Thrombomodulin Ala455Val Polymorphism and Risk of Coronary Heart Disease

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Background—Thrombomodulin (TM) is expressed on the endothelial surface and plays an important role in vasoprotection. A common polymorphism of TM at amino acid position 455 with an alanine (A) to valine (V) transition was previously reported to be associated cross-sectionally with acute myocardial infarction. Whether this single nucleotide polymorphism predicts risk of developing coronary heart disease (CHD) is unclear.

Methods and Results—Within a large cohort study, we identified 467 incident CHD cases during an average of 5 years of follow-up. We determined TM-455 genotypes on 376 CHD cases (23% black, 77% white) and a reference sample of 461. The AA genotype was significantly more prevalent in noncases than in cases ($P=0.016$). The prevalences of the AA genotype in noncase blacks and whites were 93% and 67%, respectively. The AA genotype frequency was significantly reduced in black cases versus noncases ($P=0.018$). It was also lower in white cases than in noncases, but the difference was not statistically significant ($P=0.066$). Weighted proportional hazards regression analysis after adjustment for age, sex, and other CHD risk factors showed that having the V allele increased risk of CHD by 6.1-fold (risk ratio 6.1, 95% CI 1.7 to 22.9) in blacks but did not significantly increase the risk in whites.

Conclusions—The TM A455V polymorphism predicts risk of developing CHD in blacks. (Circulation. 2001;103:1386-1389.)

Key Words: thrombomodulin ■ genetics ■ coronary disease

Thrombomodulin (TM) is a transmembrane glycoprotein expressed on the surface of endothelial cells. It is an important vasoprotective and thromboresistant molecule. It binds thrombin and alters its conformation, converting it from a potent prothrombotic to an antithrombotic enzyme. A main activity of TM-bound thrombin is cleavage of protein C to generate activated protein C, which degrades factor Va and factor VIIIa, thereby reducing coagulation reactions. TM is expressed on the surface of basal, unstimulated endothelial cells, and its expression is reduced by inflammatory mediators. Reduced levels of TM may contribute to susceptibility to vascular damage and thrombosis in septicemia and inflammation. The human TM gene does not contain introns. Several polymorphisms or mutations in the coding region and the promoter region of the TM gene have been identified. The influence of these polymorphisms over the level or activity of TM is unclear. However, it was reported that a dimorphism at codon 455, a cytosine transition to thymidine resulting in an alanine (A) to valine (V) substitution at amino acid position 455 (A455V), may be associated with myocardial infarction (MI). The report was based on a cross-sectional study of a relatively small number of patients. The relationship between this single nucleotide polymorphism and incidence of coronary heart disease (CHD) remains unclear. In the present report, we have analyzed the association of the A455V polymorphism with incident CHD in a prospective follow-up of the Atherosclerosis Risk In Communities (ARIC) study cohort. Our results show that this single nucleotide polymorphism of the TM gene is an independent risk factor for CHD in blacks.

Methods

Subjects

The ARIC study is a population-based cohort study that recruited 15,792 men and women aged 45 to 64 years from Forsyth County, North Carolina; Jackson, Miss (blacks only); the northwest suburbs of Minneapolis, Minn; and Washington County, Maryland. A previous report described the recruitment and examination methods. The baseline examination, including collection of blood and DNA samples, was performed between 1987 and 1989 as described previously. These participants were reexamined on a 3-year cycle;
93% of the initial cohort were reexamined in 1990 to 1992, 86% in 1993 to 1995, and 80% in 1996 to 1998 by identical procedures.

Incident CHD Cases and Cohort Random Sample
A case-cohort design was used for this nested genetic study. For this analysis, we included CHD events that occurred between the initial visit (1987 to 1989) and December 31, 1993. The mean follow-up period was 5 years. We defined CHD incidence as (1) definite or probable MI, (2) silent MI, (3) definite CHD death, or (4) coronary revascularization. The CHD events were ascertained by strategies described previously. All identified potential clinical events were reviewed and adjudicated by an ARIC morbidity and mortality classification committee using published criteria. Coronary revascularization was defined as having hospital procedure codes for coronary bypass graft, coronary angioplasty, or coronary atherectomy.

A stratified random sample of the entire ARIC cohort was selected as a reference group. For this reference group (designated as noncases), we oversampled participants with an average carotid intima-media thickness measurement at baseline less than the 30th percentile and stratified the sampling by age and sex. As a result of these varying sampling fractions, we adjusted for the sampling and the case-cohort design by Barlow’s method. Because the prevalence of the VV genotype was very low in blacks, we oversampled participants with an average carotid intima-media thickness measurement at baseline less than the 30th percentile and stratified the sampling by age and sex. As a result of these varying sampling fractions, we adjusted for the sampling and the case-cohort design by Barlow’s method.21 Because there was a significant difference in the prevalence of AA and AV/VV genotypes between whites and blacks, we performed separate weighted proportional hazards regression analyses for whites and blacks. In the weighted proportional hazards regression models, we adjusted for sex, age, and the following factors related to CHD in this sample: white blood cell count, hypertension, diabetes, total cholesterol, HDL cholesterol, and cigarette smoking.

Baseline Measurements
Trained technicians measured blood pressure 3 times using a random-zero sphygmomanometer. The mean of the last 2 measurements was used for analysis. Hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or current use of antihypertensive medication. Cigarette smoking and ethanol intake were based on questionnaire described previously.

Venous blood was collected and processed at the baseline examination according to procedures described previously. Specimens were sent by overnight courier to the Central Hemostasis Laboratory in Minneapolis, where they were stored at −20°C to −80°C until assay. Plasma total cholesterol and triglycerides were measured by enzymatic methods as described previously. HDL cholesterol was measured after dextran-magnesium precipitation to remove non-HDL lipoproteins. Erythrocyte levels were measured by the thrombin time titration method, and von Willebrand factor antigen levels were measured by enzyme immunoassay as described previously. Soluble TM levels in plasma were measured by enzyme immunoassay as described previously. The laboratory coefficients of variation for soluble TM between and within assays were 8.2% and 6.0%, respectively. Serum glucose was measured by the hexokinase method. Diabetes mellitus was defined as a fasting glucose level ≥126 mg/dL or a nonfasting glucose level ≥200 mg/dL or a history of, or treatment for, diabetes. White blood cells were counted by local laboratories.

Determination of TM-455 Genotypes
Genomic DNA was isolated from buffy-coat white blood cells by phenol-chloroform extraction and ethanol precipitation. Genotypes of the TM-455 dimorphism were determined by single strand conformation polymorphism (SSCP) according to a procedure described previously. Amplification of the TM gene by polymerase chain reaction was performed with forward primer, 5’-CCGTACCTTCCAGGCTATTCG-3’, and reverse primer, 5’-ACGGCCGGGAGGCTACAGGTCCTAG-3’. This resulted in the generation of a 153-bp fragment. SSCP for detecting the TM-455 dimorphism was done with the amplified polymerase chain reaction product as described previously.

Data Analysis
To determine the relation of TM dimorphism with other variables, some of which may be confounders in this analysis, we used ANCOVA to compute age-, race-, and sex-adjusted mean levels or percentages of the variables of interest for the AV/VV and AA genotypes. We also used ANCOVA to compute age-, race-, and sex-adjusted mean or percentage values of study variables for CHD cases versus noncases after appropriate weighting for the stratified case-cohort sampling design. We computed the risk ratios (RRs) and 95% CIs for the time to development of CHD using a weighted proportional hazards regression, accounting for the stratified random sampling and the case-cohort design by Barlow’s method. Because there was a significant difference in the prevalence of AA and AV/VV genotypes between whites and blacks, we performed separate weighted proportional hazards regression analyses for whites and blacks. In the weighted proportional hazards regression models, we adjusted for sex, age, and the following factors related to CHD in this sample: white blood cell count, hypertension, diabetes, total cholesterol, HDL cholesterol, and cigarette smoking.

Results
Because the prevalence of the VV genotype was very low in cases (1.4%) and noncases (0.6%), we combined VV and heterozygous AA in our analysis. Table 1 shows the distribution of AA and AV/VV genotypes in 376 cases and 461 noncases. The prevalence of AV/VV was higher, and conversely, the prevalence of AA was lower in cases than in noncases (P=0.016). When these data were analyzed separately by race, the prevalence of AA genotype was higher in black noncases than in white noncases (Table 1). The frequency of cases with AV/VV was significantly higher than that of noncases among blacks (P=0.018) (Table 1). The percent of cases with AV/VV was also higher than that of noncases in whites, and the difference was close to being statistically significant (P=0.066) (Table 1). Table 2 shows that other than a significant association of TM genotype with race, the TM genotypes were not associated with age, sex, or other CHD risk factors.

The RR of AV/VV versus AA for developing CHD was analyzed by weighted proportional hazards regression analysis. In the entire sample, regardless of race, the combined AV/VV genotype group was associated with significantly higher CHD risk by 60% over the AA genotype (RR 1.6, 95%
CI 1.1 to 2.3) after adjustment for age, race, and sex. However, the RR was reduced to 1.2, which was statistically insignificant, after adjustment for additional CHD risk factors. When the RR was determined according to race, the AV/VV genotype group increased the risk of CHD in blacks by 4.4-fold after adjustment for age and sex and by 6.1-fold when adjusted for other CHD risk factors. In contrast, genotypes were not significantly associated with risk of CHD in whites (Table 3).

Soluble TM in plasma is a degradation product of endothelial TM. Results from a recent ARIC report suggest that the plasma concentration of soluble TM may reflect the level of endothelial TM expression. We therefore compared mean plasma soluble TM concentrations between the AA and AV/VV genotypes in the cohort random sample. The mean soluble TM level in the AA genotype (42.7 ng/mL) was not significantly different from that in the AV/VV genotype (39.2 ng/mL, P = 0.31). This was also true for both whites (P = 0.20) and blacks (P = 0.22).

**Discussion**

An important finding from this prospective case-cohort study is that an alanine-to-valine mutation at amino acid residue 455 of TM increases risk of CHD by 6-fold in blacks, whereas this same mutation does not significantly increase CHD risk in whites. This result underscores the importance of investigating genetic risk factors for CHD by ethnic origin.

TM residue 455 is located at the epidermal growth factor (EGF)-like domain of the extracellular region of endothelial membrane TM. There are 6 EGF-like repeats, and the last 3 repeats are functionally important for protein C activation and thrombin binding. TM-455 is located in the last EGF-like repeat. A valine substitution for alanine was therefore considered to have the potential of altering the TM activity. However, a recent report showed that TM-455A and TM-455V expressed in COS-1 cells by transient transfection possessed similar kinetic properties for activation of protein C and thrombin binding. We recently observed that the plasma soluble TM level was inversely associated with the risk of CHD. These results led us to postulate that the plasma soluble TM concentration reflects the expression level of TM on endothelial surface. Because the endothelial TM level may be suppressed by proinflammatory cytokines, which have been shown to play an important role in CHD, we chose to analyze the correlation of TM-455 genotypes with soluble TM concentrations only in the random cohort sample. Our results did not show a statistically significant difference in plasma soluble TM between AA and AV/VV genotypes in the entire random cohort sample nor in blacks or whites. A previous report on whites also did not show a difference in soluble TM between these 2 genotypes.

The functional basis for the marked difference in the influence of the TM-455 polymorphism on the risk of CHD between blacks and whites is unclear. One possible explanation for these race-specific effects is that the TM-455 mutation causes a subtle reduction in TM function in vivo and that endothelial TM levels in blacks are more sensitive to inhibition by proinflammatory mediators than those in whites, which would further compromise the TM activity in blacks carrying the VV + AV genotypes. Another possible explanation is that risk factors for CHD differ between blacks and whites and this TM mutation occupies a more important position in determining CHD relative risk in blacks than in whites. Finally, the TM-455 polymorphism could be a marker for a neighboring polymorphism that confers a true biological difference to account for CHD risk.

There are 2 previous reports on the association of the TM-455 polymorphism with thrombotic disorders. Both reports were based on cross-sectional studies. One study from the Netherlands examined TM-455 genotype frequency in 25 patients with venous thromboembolic diseases and 26 controls and did not find a significant difference between cases

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<th>TABLE 3. Weighted Proportional Hazards Regression Analysis of AV+VV Genotypes for the Time to Development of CHD in Blacks and Whites</th>
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<td>Model Adjustment</td>
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<td>Age, race, sex</td>
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<td>Age, race, sex, and other risk factors*</td>
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*Other risk factors include hypertension, diabetes, total cholesterol, HDL cholesterol, cigarette smoking, and white blood cell count.
and controls. Another cross-sectional study from Sweden analyzed genotype frequency in 91 patients who had at least 1 episode of acute MI before the age of 50 years and 159 healthy volunteers and reported a significantly higher A allele frequency in patients. The control group and patient groups were not matched, and confounding risk factors were not adjusted for in this study. Our report represents the first prospective study on the association of the TM-455 polymorphism with CHD. Contrary to the results from the cross-sectional study, our results show a lower A allele frequency in white CHD cases, with an age- and sex-adjusted $P$ value of 0.066. However, neither univariate nor multivariate analysis shows a significant association of this polymorphism with incident CHD in our white group, despite the inclusion of a relatively large number of white CHD cases ($n=289$) from 3 US communities (Washington County, Maryland; northwest Minneapolis suburb, Minn; and Forsyth County, North Carolina). It is thus unlikely that the TM-455 polymorphism is an independent risk factor for incident CHD in American whites.

The genotype frequency in our white group differs from that reported by the Swedish study. The AA genotype frequency in the Swedish healthy subjects was 56% compared with 67% in our white noncases. It is unclear whether the difference in genotype frequency between the Swedish whites and our American whites influences the association of TM-455 polymorphism with CHD. This important issue should be further investigated by prospective studies. The TM-455 genotype frequency in other ethnic groups was not reported previously. In the present study, we showed that the AA genotype was much more prevalent in blacks than in American whites (93% versus 67%). The black cohort in the ARIC study was recruited from Jackson, Miss, and Forsyth County, North Carolina. There is no significant difference in the genotype frequency between these 2 communities. Whether the genotype frequency is identical among all blacks in the United States will need to be documented. Likewise, it will be important to determine this genotype frequency and the association of this genetic polymorphism in blacks and other ethnic groups.

Our results should be interpreted with caution because the number of black cases was relatively small compared with that of whites. They should be confirmed with a larger sample size of black cases. It will be possible to do this in the next several years when additional black CHD cases are identified during continuing follow-up of the ARIC cohort.

Acknowledgments

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