Ubiquitous Elevation of Matrix Metalloproteinase-2 Expression in the Vasculature of Patients With Abdominal Aneurysms

Stephen Goodall, BSc; Mat Crowther, PhD; David M. Hemingway, MD, FRCS; Peter R. Bell, MD, FRCS; Matt M. Thompson, MD, FRCS

Background—Patients with abdominal aortic aneurysms (AAAs) exhibit arterial dilation and altered matrix composition throughout the vasculature. Matrix metalloproteinase-2 (MMP-2) is the dominant elastase in small AAAs, and overexpression of MMP-2 in vascular smooth muscle cells (SMCs) may be a primary etiological event in aneurysm genesis. The aim of this study was to investigate MMP-2 production in vascular tissue remote from the abdominal aorta.

Methods and Results—Inferior mesenteric vein (IMV) was harvested from patients undergoing aneurysm repair (n = 21) or colectomy for diverticular disease (n = 13, control). Matrix composition of the vessels was determined by stereological techniques. MMPs were extracted from tissue homogenates and quantified by gelatin zymography and ELISA. MMP-2, membrane type-1 MMP (MT1-MMP), and tissue inhibitor of metalloproteinases type 2 (TIMP-2) expression were determined by Northern analysis. SMCs were isolated from IMV, and the production and expression of MMP-2 and TIMP-2 in the SMC lines were quantified. Tissue homogenates and isolated inferior mesenteric SMCs from patients with aneurysms demonstrated significantly elevated MMP-2 levels, with no difference in TIMP-2 or MT1-MMP. These differences were a result of increased MMP-2 expression. Histological examination revealed fragmentation of elastin fibers within venous tissue obtained from patients with AAA and a significant depletion of the elastin within the media. In situ zymography localized elastolysis to medial SMCs.

Conclusions—Patients with AAA have elevated MMP-2 levels in the vasculature remote from the aorta. This finding is due to increased MMP-2 expression from SMCs, a characteristic maintained in tissue culture. These data support both the systemic nature of aneurysmal disease and a primary role of MMP-2 in aneurysm formation. (Circulation. 2001;104:304-309.)

Key Words: aneurysm ▪ aorta ▪ cells ▪ enzymes ▪ metalloproteinases

The biochemical and pathological changes associated with aortic aneurysms have been well characterized. Aneurysms demonstrate arterial dilation, wall thickening, and dramatic reduction in the elastin/collagen ratio.1-3 These changes are accompanied by an inflammatory infiltrate4,5 and excessive production of matrix metalloproteinases (MMPs),6-8 which regulate widespread matrix degradation.

Despite extensive research aimed at characterizing aneurysmal tissue, the agents responsible for initiating aneurysm formation remain elusive. Exact determination of the influence of any etiological agent has proved difficult, because there is no reliable model of spontaneous aneurysm formation, and human studies rely on the use of established aneurysmal tissue, at the end stage of a complex pathological process.

Recent studies have postulated a pivotal role for MMP-2 in early aneurysm formation. MMP-2 is constitutively expressed by many cell types, activated by membrane-bound membrane-type-1 MMP (MT1-MMP), and inhibited by tissue inhibitor of metalloproteinases type 2 (TIMP-2). MMP-2 has substrate specificity for elastin and fibrillar collagen9 and is found in the normal and aneurysmal aorta in association with MT1-MMP and TIMP-2.10

Freestone et al11 demonstrated that the principal metalloproteinase in small aneurysms was MMP-2 and suggested that MMP-9 was involved in aneurysm expansion at a later stage, a finding confirmed by other investigators.12 McMillan et al13 reported increased levels of MMP-2 mRNA synthesized by mesenchymal cells in aneurysmal tissue. Additional evidence suggesting an etiological role for MMP-2 was established by culturing aortic mesenchymal cells.14,15 Crowther et al16 demonstrated smooth muscle cells (SMCs) derived from aneurysmal aorta produced 3-fold higher levels of MMP-2 than cells from age-standardized atherosclerotic tissue. This increased MMP-2 production resulted from increased MMP-2 transcription,
with comparable levels of TIMP-2 and MT1-MMP mRNA. These data suggested the regulation of MMP-2 gene expression was altered in aortic SMCs from patients with abdominal aortic aneurysm (AAA).

Recent evidence suggested that patients with AAA have a systemic disease of their vasculature that manifests as local aneurysmal dilation. Ward\textsuperscript{17} revealed that the mean diameters of all peripheral arteries were significantly greater in patients with aortic aneurysms. Baxter et al\textsuperscript{18} demonstrated a reduction in the elastin/collagen ratio throughout the arterial vasculature of AAA patients. Interestingly, these findings do not appear to be unique to the arterial system. Loftus et al\textsuperscript{19} examined the prevalence of true aneurysms in infragenicular vein bypass grafts. Their study demonstrated that 40% of bypass grafts performed for popliteal aneurysm repair subsequently formed true vein graft aneurysms, compared with an incidence of only 2% in patients treated for lower-limb atherosclerotic occlusive disease.

These previous studies suggested any biological process initiating localized aneurysm formation might be manifest in the entire vascular tree. The hypothesis of the present study was that tissue remote from the abdominal aneurysm might be used to identify factors initiating aneurysm formation. The aim was to investigate MMP-2 expression and matrix structure in venous tissue from patients with aneurysmal disease.

### Methods

#### Study Design

This study was performed with the approval of the Leicestershire Research Ethics Committee. Two groups of patients were recruited, those having transperitoneal AAA repair and those undergoing left colonic resection for benign colonic pathology. Patient demographic data are given in Table 1. No difference with regard to age (Mann-Whitney test \( P=0.5491, U=109.5 \)) or sex (Fisher exact test \( P=0.4196 \)) was noted.

#### Tissue Collection

At operation, a segment of inferior mesenteric vein (IMV) was excised from a standard location immediately anterior to the abdominal aorta. This was part of the standard surgical procedure in both groups of patients. This vein was immediately placed in minimal ainal aorta. This was part of the standard surgical procedure in both

#### Gel Enzymography

Metalloproteinases were extracted from tissue by the method of Vine and Powell.\textsuperscript{7} The protein concentration in each sample was standardized to 1 mg/mL with protein assay (Bio-Rad). Gelatin zymography was performed as described by Crowther et al\textsuperscript{16} with 1 mg/mL gelatin (Sigma) in a 10% SDS-polyacrylamide gel. Cultured medium from HT1080 human fibrosarcoma cells (ECACC No. 85111505) was used as a positive control. Each sample was quantified by densitometric methodology.

Characterization of the proteases causing gelatinous activity was achieved by addition of 1,10-phenanthrolene (10 mmol/L). Further confirmation of the identity of MMP-2 in tissue homogenates and SMC culture media was achieved by Western blot analysis.\textsuperscript{16} Levels of MMP-2 and TIMP-2 were quantified by ELISA (Amersham).

#### Histology

Three rings of vein 3 mm in diameter were prepared and stained with hematoxylin and eosin or Miller’s elastin and Van Gieson’s stain (EVG; RA Lamb).\textsuperscript{10} Immunohistochemical staining of venous tissue allowed the localization of MMP-2, MT-MMP, and TIMP-2 to be determined. An indirect staining protocol was used that incorporated 3 steps. Primary antibodies specific to each antigen were obtained (Chemicon), and staining was performed as described previously.\textsuperscript{10}

#### Stereological Tissue Analysis

The relative proportions of elastin, collagen, and SMCs in the extracellular matrix were determined by stereological analysis of EVG sections, as described by Wills et al.\textsuperscript{20} Black represented elastin, pink represented collagen, and yellow represented SMCs. Eight fields of 100 points were chosen at random within the medial, intimal, and adventitial layers for all slides. Twelve patients were chosen from each group.

#### In Situ Zymography

Frozen tissue specimens were cut into 10-\( \mu \)mol/L sections and applied to silane-coated slides. These were submerged in 4 \( \mu \)g/mL fluororesceinated porcine gelatin (Molecular Probes) in 50 mmol/L Tris HCl, pH 7.6, containing 5 mmol/L CaCl\textsubscript{2}, 0.2 mmol/L sodium azide, for 18 hours at 37°C. Positive control slides were pretreated with 200 mmol/L phorbol 12-myristate 13-acetate for 30 minutes at 25°C, and negative controls were prepared by including 10 mmol/L 1,10-phenanthrolene. Gelatinolysis was visualized under mercury vapor lamp illumination as green zones against a black background. We determined the precise location of proteolytic activity by comparing serial sections under light illumination.

#### RNA Extraction

RNA was extracted by the Trizol method supplied by Gibco. The concentration of cells used was \( 2\times10^{5} \) cells/mL of Trizol, and the concentration of tissue was 100 mg/mL.

#### Northern Blotting

Northern analysis compared levels of expression of MMP-2, TIMP-2, and MT1-MMP between samples. Northern blotting was performed as described previously.\textsuperscript{16} Completed blots were analyzed by laser densitometry, with sample normalization being achieved by comparison with GAPDH. Probes were prepared with a reverse transcriptase polymerase chain reaction and the following sequences: MMP-2 reverse, GCCACCGTGGAAGATAGC; MT1-MMP reverse, TGATGATCACCTCCTTCC; TIMP-2 reverse, ACCCTGTGGTTCAGGCTCTTC; and GAPDH reverse, GCCAAAATCGTTTGATCATCC.

#### Cell Culture

The vein was immediately freed of blood and adipose tissue by washing with cold minimal essential medium. Thirty patients from each group were used, and SMCs were grown by an explant technique.\textsuperscript{16} Cultures were passaged after cellular outgrowths from the explants had reached 80% confluence (20 to 30 days). SMC identity was confirmed by immunostaining to \( \alpha \)-actin.

SMCs were used once they had reached the third passage. Trypsinized cells were plated into 6-well plates (Nunclon, Gibco) at a density of \( 1\times10^{5} \) cells per well, where they were allowed to adhere. After 24 hours, the media were replaced with quiescence media containing 0.4% fetal bovine serum. After an additional 24 hours, these media were replaced with QBSF56 serum-free media (Sigma). After 48 hours, the media were transferred to freezing vials and snap-frozen for future assays.

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### Table 1. Demographic Data of Patients Entering Study

<table>
<thead>
<tr>
<th>Demographic Data</th>
<th>AAA</th>
<th>Control</th>
<th>( P )</th>
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<tr>
<td>Age, y</td>
<td>72 (range 63–83)</td>
<td>68 (range 58–86)</td>
<td>0.5491</td>
</tr>
<tr>
<td>Sex, % male</td>
<td>81</td>
<td>67</td>
<td>0.4196</td>
</tr>
<tr>
<td>Aortic diameter, cm</td>
<td>5.6 (range 4.6–7.5)</td>
<td>&lt; 2.5</td>
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Results

Enhanced MMP-2 Activity in IMV Tissue Homogenates From Patients With AAA

Gelatinolytic activity in IMV tissue homogenates was measured by gelatin zymography and ELISA. The proform of MMP-2 was demonstrated as a lytic band at 70 kDa, and the active form was present at 66 kDa; an MMP-9 band was present at 92 kDa (Figure 1A). Scanning densitometry revealed a significant increase in MMP-2 levels in AAA patients (n=21, median OD=1.362) compared with controls (n=12, median OD=0.897; Mann-Whitney U=33, P=0.0005). No such difference was noted for MMP-9 (AAA: n=21, median OD=0.319; control: n=12, median OD=0.427; Mann-Whitney U=93, P=0.2311).

ELISA data confirmed the increase in total MMP-2 in the aneurysmal group (Mann-Whitney U=20.5, P=0.0018; Table 2). Interestingly, ELISA data for TIMP-2 revealed no significant difference in the levels produced by the AAA group (Mann-Whitney U=25.5, P=0.4817).

Increased MMP-2 Expression in IMV Tissue Homogenates From Patients With AAA

Confirmation of homologous MMP-2 overexpression within the IMV was demonstrated by Northern blotting. RNA-standardized tissue extracts from IMV clearly showed that the site of MMP-2 production was the tissue. Densitometric analysis demonstrated significantly increased expression of MMP-2 in patients with aneurysms compared with controls (Wilcoxon W=61, P=0.0327; Figure 2). This was consistent with the increased production of MMP-2 revealed by both ELISA and protein zymography. No difference in the expression of TIMP-2 (Wilcoxon W=−26, P=0.2783) or MT1-MMP (Wilcoxon W=−27, P=0.3013) was revealed.

Reduced Elastin in IMV Tissue Sections From Patients With AAA

Disruption and degradation of elastin fibers within the arterial media are key features of the aneurysmal aorta. To investigate whether similar changes were present in the venous circulation, IMV sections were stained with EVG (Figure 3). Examination revealed a visible reduction in the amount and integrity of elastin in the IMV from AAA patients compared with controls. The elastin lamellae of the control tissue were clearly visible, and the basic architecture of the vein was maintained. Tissue harvested from patients with AAA had fragmented elastin lamellae, and the overall architecture appeared to show signs of matrix degradation.

Stereological analysis confirmed that IMV from patients with AAA showed elastin degradation. The relative proportion of elastin in the medial layer of the IMV from the AAA group was 19.4%
(IQR 17.2% to 24.8%) compared with 26.8% (IQR 23.7% to 29.9%) in the control group. This represents a significant decrease in elastin (Mann-Whitney U = 24, P = 0.0181).

MMP2-Activity Was Localized to Elastin Fibers in IMV Tissue Sections

In situ zymography allowed visualization of gelatinolytic activity in the vein wall. The areas of lysis colocalized with elastin lamellae within venous media (Figure 4).

Immunohistochemistry demonstrated that the site of MMP-2/TIMP-2/MT1-MMP in the IMV tissue colocalized with the SMCs. Staining for macrophages was negative.

Enhanced MMP-2 Production and Expression in SMCs Derived From IMV From Patients With AAA

From the previous findings, increased MMP-2 activity in the IMV of patients with AAA appeared to derive from venous SMCs. To test this observation, SMC lines were cultured from IMV, and MMP-2 production and expression were determined.

Scanning densitometry of gelatin zymograms revealed significantly increased production of MMP-2 by IMV SMCs from patients with AAA compared with controls (Mann-Whitney U = 20, P = 0.001; Figure 1B). ELISA data confirmed this increase in MMP-2 in the aneurysmal group (Mann-Whitney U = 41.5, P = 0.0293); no difference in TIMP-2 was noted (Table 3). No MMP-9 was produced.

Confirmation of a homologous increase in MMP-2 expression in IMV SMCs from AAA patients was demonstrated by Northern blot analysis. RNA extracts demonstrated significantly increased expression of MMP-2 compared with RNA from IMV SMCs derived from the control group (Wilcoxon W = 69, P = 0.134; Figure 5). No significant differences were noted for TIMP-2 (Wilcoxon W = −18, P = 0.5186) or MT1-MMP (Wilcoxon W = −26, P = 0.2783).

TABLE 3. Production of MMP-2 and TIMP-2 by SMCs Harvested From IMV

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<thead>
<tr>
<th></th>
<th>Concentration, ng/mL</th>
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<tr>
<td>AAA</td>
<td>188.8 (143.2–243.7)</td>
<td>126.8 (115.8–160.3)</td>
<td>0.0293</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>16.10 (14.20–22.55)</td>
<td>14.95 (12.55–21.20)</td>
<td>0.5887</td>
</tr>
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</table>

There were significantly increased MMP-2 levels in the AAA group. Values are medians (interquartile ranges).
Discussion

Any hypothesis aiming to explain the etiology of AAA must address several issues. Biochemical and pathological features of the established aneurysm must be incorporated, as must familial tendencies and the propensity for some patients to develop multiple aneurysms at separate sites. Previous studies have established that the vasculature of patients with AAA exhibited widespread extracellular matrix changes and arterial dilation. Recent evidence has suggested that MMP-2 may play an integral role in early aneurysm formation. The present study was designed to investigate MMP-2 production and expression in the veins of patients with AAA.

The underlying hypothesis was that an overexpression of MMP-2 might be responsible for early elastolysis observed in small aneurysms and might be demonstrable in blood vessels remote from the abdominal aorta. The concept of using remote tissue was important, because although these vessels might demonstrate subtle changes associated with the aneurysmal process, they do not undergo the dramatic secondary degeneration that occurs in the aneurysmal aorta. IMV was used as a source of tissue because it was easily obtainable in patients undergoing elective, transperitoneal aneurysm repair and age-matched control subjects who were having left colonic resection. One possible disadvantage to the use of the IMV is its proximity to the abdominal aorta. This proximity may allow any inflammatory process affecting the aorta to influence the mesenteric vein. To negate this effect, we did not study any inflammatory aneurysms, and all patients had a mesenteric vein that was not adherent to the posterior peritoneum. However, it will be important for MMP-2 levels to be determined in different vascular tissues in patients with abdominal aneurysms.

The present study demonstrated that IMV of patients with AAA had a significantly higher MMP-2/TIMP-2 ratio than tissue from age-matched controls. Northern blotting revealed this was due to increased expression of the MMP-2 gene, because there was no difference in TIMP-2 and MT1-MMP transcription. These findings suggest the vasculature of patients with AAA exhibits a systemic tendency toward increased proteolysis. This tendency was confirmed when venous tissue was examined histologically.

Stereological analysis established that IMV harvested from patients with AAA had a significantly higher MMP-2/TIMP-2 ratio than tissue from age-matched controls. Northern blotting revealed this was due to increased expression of the MMP-2 gene, because there was no difference in TIMP-2 and MT1-MMP transcription. These findings suggest the vasculature of patients with AAA exhibits a systemic tendency toward increased proteolysis. This tendency was confirmed when venous tissue was examined histologically.

In previous studies, SMCs derived from the aneurysmal wall were demonstrated to express MMP-2 at a higher level than control SMCs from patients with aortic atherosclerosis. The final part of the present study quantified MMP-2 expression from cells derived from IMV of patients with aneurysmal disease. These data proved venous SMCs from patients with AAA exhibited significantly higher concentrations of MMP-2 protein and mRNA than cells from age-matched controls. Findings from the present investigation supported the concept of widespread proteolysis in the vasculature of patients with AAA, suggesting that this may be due to overexpression of MMP-2 from vascular SMCs.

Previous studies have demonstrated that some AAA may be genetically determined. Many epidemiological reports demonstrated a familial tendency to aneurysmal formation, and evidence now exists through segregation analysis of multigenerational aneurysmal pedigrees that AAA susceptibility is controlled by a single gene defect. To date, most obvious candidate genes for AAA (elastin, collagen, fibrillin, and TIMP-1) have been examined with little evidence of causality. On the basis of findings from the present study, it may be speculated that overexpression of the MMP-2 gene may be important in
aneurysm formation and that MMP-2 may be regarded as a candidate gene for aneurysm development.

Previous investigations into the structure of the MMP-2 gene have not immediately suggested features associated with susceptibility genes. The level of expression of MMP-2 is not readily modulated, because it is constitutively expressed and exhibits the characteristics of a housekeeping gene. The MMP-2 promoter lacks a TATA box and the AP-1 transcriptional factor binding motif common to other inducible MMPs. Despite these features, examples have been reported of tissue-specific and developmentally related modulation of MMP-2. Additionally, the regulation of MMP-2 expression in rodent mesangial glomerular cells has been shown to be responsive to exogenous cytokinetic stimuli and is constitutively elevated. Harendza et al and Mertens et al identified and characterized a cis-acting enhancer element in the rat MMP-2 gene, which binds the transcription factors AP-2 and YB-1.

Contemporary evidence suggested that the MMP-2 gene may be differentially expressed and inducible and may therefore be more likely to be implicated in pathological conditions. Price et al recently documented six polymorphisms in the human MMP-2 promoter region, and one of the transitions, C-T at −1306, was associated with differential promoter activity. Results from the present study suggest that patients with AAA have a widespread and increased expression of MMP-2 within their vasculature that is possibly responsible for early elastolysis and aneurysmal degeneration of the abdominal aorta. Recent investigations into the MMP-2 gene structure suggest MMP-2 polymorphism analysis in patients with AAA is warranted.

Acknowledgment

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References

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