Matrix Metalloproteinase-9 Genotype Influences Large Artery Stiffness Through Effects on Aortic Gene and Protein Expression

Tanya L. Medley, Timothy J. Cole, Anthony M. Dart, Christoph D. Gatzka, Bronwyn A. Kingwell

Objective—Because large artery stiffening contributes to myocardial ischemia, its determinants are of relevance as potential risk markers. This study examined whether matrix metalloproteinase (MMP)-9 (gelatinase B) genotype is associated with large artery stiffening and aortic MMP-9 gene and protein expression.

Methods and Results—MMP-9 genotype (C-1562T promoter polymorphism) was determined in 84 patients (73 male) with angiographically defined coronary artery disease (CAD). Carotid applanation tonometry was used to assess central blood pressures and, with Doppler velocimetry, to assess aortic stiffness (input and characteristic impedance). Gene expression real-time polymerase chain reaction (RT-PCR) and protein levels (Western blotting) were assessed in relation to genotype in aortic samples from a separate population. T-allele carriers (C/T and T/T) had stiffer large arteries (higher input and characteristic impedance) and higher carotid pulse and systolic blood pressure (all \( P < 0.05 \)) than C/C homozygotes. In aortic samples, gene expression was 5-fold higher and active protein levels were >2-fold higher in T-allele carriers.

Conclusions—Because the T allele was associated with greater MMP-9 mRNA and protein levels, the greater large artery stiffness in T-allele carriers may be secondary to excessive degradation of the arterial elastic matrix. The consequent higher pulse pressure may increase susceptibility to myocardial ischemia. (Arterioscler Thromb Vasc Biol. 2004; 24:1479-1484.)

Key Words: matrix metalloproteinase ■ coronary artery disease ■ large artery stiffness ■ pulse pressure

Large artery stiffness predicts coronary outcome independently of conventional risk factors.\(^1\) This may relate to similar atherosclerotic progression in the coronaries and aorta, with the latter leading to large artery stiffening.\(^2\) In addition, large artery stiffening, whether a primary cause or as a consequence of atherosclerosis, may exacerbate coronary ischemia and events through widening of pulse pressure.\(^3\) This effect is mediated by increased cardiac afterload secondary to systolic pressure elevation and reduced coronary perfusion as a consequence of lower diastolic pressure.\(^4\)–\(^6\) The mechanisms contributing to large artery stiffening in the context of coronary disease are multiple; however, given their important role in disease-related vascular remodeling, matrix metalloproteinases (MMPs) are likely to be important.

The MMPs are a family of enzymes required for the degradation of extracellular matrix during embryonic development, morphogenesis, and tissue remodeling. MMP-9, also known as gelatinase B or 92-kDa type IV collagenase, may be particularly relevant to large artery stiffening associated with vascular disease. MMP-9 expression is increased after vascular injury\(^7\) and is particularly evident in inflammatory atherosclerotic lesions. High expression of MMP-9 has been associated with coronary plaque destabilization\(^8\)–\(^10\) and with outward aortic remodeling and aneurysm formation.\(^11\)–\(^14\) These phenomena presumably occur as a consequence of excessive degradation of extracellular matrix components. MMP-9 substrates include denatured collagens (gelatin), collagen types II, IV, and V, elastin, entactin, and vitronectin.\(^15\) Excessive degradation of important matrix components may influence aortic stiffening. Genetic variations may exacerbate the overexpression of MMP-9 in the setting of coronary artery disease (CAD). One such functional polymorphism is in the promoter of the MMP-9 gene and is characterized by a single nucleotide change from cytosine to thymidine 1562-bp upstream from the start of transcription (C/T). In vitro studies using mammalian cell transient transfection experiments and DNA–protein interaction assays have shown that the T allele results in a higher promoter activity compared with the C allele because of preferential binding of a transcription repressor protein to the C allele.\(^16\) The T allele has also been associated with higher MMP-9 plasma levels in CAD patients,\(^17\) a-
TABLE 1. Characteristics of CAD Patients

<table>
<thead>
<tr>
<th>Genotype</th>
<th>C/C</th>
<th>T-Allele Carrier</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>59</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Frequency, %</td>
<td>70</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>60±9</td>
<td>63±7</td>
<td>0.15</td>
</tr>
<tr>
<td>M/F</td>
<td>50/9</td>
<td>23/2</td>
<td>0.30</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.6±5.0</td>
<td>27.9±3.3</td>
<td>0.50</td>
</tr>
<tr>
<td>Resting HR, min⁻¹</td>
<td>60±10</td>
<td>59±8</td>
<td>0.79</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L⁻¹</td>
<td>5.0±1.2</td>
<td>4.4±0.8</td>
<td>0.06</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L⁻¹</td>
<td>2.9±1.0</td>
<td>2.4±0.7</td>
<td>0.07</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L⁻¹</td>
<td>1.0±0.5</td>
<td>1.0±0.3</td>
<td>0.68</td>
</tr>
<tr>
<td>Triglycerides, mmol/L⁻¹</td>
<td>1.8±0.8</td>
<td>1.6±0.7</td>
<td>0.35</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>36</td>
<td>17</td>
<td>0.36</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>9 (15)</td>
<td>3 (12)</td>
<td>0.49</td>
</tr>
<tr>
<td>Maximum stenosis (%)</td>
<td>94±7</td>
<td>93±8</td>
<td>0.65</td>
</tr>
<tr>
<td>Previous angioplasty, n (%)</td>
<td>13 (22)</td>
<td>3 (12)</td>
<td>0.21</td>
</tr>
<tr>
<td>Aortic area (cm²)</td>
<td>3.3±0.7</td>
<td>3.6±0.7</td>
<td>0.60</td>
</tr>
</tbody>
</table>

M indicates male; F, female; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Data are mean±SD unless otherwise stated.

*P<0.05 from ANOVA.

though this has not been confirmed in human vascular tissue. This study sought to investigate the influence of the MMP-9 (C/T) polymorphism on aortic gene expression, protein levels, and large artery stiffening in patients with CAD.

**Methods**

**Subjects and Study Design**

Eighty four (73 male) CAD patients aged 61±8 years (mean±SD; range, 30 to 75) gave informed consent to participate in the study, which was approved by the Ethics Committee of the Alfred Hospital. Patients with previous bypass surgery were excluded because of the possible confounding effects of surgery on large artery stiffness. CAD patients were defined angiographically as having stenoses ≥80% in the major coronary vessels at the time of the study. The major coronary vessels were the left anterior descending, the left circumflex, or its marginal branch when the branch constituted the main continuation of the circumflex, and the right coronary artery. Percentage narrowing was determined from views of the vessels in 2 planes. The most severe lesion in the 3 major vessels was reported as the maximum coronary stenosis. Left ventricular outflow tract area was measured by 2-dimensional echocardiography (Hewlett-Packard Sonos 1500). Detailed patient characteristics are shown in Table 1.

Patients attended the laboratory after an overnight fast. Blood was drawn for fasting lipids and DNA extraction. Patients then rested in a quiet temperature-controlled room (22°C) for at least 15 minutes and until blood pressure had stabilized. Aortic input and characteristic impedance were then assessed noninvasively. To investigate gene expression and protein levels, 12 aortic samples from a separate population (cardiac transplant patients) were also analyzed. Five samples were from patients with cardiac disease of ischemic cause and 7 were idiopathic cardiomyopathy. After harvesting, hearts were placed immediately on ice for a maximum of 4 hours until dissection and freezing for later DNA, RNA (n=8), and protein extraction (n=12).

**Resting Blood Pressure**

Resting brachial arterial blood pressure and heart rate were measured 3 times, at 3-minute intervals, using a Dinamap vital signs monitor (1846 SX; Critikon) after subjects rested undisturbed in a quiet temperature-controlled room (22°C) for ≥10 minutes, or until blood pressure had stabilized.

**Aortic Impedance**

Aortic flow velocity was measured using a Doppler velocimeter (3.5-Hz Multi-Dopplex MD1, Huntleigh Technology), and right carotid pressure was measured simultaneously by applanation tonometry (SPT-301, Millar Instruments) as previously described. Ensemble average time series of both flow and pressure (10 cardiac cycles aligned to the foot of the pressure waveform) were used to calculate aortic input impedance. Input impedance was calculated at 1.75 Hz as a measure of large artery stiffness at frequencies incorporating pressure wave reflection. Characteristic impedance, in the absence of wave reflection, was calculated as the arithmetic mean of moduli ≥2 Hz. This index varies directly with elastic modulus and is therefore a measure of intrinsic vascular stiffness.

**Genotyping**

Genomic DNA was prepared from whole blood using routine procedures. The MMP-9 promoter polymorphism was amplified from 100 ng of genomic DNA in a 25-µL PCR reaction using the following primers: forward, 5’ FAMGCC TGG CAC ATA GTA GGC CC 3’; and reverse, 5’ CCT CTT AGC CAG CCG GCA TC 3’. PCR conditions included pre-denaturation at 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 53°C for 1 minute, and 72°C for 1.5 minutes, and a final extension at 72°C for 10 minutes. PCR fragments were sized using a 3100 Genetic Analyser (PE Biosystems) and analyzed using Genescan Analysis Software version 3.7. Both alleles were verified by DNA sequencing.

**Gene Expression**

There was sufficient tissue to determine MMP-9 mRNA levels in 8 of the 12 aortic samples (C/C, n=4; C/T or T/T, n=4) using real-time RT-PCR sequence detection (ABI Prism 7700; Applied Biosystems). Total RNA was extracted using the trizol reagent method (Life Technologies), treated with DNase I (Ambion) and stored at −70°C. cDNA was synthesized using PE Biosystems RT kit with random hexamer primers. Real-time RT-PCR amplifications were prepared in duplicate on 2 separate occasions (ie, 4 times) using the following primers and probe: forward primer, 5’ GCT CGG CCA CAC AAA ACT 3’; reverse primer, 5’ CCC TCT CAC TCG CTT GTA CAG 3’; and probe, 6FAM TGA CGA TGT CTT CGT CCT CC TAMRA. MMP-9 mRNA levels were normalized to 18S rRNA (Applied Biosystems) and fold expression was determined as previously described.

**Protein Levels**

Total protein was extracted in 12 aortic samples and concentrations were determined using the Bio-Rad Protein Assay. Exactly 15 µg of total protein was separated by SDS-PAGE on 7.5% polyacrylamide gels, blotted onto nitrocellulose membranes, and immunostained with a human MMP-9 primary antibody (1:1000 for 1.5 hours, Sigma, St Louis, Mo). After addition of a peroxidase-conjugated secondary antibody (1:1000 for 1 hour), visualization was achieved using the ECL technique (Amer sham Pharmacia Biotech). Protein levels were quantitated from digitized films as the product of band density and area using Optimas 6.1 (Media Cybernetics, LP).

**Biochemical Analyses**

Total and low-density lipoprotein cholesterol and triglycerides were determined enzymatically with a Cobas-BIO centrifugal analyzer (Roche Diagnostic Systems).

**Statistical Analyses**

Analysis of variance was used to compare data stratified by genotype. Potential confounders were entered into these analyses as covariates. χ² Analysis was used to compare categorical variables. All data were analyzed using SPSS for Windows Version 11.0 (SPSS
Results

Allele frequencies were 0.83 and 0.17 for the C and T alleles, respectively, and were not significantly different from previous studies or from Hardy–Weinberg equilibrium. Because of the low frequency of the T allele (only 2 homozygotes), C/C homozygotes were compared with T-allele carriers (C/T and T/T) for all analyses. Age, gender ratio, body mass index, blood lipids, and heart rate were not different between C/C homozygotes and T-allele carriers (Table 1). There was no difference in severity of coronary disease, the number of ex-smokers, the prevalence of previous angioplasties, or diabetes (Table 1) or in medication profile (Table 2) between C/C homozygotes and T-allele carriers. In particular, 52% of the population were using statin medication; however, there was no difference in coronary disease, number of ex-smokers, the prevalence of previous angioplasties, or diabetes between C/C homozygotes and T-allele carriers (P=0.22). Only 1 patient was not of European descent.

Large Artery Stiffness and MMP-9 Genotype

CAD patients that carried a T-allele had significantly higher aortic stiffness as assessed by both input impedance (P=0.008) and characteristic impedance (P=0.025, Table 1, Figure 1). The higher characteristic impedance indicates that such differences were independent of wave reflection and caused by differences intrinsic to the walls of conduit arteries. These relationships remained significant after age, gender, mean arterial pressure (MAP), total cholesterol, low-density lipoprotein cholesterol, and triglycerides were entered into the analysis as covariates (P=0.034 and P=0.035 for input and characteristic impedance, respectively). In multivariate analysis incorporating the aforementioned factors, MMP-9 genotype was an independent predictor of aortic input (partial r=0.21, P=0.03) and characteristic (partial r=0.22, P=0.05) impedance. Other important predictors were sex, age, and MAP.

Blood Pressure and MMP-9 Genotype

The T allele was associated with higher brachial systolic (P=0.048) and pulse pressure (P=0.035), as well as carotid systolic (P=0.05) and pulse pressure (P=0.04, Figure 2). There was no difference in MAP and diastolic blood pressure between C/C and T-allele carriers (P=0.24 and P=0.45, respectively; Figure 2).

MMP-9 mRNA and Protein Levels

Gene expression (normalized to 18s rRNA) in the aortic samples was 5-fold higher in T-allele carriers compared with C/C homozygotes (Figure 3; P=0.02). At the protein level, there was a trend for increased total protein level in T-allele carriers (Figure 4; P=0.26), and active MMP-9 protein was significantly higher in this group (P=0.01; Figure 4). Importantly, there was no difference in mRNA or protein levels between samples from patients with ischemic versus idopathic presentations. When the 2 groups were compared statistically, the respective P values for gene expression and latent, active, and total protein expression were 0.42, 0.90, 0.54, and 0.60.

Discussion

In this study, the MMP-9 C-1562T promoter polymorphism was associated with large artery stiffening in patients with CAD. Specifically, the T allele was linked with stiffer large arteries and increased levels of MMP-9 mRNA and active protein compared with C/C homozygotes. The greater large artery stiffness in T-allele carriers may thus be secondary to excessive degradation of the arterial elastic matrix. That the higher stiffness of T-allele carriers was of structural origin is supported by the fact that characteristic impedance (intrinsic vascular stiffness in the absence of wave reflection) was higher in this group but MAP did not differ with genotype.

Table 2: Medications

<table>
<thead>
<tr>
<th></th>
<th>C/C</th>
<th>T-allele Carrier</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE inhibitors, n (%)</td>
<td>13 (22)</td>
<td>10 (40)</td>
<td>0.06</td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>53 (90)</td>
<td>19 (79)</td>
<td>0.17</td>
</tr>
<tr>
<td>Beta antagonists, n (%)</td>
<td>38 (64)</td>
<td>12 (48)</td>
<td>0.16</td>
</tr>
<tr>
<td>Calcium antagonists, n (%)</td>
<td>20 (34)</td>
<td>6 (24)</td>
<td>0.30</td>
</tr>
<tr>
<td>Diuretics, n (%)</td>
<td>6 (10)</td>
<td>2 (8)</td>
<td>0.57</td>
</tr>
<tr>
<td>Lipid lowering, n (%)</td>
<td>35 (59)</td>
<td>18 (72)</td>
<td>0.19</td>
</tr>
<tr>
<td>Nitrates, n (%)</td>
<td>26 (44)</td>
<td>9 (36)</td>
<td>0.38</td>
</tr>
<tr>
<td>Antihypertensives, n (%)</td>
<td>32 (54)</td>
<td>16 (64)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

ACE indicates angiotensin-converting enzyme. All data are presented as number of patients and (percent of genotype).
Because large artery stiffening is a predictor of myocardial ischemic threshold in CAD patients, these data suggest that T-allele carriers may be particularly susceptible to myocardial ischemia.

MMP-9 Expression and Arterial Remodeling

Despite the modest sample size, there was a significant and consistent difference in aortic gene expression and protein levels in relation to presence of the T allele. MMP-9 mRNA levels were 5-fold higher in aortic samples from T-allele carriers compared with C/C homozygotes. Gene expression was highly consistent for a given genotype despite differences in etiology between samples. This finding suggests that genotype is a stronger influence than disease etiology on gene and protein expression. The current study is the first examination of the relationship between MMP-9 C-1562T genotype and gene expression in human tissue. Although it was not possible to obtain aortic tissue from the patients in whom we related genotype to arterial stiffness, our data are consistent with previous cell culture experiments that associated the T allele with a 2-fold higher promoter activity than the C allele. The reason for the difference in the magnitude of expression between alleles/genotypes in tissue versus cell culture most likely reflects differences in growth conditions between cultured cells and aortic cells in vivo. Furthermore, the reporter gene expression studies were performed in murine lung macrophages, which may show different regulatory patterns compared with human aortic tissue.

The T allele has previously been associated with higher levels of MMP-9 in plasma, but this relationship has not been examined in vascular tissue. In the current study, the T allele was associated with higher levels of active MMP-9 protein in the human aorta. The higher mRNA levels would
be expected to at least partly account for increased synthesis of pro--MMP-9 and thus greater levels of active MMP-9.

The higher expression of MMP-9 associated with the T allele may be directly involved in promoting stiffer large arteries through vascular remodeling, which disrupts the mechanical integrity of the large arteries. In support of this, studies using MMP-9--deficient cells from the carotid artery of mice have demonstrated that MMP-9 may influence arterial remodeling not only through matrix degradation but also through reorganization. Because arterial elastic properties are conveyed primarily by the matrix, it is plausible that excessive degradation and remodeling of the elastic matrix associated with MMP-9 overexpression will result in vessel wall stiffening. An example of an association between large artery stiffening and MMP-9 overexpression is seen in Marfan syndrome.23,24 In this condition, aortic stiffening is associated with MMP-9 overexpression will result in vessel wall stiffening. An example of an association between large artery stiffening and MMP-9 overexpression is seen in Marfan syndrome.23,24 In this condition, aortic stiffening is thought to be the incipient event that causes subsequent compensatory outward aortic remodeling that may deteriorate to aneurysm and rupture. There is also increasing evidence for a relationship between overexpression of MMP-9 and the development of non-Marfan aortic aneurysms.25,26 Degradation of the elastic extracellular matrix within the aortic wall is a hallmark of aortic aneurysms, and the increased expression of MMP-9 mRNA found in the aortic tissue of these patients further supports a role for MMP-9 in outward remodeling of the aorta. In addition, MMP-9 knockout mice that underwent abdominal aortic aneurysm induction by abluminal application of calcium chloride were resistant to aortic dilation after 10 weeks.27

Clinical Implications

The T allele was associated with stiffer large arteries, which would have contributed to the higher pulse pressure in this group. Both arterial stiffness and pulse pressure have been related positively to cardiovascular and, in particular, coronary outcome.1,29 The mechanism underlying this relationship likely relates to the mismatch in cardiac blood supply and demand that occurs when the heart ejects into a stiff circulation. This effect is mediated by increased cardiac afterload, secondary to systolic pressure elevation, and reduced coronary perfusion as a consequence of lower diastolic pressure.4-6 Clinically, this relationship is manifested by a reduction in ischemic threshold in CAD patients with stiffer large vessels.3 T-allele carriers may thus be predisposed to a reduced ischemic threshold.

The T allele has been associated with an increased risk for cardiovascular events in patients with CAD.17 This elevated risk relates at least in part to plaque destabilization associated with increased MMP-9 activity and erosion of the plaque fibrous cap.30 The current study suggests that large artery stiffening may represent another mechanism contributing to coronary risk associated with the T allele. As discussed, this mechanism relates to reduction in ischemic threshold caused by pulse pressure elevation. In addition, elevated pulse pressure could also contribute to plaque rupture.31

MMP-9 Genotype and Medication

Statins generally decrease MMP-9 expression in association with a decrease in inflammation and increased plaque stability.32 Statins may therefore represent a potential confounding factor; 52% of patients in the current study were using statins. There was not, however, any difference between genotypes in the proportion of patients using statins. MMP-9 genotype was thus associated with arterial stiffness despite moderate statin usage, suggesting that genotype is a relatively important modulator of vascular properties. There was a trend for higher angiotensin-converting enzyme inhibitor use in T-allele carriers versus C/C homozygotes (40% versus 22%; Table 2). There is some evidence that ACE inhibitors exert direct effects on the arterial wall to reduce stiffness.2 Thus, the higher stiffness in T-allele carriers has likely been under-estimated in the current study.

Conclusion

MMP-9 genotype is associated with large artery stiffening in patients with CAD. In particular, patients that carry at least 1 copy of the T allele have stiffer large arteries compared with C/C homozygotes and may be more susceptible to myocardial ischemia. This relationship may result from increased degradation of important elastic components of the extracellular matrix secondary to elevated MMP-9 gene expression and protein levels in the aorta.

Acknowledgments

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References


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