Marburg I Polymorphism of Factor VII–Activating Protease
A Prominent Risk Predictor of Carotid Stenosis

Johann Willeit, MD; Stefan Kiechl, MD; Thomas Weimer, PhD; Artur Mair, MD; Peter Santer, MD; Christian J. Wiedermann, MD; Juergen Roemisch, PhD

Background—Atherothrombosis is a main pathomechanism in the evolution of vessel stenosis and is counteracted by endogenous fibrinolysis. Recently, the plasmatic serine protease “factor seven–activating protease” (FSAP) was recognized as a potent activator of prourokinase in vitro. The Marburg I polymorphism of FSAP impairs this potential and may thus facilitate arterial thrombosis.

Methods and Results—This analysis of the Bruneck Study involved 810 men and women aged 40 to 79 years. The ultrasound-based atherosclerosis progression model (5-year follow-up) permits differentiation between early atherogenesis and the advanced stenotic stages of carotid artery disease. The FSAP Marburg I polymorphism was found in 37 subjects (carriage rate 4.4%). Individuals with this genetic variant showed a prominently reduced in vitro capacity to activate prourokinase. No relation was found to exist between the Marburg I polymorphism and early atherogenesis. In contrast, it emerged as a strong and independent risk predictor of incident/progressive carotid stenosis (multivariate odds ratio [95%CI], 6.6 [1.6 to 27.7]). This finding equally applied to subjects with and without co-segregation of the Marburg II polymorphism. The risk profile of advanced atherogenesis further includes cigarette smoking, high lipoprotein(a), the factor V Leiden mutation, low antithrombin III, high fibrinogen, and diabetes.

Conclusions—In concert with other genetic and acquired conditions known to interfere with coagulation or fibrinolysis, the Marburg I polymorphism of FSAP, which attenuates its capacity to activate prourokinase, is a significant risk predictor for the evolution and progression of carotid stenosis. (Circulation. 2003;107:667-670.)

Key Words: atherosclerosis ■ risk factors ■ thrombosis ■ thrombolysis

atherothrombosis is a main pathomechanism in the evolution of vessel stenosis and the manifestation of vascular disease. Fissuring of vulnerable plaques is a key event initiating local atheroma-associated thrombus formation, whereas excessive clot propagation relies on the failure of usually efficacious antithrombotic and fibrinolytic systems.

The plasmatic serine protease factor seven–activating protease (FSAP) has been recognized as a novel potent activator of prourokinase-dependent fibrinolysis.1–6 We recently characterized a single nucleotide polymorphism (SNP) of FSAP, termed “Marburg I polymorphism,” which impairs the capacity of FSAP to activate prourokinase without attenuating its potential contribution to the extrinsic coagulation pathway.1–6 This may drive hemostasis toward a prothrombotic state.

See p 654

In the present prospective population-based study we investigated the impact of this polymorphism on the evolution and progression of carotid stenosis, thereby utilizing an ultrasound model of atherosclerosis progression capable of differentiating early from advanced stages of vessel disease.7,8

Methods

Study Subjects
In 1990, the Bruneck Study population was recruited as a sex- and age-stratified random sample of all inhabitants of Bruneck, Italy, aged 40 to 79 years (125 women and 125 men in each decade of age). A total of 93.6% of the population participated, with data assessment completed in 919 persons. During the follow-up periods between 1990 to 1995 and 1995 to 2000, 63 and 94 individuals, respectively, died or moved away. In the remaining population, follow-up was 96.5% (n=826) and 95.6% (n=684) complete, respectively.1–3

Blood specimens for DNA extraction were drawn as part of the 1995 follow-up. Adequate polymerase chain reaction products were not obtainable in 16 samples, which left 810 (1995) and 678 (2000) subjects for the main analysis.

Risk Factors and Laboratory Methods
Standard criteria were applied to define vascular risk factors.9 Blood samples were taken after subjects had fasted for ≥12 hours.
Lipoprotein(a) concentrations were determined with an ELISA (Immunno), fibrinogen according to the method of Clauss, and antithrombin III with a chromogenic assay. The factor V Leiden mutation was detected by allele-specific PCR amplification. FSAP antigen concentrations and prourokinase-activating potencies were assessed as recently described.3–5

Genomic DNA was prepared from frozen whole blood with the GenomicPrep blood DNA isolation kit (Amersham Pharmacia Biotech).

Marburg I polymorphism (Gly511Glu): 5 μL DNA was amplified in 1X TaqGold reaction buffer containing 50 pmol of forward (5’CTGGCTCATTTGGCCACAGG3’) and reverse primer (5’CAGATGTTCTGGTTCCAGGAGGAGG3’), 2.5 pmol of wild-type (5’Fam-CTGGAGGTTGGGAAAGGC-GGTGGC3’) and mutant-specific (5’Tet-CTGGAGGTTGGGAAAGGC-GGTGGC3’) probe, 3 mM/L MgCl2, 200 μM/L deoxynucleoside triphosphate, and 1.25 U TaqGold DNA polymerase (Perkin Elmer) in a total volume of 50 μL. Initial denaturation (10° 95°C) was followed by 50 cycles of alternating denaturation (1° 95°C) and combined annealing and elongation (1° 63°C) in a Perkin Elmer 7700 real-time PCR machine.

Marburg II polymorphism (Glu370Gln): protocol as above, with the following modifications: 22.5 pmol of forward (5’AAATCT-CATCTCTTTACGTTGATGTGGC3’) and reverse primer (5’GGACCCAGACGTCAAGAAGAG3’), 5 pmol of wild-type (5’Fam-ATATCCTTCCACCCCTTAA-TATCCCTTCAAGAAGAG3’) and mutant-specific (5’Vic-TATCTCTTCTCGACCTTAAA-mgb3’) probe, and 0.63 U TaqGold DNA polymerase in a total volume of 25 μL. Initial denaturation was 10° 95°C, alternating denaturation 15° 92°C, and annealing and elongation 1° 60°C.

Evaluation of results was performed in comparison to cloned wild-type and mutant fragments and equimolar mixes thereof.

Scanning Protocol
The ultrasound protocol involved scanning of the internal and common carotid arteries of either side with a 10-MHz imaging probe and a 5-MHz Doppler.5,8 Atherosclerotic lesions were defined by two ultrasound criteria: wall surface (protrusion into the lumen) and texture (echogenicity). The maximum diameter of plaques was assessed in each vessel segment (intravascular CV 10% to 15%).

Incident atherosclerosis was defined by the occurrence of new plaques in previously normal segments and progression of nonsteno tic lesions by a relative increase in the plaque diameter exceeding twice the measurement error.7,8 Both processes were combined to a single outcome category (early atherogenesis). “Advanced atherosclerosis” was assumed whenever the progression criterion was met and a lumen narrowing of >40% occurred. The 40% cutoff appears to be a biological threshold in our population, at which marked changes in the growth kinetics of plaques, risk profiles, and vascular remodeling process occurred as indicative of a switch in the underlying pathogenetic mechanisms from conventional atherogenesis to atherothrombosis.7–9

Statistical Analysis
Associations between FSAP polymorphisms and the various stages of atherogenesis were assessed by logistic regression analysis. A base model was adjusted for age and sex. Multivariate equations were fitted by a forward stepwise selection procedure (probability values for entry and removal, 0.10 and 0.15). Age and sex were additionally forced into these models to account for the age and sex structure of the population sample. Regression-standardized risks of atherogenesis according to the number of risk predictors were calculated with the marginal method of regression adjustment.

Results
In the Bruneck study cohort (n = 810), 36 subjects were heterozygous for the FSAP Marburg I polymorphism and one was homozygous (17 men, 20 women), corresponding to a carriage rate in the general community of 4.4% (3.0%–5.8%).

Cos segregation of the Marburg II polymorphism was observed in 16 (8 men, 8 women) of the 37 individuals.

In a subpopulation of the Bruneck cohort (n = 82) and in blood donors from another geographic region (n = 200),6 plasma samples were analyzed for in vitro profibrinolytic potencies of FSAP. As visualized in Figure 1, carriers of the Marburg I polymorphism showed a prominently reduced FSAP activity and activity-to-antigen ratio.

During the 5-year follow-up (1990 to 95), 384 of the 810 study subjects (47.4%) experienced early atherogenesis and 92 of 326 individuals with preexisting plaques (28.2%) showed stenotic transformation (advanced atherogenesis). No relation was found to exist between the Marburg I polymorphism and early atherogenesis (age/sex-adjusted and multivariate odds ratios [95% CIs] 0.6 [0.3 to 1.4] and 0.7 [0.3 to 1.7], respectively). However, the polymorphism emerged as a strong risk predictor of advanced atherogenesis (age/sex-adjusted odds ratio [95% CI] 3.5 [1.1 to 11.4], P = 0.036). The association remained statistically significant when adjusting the logistic regression model for other risk predictors (Table), all of which have been shown to interfere with coagulation or fibrinolysis. There was no evidence of differential effects of the Marburg I polymorphism in subpopulations according to age, sex, or levels of lifestyle attributes. The risk of advanced atherogenesis amplified with an increasing number of procoagulant risk factors clustering in individuals (Figure 2).

The FSAP Marburg II polymorphism was not associated with atherosclerosis development in our population sample. On comparing subjects with wild-type FSAP and those carrying either the Marburg II polymorphism, the Marburg I polymorphism or both genetic disorders multivariate odds ratios [95% CIs] of advanced atherogenesis were as follows: 1.6 [0.2 to 13.7] (P = 0.67); 6.2 [1.1 to 36.0] (P = 0.048); and 7.1 [1.1 to 45.1] (P = 0.037).

To demonstrate consistency of our findings over a longer time period, computations were repeated with data from the 5-year follow-up between 1995 and 2000. In these equations, the relation between the Marburg I polymorphism and advanced atherogenesis again achieved statistical significance (multivariate odds ratio [95% CI] 4.0 [1.1 to 15.7], P = 0.045).

Discussion
FSAP is a serine protease with a putative role in fibrinolysis and contact activation.3–5 It was initially described as a contributor to the extrinsic coagulation pathway and was recently recognized as a potent activator of prourokinase,3–6 which together with t-PA constitutes a dominant role in human fibrinolysis.10 The fibrinolytic action of both plasminogen activators has been proposed to be complementary and synergistic.10 In the setting of atherothrombosis prourokinase may originate from within the plaque and from circulation. Actually, prourokinase activity is substantially upregulated in advanced atherogenic lesions prone to rupture11 and evidence has recently emerged in favor of a significant contribution of prourokinase to intravascular lysis.12

We recently characterized an SNP in exon 13 of FSAP (Marburg I) which causes a Gly511Glu amino acid change near the protease active site and selectively impairs its
profibrinolytic capacity. As the outstanding findings of the present study, carriers of the polymorphism exhibited a substantially reduced capacity to activate prourokinase in vitro (Figure 1) and faced a high risk of advanced atherogenesis in the carotid arteries, with the strength of association being comparable to that assessed for high Lipoprotein(a), diabetes, or the factor V Leiden mutation (Table). A combination of two or more procoagulant factors predicted a risk of

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**Figure 1.** A, FSAP activity and activity-to-antigen ratio in the Bruneck population and B, in healthy blood donors. Bars are means and confidence intervals.

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### Advanced Atherogenesis

<table>
<thead>
<tr>
<th>Variable</th>
<th>No (n=234)</th>
<th>Yes (n=92)</th>
<th>Odds ratio (95% CI)</th>
<th>P</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>64.9±9.2</td>
<td>67.8±8.0</td>
<td>1.87 (1.19–2.92)</td>
<td>0.0064</td>
<td>0</td>
</tr>
<tr>
<td>Female sex</td>
<td>109 (46.6)</td>
<td>32 (34.8)</td>
<td>0.56 (0.25–1.25)</td>
<td>0.1555</td>
<td>0</td>
</tr>
<tr>
<td>Glucose tolerance</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
<td>1</td>
</tr>
<tr>
<td>IGT</td>
<td>20 (8.5)</td>
<td>16 (17.4)</td>
<td>3.31 (1.37–7.99)</td>
<td>0.0081</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>10 (8.1)</td>
<td>21 (22.8)</td>
<td>6.38 (2.71–14.99)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Cigarettes per day</td>
<td>3.2±7.2</td>
<td>6.6±9.6</td>
<td>1.77 (1.30–2.40)</td>
<td>0.0003</td>
<td>2</td>
</tr>
<tr>
<td>Lipoprotein(a) &gt;0.32 g/L</td>
<td>36 (15.4)</td>
<td>25 (27.2)</td>
<td>4.06 (1.83–8.96)</td>
<td>0.0005</td>
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</tr>
<tr>
<td>Alcohol consumption</td>
<td></td>
<td></td>
<td></td>
<td>0.0043</td>
<td>4</td>
</tr>
<tr>
<td>&lt;1 g/d</td>
<td>114 (48.7)</td>
<td>42 (45.6)</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–50 g/d</td>
<td>60 (25.7)</td>
<td>15 (16.3)</td>
<td>0.26 (0.10–0.66)</td>
<td>0.0046</td>
<td></td>
</tr>
<tr>
<td>51–99 g/d</td>
<td>37 (15.8)</td>
<td>17 (18.5)</td>
<td>1.03 (0.40–2.70)</td>
<td>0.9475</td>
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<tr>
<td>≥100 g/d</td>
<td>23 (9.8)</td>
<td>18 (19.6)</td>
<td>1.90 (0.63–5.69)</td>
<td>0.2535</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>2.7±0.6</td>
<td>2.9±0.6</td>
<td>1.53 (1.12–2.09)</td>
<td>0.0083</td>
<td>5</td>
</tr>
<tr>
<td>Marburg I FSAP polymorphism</td>
<td>5 (2.1)</td>
<td>8 (8.7)</td>
<td>6.63 (1.58–27.72)</td>
<td>0.0099</td>
<td>6</td>
</tr>
<tr>
<td>Factor V mutation</td>
<td>5 (2.1)</td>
<td>7 (7.6)</td>
<td>4.70 (1.19–18.55)</td>
<td>0.0291</td>
<td>7</td>
</tr>
<tr>
<td>Antithrombin III, %</td>
<td>96.3±13.0</td>
<td>92.8±16.4</td>
<td>0.74 (0.55–1.00)</td>
<td>0.0500</td>
<td>8</td>
</tr>
<tr>
<td>Platelet count, ×10⁸/L</td>
<td>217.4±56.5</td>
<td>230.3±56.6</td>
<td>1.32 (0.98–1.77)</td>
<td>0.0769</td>
<td>9</td>
</tr>
</tbody>
</table>

Values presented are mean±SD or number (%). The logistic regression model was fitted with a forward stepwise selection (Step...Step of entry). ORs were calculated for a 1-SD unit change of given variables.
carotid stenosis much higher than that calculated for single abnormalities (Figure 2).

Notably, another SNP of FSAP (Marburg II), which is commonly co-segregated with the Marburg I polymorphism but, per se, lacks in vitro effects on the fibrinolytic system (Figure 1),5 has been shown to be unrelated with atherosclerosis development and progression.

Strengths of our study include the prospective design, representation of the general community, accordance of experimental and epidemiological results, and the ultrasound model capable of studying advanced putatively atherothrombotic stages of vessel pathology in vivo, whereas the small number of subjects with FSAP polymorphism represents a limitation.

In conclusion, our findings suggest a physiologically pro-fibrinolytic role of FSAP based on its capacity to activate prourokinase. Impairment of this potential by the Marburg I polymorphism is associated with a high risk of progressive carotid stenosis, which was previously shown to arise primarily from atherothrombosis.7–9 Whether or not the Marburg I polymorphism is relevant to therapeutic thrombolysis with prourokinase cannot be inferred from our data.

References
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