Genetic Variation in Human Stromelysin Gene Promoter and Common Carotid Geometry in Healthy Male Subjects

Agostino Gnasso, Corradino Motti, Concetta Irace, Claudio Carallo, Laura Liberatoscioli, Sergio Bernardini, Renato Massoud, Pier Luigi Mattioli,† Giorgio Federici, Claudio Cortese

Abstract—A common variant in the promoter of the human stromelysin gene, causing reduced enzyme expression, has been associated with the progression of coronary atherosclerosis. On the other hand, increased stromelysin activity may promote plaque rupture. The present study was undertaken to investigate the relationship between the genetic variation in the human stromelysin gene promoter and common carotid geometry. Forty-two healthy male subjects without major coronary heart disease risk factors were investigated. The polymorphism in the stromelysin gene promoter was studied through polymerase chain reaction amplification with the use of mutagenic primers. Age, blood pressure, lipids, glucose, viscosity, and body mass index were similar in homozygotes for the 5A allele (5A/5A), heterozygotes (5A/6A), and homozygotes for the 6A allele (6A/6A). Serum matrix metalloproteinase-3 levels did not differ significantly among genotypes. Common carotid diameters and intima-media thickness, measured by noninvasive ultrasonography, were significantly larger in 6A/6A subjects (for respective 6A/6A, 5A/6A, and 5A/5A subjects, diameter at the R wave was 0.63±0.09, 0.55±0.06, and 0.53±0.04 cm [mean±SD], P<0.005 by ANOVA; intima-media thickness was 765±116, 670±116, and 630±92 μm [mean±SD], P<0.05 by ANOVA). Wall shear stress, calculated as blood velocity×blood viscosity/internal diameter, was significantly lower in 6A/6A subjects (for respective 6A/6A, 5A/6A, and 5A/5A subjects, mean wall shear stress was 10.4±2.9, 13.5±3.5, and 12.6±1.9 dyne/cm² [mean±SD], P<0.05 by ANOVA). The results demonstrate that the gene polymorphism in the promoter region of stromelysin is associated with structural and functional characteristics of the common carotid artery in healthy male subjects without major risk factors for atherosclerosis. Individuals with the 6A/6A genotype (associated with lower enzyme activity) show a triad of events, namely, increased wall thickness, enlarged arterial lumen, and local reduction of wall shear stress, which might predispose them to atherosclerotic plaque localization. (Arterioscler Thromb Vasc Biol. 2000;20:1600-1605.)

Key Words: carotid arteries ■ atherosclerosis ■ ultrasonics ■ metalloproteinases ■ polymerase chain reaction

Stromelysin is a metalloproteinase (MMP-3) involved in the turnover of the extracellular matrix components. This enzyme exhibits a wide spectrum of activity: it is able to degrade proteoglycans, collagens III, IV, V, and IX, laminin, fibronectin, gelatin, and elastin. Furthermore, stromelysin activates other members of the MMP family, such as the collagenases. Thus, it can play a pivotal role in the physiological and pathological events associated with connective tissue metabolism, including changes in arterial wall composition and/or function.1,2

Stromelysin production is regulated in response to several stimuli.3–5 A common variant in the promoter region of the human stromelysin gene with 1 allele having a run of 5 adenosines (5A) and the other having 6 adenosines (6A) has been recently reported.6 In in vitro experiments, it has been demonstrated that the 6A allele has a lower promoter activity, which is probably attributable to preferential binding of a putative transcriptional repressor protein.7 Therefore, subjects carrying the 6A allele accumulate extracellular matrix because of decreased degradation.

MMP-3 has been found to be extensively expressed in atherosclerotic plaques by foam cells of macrophage origin and smooth muscle cells in the plaque cap, intima, and adventitia. This finding has led to the hypothesis that MMP-3 might be involved in plaque rupture, followed by platelet activation and initiation of the coagulation cascade.8–12 However, data from studies involving MMP-3 5A/6A promoter polymorphism indicate that a condition associated with reduced enzyme activity (6A allele) predisposes the subject to the progression of atherosclerotic lesions in the coronary district.6 Indeed, matrix accumulation is a major feature of atheromatous lesions.

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A rearrangement of the arterial wall, involving cellular and matrix elements, is triggered by hemodynamic factors, such as shear stress and tensile stress. Variations in these forces cause the endothelium-mediated release of active substances, such as NO and endothelin, which in the short term modulate the vascular tone by restoring basal stress levels and in the long term induce vascular remodeling.13–19

The present study was designed to investigate the relationships between the MMP-3 polymorphism, vessel geometry, and shear stress in the common carotid artery of healthy male subjects selected for the absence of major risk factors for atherosclerosis.

**Methods**

One hundred nine presumed healthy male subjects were selected among hospital staff and their relatives for participation in the study. Subsequently, 62 subjects were excluded from the study because of the presence of major risk factors for atherosclerosis (36 subjects with hyperlipidemia, defined as plasma cholesterol and/or triglycerides >6.46 and 2.66 mmol/L, respectively; 4 subjects with diabetes, defined as fasting blood glucose >6.11 mmol/L; 18 subjects who were current smokers; and 4 subjects with elevated blood pressure level, defined as ≥140/90 mm Hg). Of the remaining 47 subjects, 5 refused blood withdrawal. Finally, 42 healthy male subjects, who were nonsmokers with an absence of hyperlipidemia, blood hypertension, or diabetes and not currently using any drug, gave informed consent and were enrolled.

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured on the right arm after the participant had been resting for at least 5 minutes; a standardized Hawksley zero-random sphygmomanometer was used. The average of the second and third of 3 readings was computed. Height and weight were measured by routine methods. Body mass index (BMI) was computed as weight (in kilograms) divided by height squared (in meters). Blood lipids and glucose were measured by commercially available kits. LDL cholesterol was calculated by the Friedewald formula (LDL cholesterol = total cholesterol − HDL cholesterol − [triglycerides/5]).

Echo-Doppler examination for the measurement of arterial diameter, intima-media thickness (IMT), and blood flow velocity was performed by using a ECG-triggered high-resolution ATL Ultramark 9 HDI Instrument (Advanced Technology Laboratories, Inc) equipped with a 5- to 10-MHz multifrequency linear probe, as previously described.20 All measurements were performed in the left common carotid artery, 1 to 2 cm proximal to the bulb. The left carotid artery was chosen because it was 2 to 2.5 cm longer than the right carotid artery, thus allowing the blood flow to develop a nearly parabolic profile. This is particularly relevant when measuring blood flow velocity and calculating wall shear stress. The common carotid artery was studied in longitudinal and transverse planes with anterior, lateral, and posterior approaches. A sonographer measured blood flow velocities and recorded the examination on a videotape. A reader, blinded with regard to the Doppler and physical examination results, performed the measurement of diameters and IMT. Reader and sonographer were unchanged during the course of the study. Internal diameter (ID) was defined as the distance between the leading edge of the echo produced by the intima-lumen interface of the near wall and the leading edge of the echo produced by the lumen-intima interface of the far wall. ID was measured at the R wave (ID_R) and T wave (ID_T) of the ECG.

The distance between the leading edge of the echo produced by the lumen-intima interface of the far wall. ID_T, obtained just before the systolic wave passage, is the narrowest luminal diameter, whereas ID_R, obtained during the systolic wave passage, is the largest one. IMT was measured from images displayed on a computer screen by the use of a Video Maker Card (Vitec) and analyzed by software that allows quantitative evaluation of the IMT. For each participant, 3 measurements pertaining to anterior, lateral, and posterior projections of the far wall were performed. The average of the 3 measurements was used to calculate the IMT. Blood flow velocity was detected with the sample volume reduced to the smallest possible size (1 mm) and placed in the center of the vessel. The angle between the ultrasound beam and the longitudinal vessel axis was kept between 44° and 56°. Systolic peak velocity (V_sp) and mean velocity (V_m) were recorded as the mean of 3 cardiac cycles.

Blood viscosity at a shear rate of 225/s (η) was measured in vitro, at 37°C, on the same day as the echo-Doppler examination with the use of a cone/plate viscometer (Wells-Brookfield DV III).

Detection of the stromelysin polymorphism after PCR amplification and restriction analysis with Tth111I in 3 subjects. Digested products are separated by electrophoresis on 2% agarose gel and visualized with use of ethidium bromide. M denotes DNA molecular weight marker V (Boehringer-Mannheim). The 6A/6A subject exhibits a unique undigested 130-bp band, whereas the 5A/5A subject exhibits one 97-bp digestion fragment. The 5A/6A subject displays 130- and 97-bp bands.

where τ_s, τ_m, and τ_m are expressed in dynes per centimeter squared; V_sp and V_m, in centimeters per second; ID_R and ID_T, in centimeters, and η, in poise.

The common polymorphism in the stromelysin gene promoter (5A/6A) was studied through polymerase chain reaction (PCR) amplification with the use of mutagenic primers (Figure). A 130-bp fragment was amplified from genomic DNA with use of the following primers (in parentheses, the exact positions within the promoter follow the numbering in Reference 21): forward primer (−1201 to −1172, mismatch at nucleotide −1173), 5′-GGTTCTCCATTCTTTTGATGGGGGGAAAgA-3′; reverse primer (−1072 to −1101), 5′-CTTCCGTGAATTTCACATCAC-TGCCACCACT-3′. Thus, a recognition site for the enzyme Tth111I (GACN/NNGTG) is created in the case of a 5A allele. The PCR reaction mixture contained 1 μg DNA, 0.8 μmol/L of each primer, 1.5 mmol/L MgCl₂, 0.2 mmol/L 4dNTP, and 1 U Taq DNA polymerase. After denaturing the DNA for 5 minutes at 95°C, the reaction mixture was subjected to 30 cycles of denaturing for 30 seconds at 94°C, 30-second annealing at 65°C, and 1-minute extension at 72°C. The PCR product was digested with 5 U Tth111I (Promega) for 3 hours at 65°C, and the digested products were separated by electrophoresis on 2% agarose gel and visualized with the use of ethidium bromide. The 5A genotype contains a unique Tth111I restriction site that results in 97- and 32-bp products. The method was validated by genotype confirmation through direct sequencing according to described methods.21
Total serum MMP-3 concentrations were measured by a Biotrak ELISA assay (Amersham International), based on a 2-site “sandwich” format. All tests were performed in duplicate. The sensitivity of the assay was reported as 2.35 ng/mL. According to the data provided by the manufacturer, the assay specifically recognizes total MMP-3 (active MMP-3, pro-MMP-3, and MMP-3 complexed with tissue inhibitors ), and there is no cross-reaction with other MMPs.

In the determination of MMP-3 polymorphism, Hardy-Weinberg equilibrium was assessed by $\chi^2$ analysis. All considered variables had normal distribution. ANOVA and Bonferroni-Dunn post hoc tests were used to compare continuous variables among subjects with different MMP-3 polymorphisms. Multiple regression analyses were performed to adjust carotid geometry and hemodynamic parameters for age, blood pressure, blood lipids, glucose, and heart rate.

### Results

Of the 42 subjects participating in the study, 8 subjects (19.1%) were homozygous for the 5A allele, 10 (23.8%) were homozygous for the 6A allele, and 24 (57.1%) were heterozygous. The frequencies of the 5A and 6A alleles were 0.476 (95% CI 0.367 to 0.587) and 0.524 (95% CI 0.413 to 0.633), respectively. Although obtained in a highly selected sample, the frequencies were not significantly different from those found in a control sample of 155 unrelated healthy Italian individuals (5A 0.471, 6A 0.529). The genotype distribution was not significantly different from the Hardy-Weinberg prediction.

Clinical and biochemical characteristics of participants, divided according to MMP-3 polymorphism, are shown in Table 1. Age, DBP, BMI, and blood viscosity were similar in the 3 groups. SBP, total cholesterol, triglycerides, and HDL cholesterol were slightly higher in those homozygous for the 6A allele, but the difference did not reach statistical significance at the conventional 5% level. Blood glucose was slightly, but again not significantly, lower in 6A homozygotes. Serum MMP-3 levels (mean±SD) were not significantly different among genotypes: 88.9±8.1 ng/mL in 5A/5A, 93.3±15.3 ng/mL in 5A/6A, and 92.8±12.8 ng/mL in 6A/6A (F=0.317 and P=0.73 by ANOVA).

Common carotid artery diameter, at the R and T waves of the ECG, and IMT were significantly larger in 6A/6A subjects compared with heterozygotes or homozygotes for the 5A allele. No difference was observed between these last 2 groups. Mean blood flow velocity was slightly but significantly lower in 6A homozygotes. Increased arterial diameter and reduced flow velocity yielded significantly lower values of wall shear stress in 6A/6A subjects. Again, no difference between 5A/6A and 5A/5A subjects was observed (Table 2).

Table 3 reports the same variables illustrated in Table 2 after adjustment for possible confounding variables by multiple regression analysis. Although slightly attenuated, the differences between homozygotes for the 6A allele and heterozygotes or homozygotes for the 5A allele persisted even after adjustment.

### Discussion

The results of the present study suggest that a genetic polymorphism of an MMP, namely, stromelysin, which...
modulates its enzymatic activity, is associated with structural and functional characteristics of the common carotid artery in healthy male subjects without major risk factors for atherosclerosis. Individuals homozygous for the 6A allele show a triad of events, namely, increased wall thickness, enlarged arterial lumen, and local reduction of wall shear stress, which might predispose them to atherosclerotic plaque localization.

The relationship between MMP-3 polymorphism and the observed triad of vascular events might be explained in several ways. The reduced activity of stromelysin in 6A subjects could lead to matrix accumulation and thickening of the arterial wall. This would cause the lumen of the artery to narrow, thereby subjecting the endothelium to an increase in wall shear stress. The arterial enlargement to restore basal hemodynamic forces would overcompensate, thus leading to the observed arterial dilation and reduced wall shear stress. Such a mechanism has been postulated by several authors to explain the compensatory enlargement of carotid as well as coronary arteries in response to atherosclerosis.23–25

An alternative hypothesis would consider the impairment in elastic properties of the arterial wall, consequent to the uncoordinated matrix accumulation, as a primary event. This would cause reduced resistance to the dilating effect of blood pressure, arterial dilation, and reduction of wall shear stress. The thickening of the arterial wall could be an adaptive process, subsequent to the changes in hemodynamic load.24

Enlarged common carotid diameter has been observed in different physiological and pathological situations. Two population-based studies have reported an increasing common carotid artery diameter with aging.26,27 Likewise, we have observed increasing common carotid diameter with age in normal subjects without major coronary heart disease risk factors.20 Body weight and height are other situations in which a correlation with arterial diameter has been described27; we also found in previous studies a correlation between BMI and common carotid diameters. However, there are also pathological conditions associated with enlarged common carotid diameters. We have recently reported that male subjects with non–insulin-dependent diabetes mellitus have significantly larger common carotid diameters compared with age- and sex-matched controls.28 Furthermore, the presence of atherosclerotic plaque, in the coronary and carotid circulation, is often associated with larger arterial diameter.29 Whether this enlargement is primary or secondary to the presence of plaque is still a matter of debate.

As a whole, data in the literature indicate that increases in the arterial lumen and wall thickness are constant findings that accompany the process of aging as well as clinical conditions associated with atherosclerosis.30 Our present study demonstrates that middle-aged subjects homozygous for the 6A allele, although healthy and free of the major risk factors for atherosclerosis, exhibit alterations in the common carotid artery resembling those observed in aging and atherosclerosis.

Present knowledge about the influence of MMP-3 polymorphism on atherosclerosis provides evidence of a link between the presence of the 6A allele and progression of coronary lesions. In a report on 72 patients with coronary heart disease defined by angiography participating in the St. Thomas Atherosclerosis Regression Study (STARS), Ye et al6 found that those homozygous for the 6A allele showed more rapid progression of global and focal atherosclerotic stenoses over the 3-year study period; this was particularly evident in patients with mild baseline stenoses or with higher LDL cholesterol concentration. Reports from the Regression Growth Evaluation Statin Study (REGRESS), investigating symptomatic patients over a 2-year period, and data from the Lopid Coronary Angiography Trial (LOCAT) involving men after coronary bypass with low HDL cholesterol show similar results in the placebo groups, whereas no difference in vessel diameter change was observed in the groups treated with pravastatin (REGRESS) or gemfibrozil (LOCAT).31,32 The findings of the present study, which was targeted to investigate the relationship between MMP-3 polymorphism and the carotid artery in a preselional stage, are in agreement with the results of the above-mentioned studies. The design of the present study makes difficult a direct comparison of our results with the data published so far. To our knowledge, the present study is the first to report on the effects of MMP-3 polymorphism in a selected sample of healthy and relatively young male subjects free of the symptoms and signs of atherosclerosis. The extracranial carotid district has been chosen because it can be easily and reproducibly studied by noninvasive techniques.

We also measured the total serum MMP-3 levels to identify any possible relationship with the genotype. No significant association was evidenced, even though the limited sample size does not allow an adequate statistical power. Tissue MMPs may leach into the blood stream in increased amounts in several pathological conditions (eg, cancer and inflammatory disease).33 However, an increased local prod-

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**TABLE 3. ANOVA of Left Common Carotid IDs, IMT, and Blood Velocity Among Participants With Different Stromelysin 5A/6A Genotypes, After Adjustment for Possible Confounding Variables by Multiple Regression Analyses**

<table>
<thead>
<tr>
<th></th>
<th>5A/5A (n = 8)</th>
<th>5A/6A (n = 24)</th>
<th>6A/6A (n = 10)</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID, cm</td>
<td>0.53±0.04</td>
<td>0.55±0.05</td>
<td>0.60±0.07*</td>
<td>3.972</td>
<td>≤0.02</td>
</tr>
<tr>
<td>ID, cm</td>
<td>0.58±0.04</td>
<td>0.60±0.05</td>
<td>0.66±0.08†</td>
<td>5.210</td>
<td>≤0.01</td>
</tr>
<tr>
<td>IMT, μm</td>
<td>657±68</td>
<td>668±91</td>
<td>748±93*</td>
<td>3.374</td>
<td>≤0.04</td>
</tr>
<tr>
<td>Vp, cm/s</td>
<td>77.9±11.8</td>
<td>82.7±8.8</td>
<td>74.2±10.6</td>
<td>2.811</td>
<td>≤0.07</td>
</tr>
<tr>
<td>Vb, cm/s</td>
<td>36.8±3.8</td>
<td>38.9±4.3</td>
<td>35.4±2.5</td>
<td>3.075</td>
<td>≤0.05</td>
</tr>
<tr>
<td>τs, dyne/cm²</td>
<td>25.1±4.2</td>
<td>25.5±3.6</td>
<td>21.3±3.9†</td>
<td>4.470</td>
<td>≤0.02</td>
</tr>
<tr>
<td>τb, dyne/cm²</td>
<td>12.9±1.6</td>
<td>13.1±2.5</td>
<td>11.1±1.3†</td>
<td>3.165</td>
<td>≤0.05</td>
</tr>
</tbody>
</table>

Values are mean±SD. All variables were adjusted for age, SBP, DBP, BMI, HR, blood glucose, and LDL-C.

*P<0.167 vs 5A/5A; †P<0.0167 vs 5A/6A (Bonferroni-Dunn test).
tion of MMPs may not necessarily be associated with increased circulating levels, because MMPs are capable of binding to the connective matrix.34 Our data suggest that changes in vessel wall MMP-3 expression as determined by gene promoter polymorphism are not reflected in blood MMP-3 levels.

The hypothesis of a genetically determined reduced expression of stromelysin as associated with the progression of atherosclerosis is somehow in contrast to the present view that an increased activity in matrix-degrading enzymes is a potential source of plaque instability.35 Recently, Terashima et al36 reported a significantly higher prevalence of the 5A/6A+5A/5A genotype among patients with acute myocardial infarction compared with control subjects. In this ethnic group, however, the 5A allelle frequency was much lower than that observed in other European populations. Further investigation is therefore required to clarify this point.

Furthermore, one has to consider that plaque progression and plaque rupture are 2 distinct processes that recognize different pathogenetic mechanisms and moments.37 One can hypothesize that an imbalance in stromelysin expression in the arterial wall in either direction can have deleterious effects, depending on the stage of the atheromatous lesions. A reduced enzyme activity leads to the accumulation of extra-cellular matrix and the progression of atherosclerosis.6 Accordingly, Tyagi et al38 have reported that weight for weight, atherosclerotic vessels contain more collagen and less MMP activity than do normal arteries. On the other hand, focal overexpression of matrix-degrading activity in crucial plaque sites can trigger the rupture of the fibrous cap and acute thrombotic events.12

The relatively low number of enrolled subjects requires a comment. Subjects with no major coronary heart disease risk factors were selected. Although a larger number of presumed healthy subjects were initially considered, only 42 individuals fulfilled the enrollment criteria. The distribution of the MMP-3 genotypes, however, allowed us to have a sufficient number of subjects in each group. The power of the study, calculated on the basis of the difference observed in IDs and in the sample size investigated, was indeed at least 80%. Thus, the findings reported in the present study may be considered substantial, although studies with larger sample sizes are certainly needed for a confirmation.

In conclusion, the present study identifies the MMP-3 promoter polymorphism as a major genetic determinant of wall thickness and vessel dilation of the common carotid artery in the absence of confounding risk factors for atherosclerosis. Future longitudinal studies are warranted to verify the impact of this gene polymorphism on atherosclerosis-related clinical end points.

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