary to extract MMPs tightly bound to matrix membranes.\(^{21}\)

The ratio MMP-9/MMP-2 was significantly lower in large aAAAs than in rAAAs. This was due to decreased MMP-9 activity and no change in MMP-2 activity. It may indicate that decreased MMP-9 activity contributes to reduce the potential of rupture. The MMP activity in medium-sized aAAAs falls between those of rAAAs and large aAAAs. This group of aneurysms is heterogeneous and results of present analysis are difficult to interpret.

Large aAAAs may express lower MMP-9 activity because of decreased production rate and/or a low content of inflammatory cells, especially macrophages. Thus, large aAAAs contain lower levels of MMP-9 messenger RNA compared to medium-sized AAAs.\(^{14}\)

The presence of \textit{Chlamydia pneumoniae} may also affect MMP activity.\(^{22}\) MMP-9 may also be more tightly bound to tissue membranes in large aAAAs and consequently not removed by extraction. However, the increased protein extraction achieved in such aneurysms does not support this argument. There may be more inhibitors in large aAAAs. MMP-9 activity was negatively correlated to aAAA diameter because decreasing diameter reflects a natural selection of aneurysms with low risk of rupture, while those with high risk have ruptured.

MMP-9 is principally located to the macrophages of the adventitia,\(^{12,23}\) which is the zone of remodelling.\(^{20}\) MMP-2 is preferentially localised to the media of the

![Fig. 3. Total MMP-9 activity, quantified with respect to protein concentration in each sample, in medium-sized and large aAAAs and rAAAs.](image-url)

**Results**

MMP-2 and MMP-9 activities are shown in Table 2, and zymographic patterns in Figure 1.

More protein was extracted from large aAAAs (1.38 ± 0.17 mg/ml) than from rAAAs (0.86 ± 0.11 mg/ml, \(p<0.05\)) (Table 2), for which reason total activity of MMP-2 and MMP-9 in each sample was appropriately adjusted.

Multiple regression analysis showed that aAAA diameter was significantly negatively correlated to MMP-9 but not to MMP-2 activity (Table 3). There was no correlation between rAAA diameter and MMP-2 activity \((r = 0.14)\) or MMP-9 activity \((r = 0.02)\). MMP-9/MMP-2 ratio, which was not influenced by differences in protein concentrations between samples, was significantly lower in large aAAAs \((1.47 ± 0.12)\) than in rAAAs \((2.46 ± 0.34, \ p<0.05)\). There were no significant differences in the ratios between medium sized aAAAs and rAAAs or large aAAAs (Fig. 2).

The protein-adjusted activity of total MMP-9 was significantly lower in large aAAAs \((1190 \text{ au} ± 247)\) than in rAAAs \((2647 \text{ au} ± 498, \ p<0.05)\) (Fig. 3), but there was no difference in total MMP-2 activity. Consequently, the difference in MMP-9/MMP-2 balance between large aAAAs and rAAAs was caused by significantly lower MMP-9 activity in large aAAAs. No significant differences were found in protein-adjusted total MMP-2 or MMP-9 activity between medium-sized aAAAs and large aAAAs or rAAAs (Table 2).
vascular wall. High MMP-9 activity, as we found in rAAAs, may inhibit aortic remodelling, leading to higher risk of rupture. Low MMP-9 activity, on the other hand, may permit remodelling of the adventitia and maintenance of aneurysm wall strength. Collagen synthesis is required for remodelling and this may explain the higher yield of proteins in extractions from large aAAAs. Ultrasound studies of aneurysm wall compliance in non-ruptured and ruptured aneurysms show a diminished stiffness of the aneurysm wall in ruptured aAAAs compared to non-ruptured AAAs. This also suggests that insufficient remodelling of the adventitia predisposes to rupture. Patients with rAAA also have abnormally high serum levels of the aminoterminal propeptide of type III procollagen, reflecting type III collagen turnover. This suggests a highly active collagen metabolism and remodelling process, which may be opposed by MMP-9 activity.

Patients with an AAA might, theoretically, benefit from MMP-inhibiting drugs. Recent studies show that aneurysm development can be hampered by an MMP-inhibiting tetracycline (Doxycycline).

In conclusion, high MMP-9 activity in the AAA wall is associated with rupture. We can not conclude anything about the pathological development of AAAs over time. However, more studies on MMPs, matrix protein degradation and degradation products in the aneurysm wall are needed to confirm the hypothesis.

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