Heparin Cofactor II Is a Novel Protective Factor Against Carotid Atherosclerosis in Elderly Individuals

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Methods and Results—Maximum plaque thickness (MPT) in the carotid artery was measured by ultrasonography in 306 Japanese elderly individuals (154 men and 152 women; age, 40 to 91 years; 68.9 ± 11.1 years, mean ± SD). The relevance of cardiovascular risk factors including plasma HCII activity to the severity of MPT was statistically evaluated. Plasma HCII activity decreased with age. Simple linear regression analysis after adjustments for age and sex showed that lipoprotein(a), glycosylated hemoglobin A1c, and presence of diabetes mellitus significantly contributed to an increase in MPT values ($r = 0.119$, $P < 0.05$; $r = 0.196$, $P < 0.001$; and $r = 0.227$, $P < 0.0001$, respectively). In contrast, high-density lipoprotein (HDL) cholesterol and HCII activity were negatively correlated with MPT values ($r = -0.117$, $P < 0.05$, and $r = -0.202$, $P < 0.0005$, respectively). Multiple regression analysis revealed that plasma HCII activity and HDL cholesterol independently contributed to the suppression of MPT values and that the antiatherogenic contribution of HCII activity was stronger than that of HDL cholesterol ($P < 0.001$ and $P < 0.05$, respectively).

Conclusions—These results suggest that HCII can be a novel and independent antiatherogenic factor. Moreover, HCII is a stronger predictive factor than HDL cholesterol against carotid atherosclerosis in elderly individuals. (Circulation. 2004;109:2761-2765.)

Key Words: atherosclerosis ■ carotid arteries ■ ultrasonics ■ thrombin ■ heparin

Thrombin not only acts as an enzyme capable of forming fibrin clot, but it also activates platelets, vascular endothelial cells, vascular smooth muscle cells, macrophages, and fibroblasts to enhance procoagulation, chemoattraction, mitogenesis, and proliferation of these cells. Thrombin elicits its action in these cells through proteolytic processing of a specific cell-surface receptor known as proteinase-activated receptors (PARs). Nelken et al examined the expression of PAR-1 in human arteries by both in situ hybridization and immunohistochemistry and showed that it was expressed almost exclusively in the endothelial layer in normal-appearing arteries. By contrast, in human atheroma, PAR-1 was widely expressed in regions where macrophages, vascular smooth muscle cells, and mesenchymal-appearing intimal cells are abundantly present. Thus, PAR-1 activation is known to play a key role in cardiovascular disorders such as arterial thrombosis, atherosclerosis, and restenosis after percutaneous coronary intervention, and antithrombin agents as well as antagonists of thrombin receptor are shown to be effective for the treatment of these disorders.

Thrombin generated at sites of injured vascular endothelium as the result of aging, oxidative stress, hyperglycemic toxicity, abnormal lipid profile, and so forth is inactivated by two major coagulation modulators: Antithrombin (AT) and heparin cofactor II (HCII). AT elicits its optimal antithrombin actions by binding to heparan sulfate, and anticoagulatory active heparan sulfate proteoglycans is reported to be concentrated immediately beneath the aortic endothelium with only a very small amount on the luminal surface of the endothelial cells. HCII is a serine protease inhibitor (serpin)
with molecular weight of 65.6 kDa that is synthesized by the liver and circulates in plasma at a concentration of \( \approx 1.0 \mu\text{mol/L} \). HCII inactivates thrombin but not other proteases involved in blood coagulation or fibrinolysis. HCII has been detected in the intima and media of normal human arteries, where dermatan sulfate is deposited. The inactivation rate of thrombin by HCII increases by >1000-fold after binding to dermatan sulfate (DS). Because DS is secreted by smooth muscle cells and fibroblasts and is deposited in the matrix of vascular intima and media, HCII can counteract the actions of thrombin generated at injured vascular walls.

We have previously reported a 66-year-old Japanese woman with congenital HCII deficiency manifesting multiple atherosclerotic lesions, including multiple coronary artery stenosis, stenosis of internal carotid artery, renal artery stenosis, and abdominal aortic aneurysm. A similar case of a 61-year-old Japanese man with congenital HCII deficiency was also reported manifesting severe coronary artery disease. By analyzing clinical features of patients with congenital HCII deficiency reported thus far, we found that most of probands younger than 40 years had deep vein thrombosis, whereas elderly probands over 40 years of age tended to show atherosclerotic disorders including angina pectoris and cerebral infarction. Taken together, it is intriguing to hypothesize that HCII is of potential importance in the protection of atherosclerosis in elderly individuals with atherosclerotic risk factors. However, no clinical data have been reported regarding the pathological significance of HCII against the development of atherosclerotic disorders.

The present study was undertaken to clarify the association between plasma HCII activity and severity of carotid atherosclerosis in elderly individuals with or without cardiovascular risk factors. To address this issue, we used high-resolution ultrasound scanning of carotid artery for accurate assessment of the presence and extent of carotid atherosclerosis.

### Methods

#### Subjects

Three hundred six (154 men and 152 women) Japanese individuals over 40 years of age were recruited from Kondo Naika Hospital, Tokushima, Japan, between April 2002 and August 2003.

All subjects received a standardized interview and physical examination. A nonsmoker was defined as a person who had not smoked for more than 1 year. Body mass index (BMI) was calculated as an index of obesity. Blood pressure was measured twice and averaged. Hypertensive patients were defined as those with systolic blood pressure (SBP) >140 mm Hg and/or diastolic blood pressure >90 mm Hg or those receiving antihypertensive agents. The patients who were diagnosed as having white coat hypertension were categorized as having hypertension. Hyperlipidemic patients were defined as those with total cholesterol (T-chol) >220 mg/dL and/or triglyceride (TG) level >150 mg/dL or those receiving lipid-lowering agents. Patients were classified as diabetics by use of insulin and/or oral hypoglycemic agents or by glycosylated hemoglobin A1c (HbA1c) >6.5%. In this study, screening of the patients for cardiovascular risk factor(s) included one or a combination of such risk factors as current smoking, hypertension, hyperlipidemia, and diabetes mellitus. Exclusion criteria for the current study included known malignancy, renal failure, liver dysfunction, and malnutrition. The study followed the institutional guidelines of the University of Tokushima and Kondo Naika Hospital, and informed consent was obtained from all patients according to the Declaration of Helsinki.

### Biochemical Analyses

Before noon, overnight fasting blood samples were collected from the antecubital vein and were assayed immediately for HbA1c and serum lipid parameters including T-chol, high-density lipoprotein cholesterol (HDL-chol), low-density lipoprotein cholesterol (LDL-chol), lipoprotein (Lp(a)), and lipid peroxide. Serum levels of T-chol, HDL-chol, and TG were measured by the enzymatic method, and the LDL-chol value was calculated by Friedewald's formula. Serum Lp(a) was measured by turbidimetric immunoassay, lipid peroxide by hemoglobin methylene blue method, and HbA1c by latex agglutination assay. These chemistries were measured at a commercially available laboratory (FALCO Biosystems, Kyoto, Japan).

### Measurements of Plasma HCII and Antithrombin Activities

Blood was drawn as mentioned above, collected into a tube containing 1/10 volume of 3.8% sodium citrate, and centrifuged at 2000g for 20 minutes. Plasma was stored at –80°C until use. Plasma HCII activity was measured on the basis of antithrombin activity in the presence of dermatan sulfate, with the use of the Stachrom HCII assay kit (Diagnostica Stago). The intra-assay and interassay coefficients of variation of this kit were 3.9% and 4.3%, respectively.

 Plasma AT activity was measured by a chromogenic method based on antithrombin activity in the presence of heparan sulfate, with the use of the Testzyme ATIII-2 kit (Kabi Diagnostica AB).

### Ultrasound Measurements

The ultrasound apparatus used in this study was Hitachi EUB-525 with a 10-MHz B-mode transducer (Hitachi Medical Corporation). The patient’s head position, sonographer position, scanning angles, and scanning angle sequence were all standardized. All segments including both sides of the common carotid artery, carotid bifurcation, and internal carotid artery were scanned, and carotid plaque that was defined as an area of focal hyperchoic wall thickening in longitudinal arterial images was searched. The most thickened part of the plaque was recorded as maximum plaque thickness (MPT). If no plaque was identified, the most thickened distance from adventitia/media to intima/lumen (intima-media thickness, IMT) was measured, and this index was used as MPT. The greater MPT/IMT value obtained from the scan of right and left carotid arteries in each patient was used for statistical analyses. All scans were performed by the same sonographer, and images were recorded on magneto-optical discs and VHS videotape for further analyses.

### Statistical Methods

Continuous variables were averaged and values expressed as mean±SD or as a percentage for categorical parameters. Male gender and presence of hypertension, diabetes mellitus, hyperlipidemia, and current smoking were coded as dummy variables. Comparison of continuous variables between men and women was performed with the use of an unpaired t test. Gender differences of categorical variables including current smoking, hypertension, hyperlipidemia, and diabetes mellitus were assessed by the \( \chi^2 \) test. Difference of mean plasma HCII activity stratified by the age group was evaluated by I-way ANOVA. Degree of association between independent variables such as sex, age, BMI, SBP, serum lipid parameters, HbA1c, plasma AT and HCII activities, history of current smoking, hypertension, diabetes mellitus, and hyperlipidemia was measured by means of simple linear regression and multiple regression analyses. These analyses were performed on an Apple Macintosh computer with the use of Excel (Microsoft X) and Stat View statistical package (Stat View 5.0, SAS Institute Inc). Statistical significance was defined as \( P<0.05 \).

### Results

#### Baseline Characteristics

Physical and laboratory characteristics of subjects enrolled in this study are shown in Table 1. On average, women were older and had higher T-chol, LDL-chol, and HDL-chol levels.
and antithrombin activity than did men. Male patients showed higher levels of TG and lipid peroxide compared with female patients. No significant gender differences were observed in values of BMI, SBP, Lp(a), HbA1c, and plasma HCII activity. Percentages of current smoking and diabetes mellitus in male patients were significantly higher than those of female patients. In contrast, the percentage of hyperlipidemia in female patients was significantly higher than that of male patients.

According to the criteria of this study, we identified that 35 elderly subjects (17 men and 18 women; mean age, 68.7 ± 11.0 years old) had one or more cardiovascular risk factors: HCII activity, 98.9 ± 18.6% at start and after 1 month of treatment. No significant changes of its value (P = 0.0005) in the youngest age group (≤59 years old, n = 61, 101.2 ± 18.4%) than that in the oldest age group (≥80 years old, n = 58, 87.7 ± 17.2%). Thus, relations between MPT and independent variables including plasma HCII activity were evaluated after adjustments for age and sex.

**Difference of HCII Activity Among Age Groups**
Measurements of plasma HCII activity in 10 subjects at 1-month intervals revealed no significant changes of its value (89.8 ± 11.7% and 92.4 ± 13.7% at start and after 1 month of the measurements, respectively). Figure 1 shows plasma HCII activity stratified by age group. Plasma HCII activity tended to decrease with age, and HCII activity was significantly higher (<0.0005) in the youngest age group (≤59 years old, n = 61, 101.2 ± 18.4%) than that in the oldest age group (≥80 years old, n = 58, 87.7 ± 17.2%). Thus, relations between MPT and independent variables including plasma HCII activity were evaluated after adjustments for age and sex.

**Difference of HCII Activity and MPT Value Between Elderly Subjects With and Without Cardiovascular Risk Factors**
Subjects without cardiovascular risk factors had significantly higher levels of HCII activity and reduced MPT values compared with those in subjects with cardiovascular risk factors. Baseline characteristics were compared between men and women by unpaired t test or χ² test for independence.

**Correlation Between Plasma HCII Activity and Severity of Carotid Atherosclerosis**
As shown in Table 2, simple linear regression analysis on entire series of subjects showed that Lp(a) and HbA1c were positively correlated with MPT (r = 0.119, P < 0.05 and r = 0.196, P < 0.001, respectively). The presence of diabetes mellitus also contributed significantly to the increase of MPT (r = 0.227, P < 0.0001). In contrast, HDL-chol and plasma

### TABLE 1. Clinical Characteristics of Subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n = 306)</th>
<th>Men (n = 154)</th>
<th>Women (n = 152)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>68.9 ± 11.1</td>
<td>67.2 ± 10.8</td>
<td>70.6 ± 11.1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.2 ± 3.7</td>
<td>23.6 ± 3.8</td>
<td>22.8 ± 3.5</td>
<td>NS</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>138.3 ± 20.2</td>
<td>138.2 ± 20.2</td>
<td>138.5 ± 20.3</td>
<td>NS</td>
</tr>
<tr>
<td>T-chol, mg/dL</td>
<td>198.1 ± 39.6</td>
<td>187.3 ± 40.9</td>
<td>209.1 ± 35.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LDL-chol, mg/dL</td>
<td>124.5 ± 34.5</td>
<td>114.4 ± 35.5</td>
<td>134.8 ± 30.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HDL-chol, mg/dL</td>
<td>47.0 ± 13.2</td>
<td>44.2 ± 12.4</td>
<td>49.9 ± 13.4</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>131.6 ± 85.4</td>
<td>143.5 ± 104.1</td>
<td>119.5 ± 58.8</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Lipid peroxide, nmol/mL</td>
<td>0.45 ± 0.23</td>
<td>0.48 ± 0.26</td>
<td>0.41 ± 0.18</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Lipoprotein(a), mg/dL</td>
<td>23.0 ± 17.1</td>
<td>22.9 ± 15.7</td>
<td>23.1 ± 18.5</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.9 ± 1.7</td>
<td>6.1 ± 1.8</td>
<td>5.8 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>Antithrombin activity</td>
<td>93.1 ± 14.8</td>
<td>89.5 ± 14.3</td>
<td>96.6 ± 14.5</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Heparin cofactor II activity, %</td>
<td>94.0 ± 18.3</td>
<td>92.1 ± 18.2</td>
<td>95.9 ± 18.2</td>
<td>NS</td>
</tr>
<tr>
<td>Maximum plaque thickness, mm</td>
<td>2.04 ± 1.13</td>
<td>2.15 ± 1.18</td>
<td>1.93 ± 1.07</td>
<td>NS</td>
</tr>
<tr>
<td>Current smoking, %</td>
<td>28.4</td>
<td>48.7</td>
<td>7.9</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>57.8</td>
<td>55.8</td>
<td>59.9</td>
<td>NS</td>
</tr>
<tr>
<td>Hyperlipidemia, %</td>
<td>42.5</td>
<td>35.7</td>
<td>49.3</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>30.7</td>
<td>36.4</td>
<td>25</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Values are mean ± SD or percentage. Baseline characteristics were compared between men and women by unpaired t test or χ² test for independence.
HCII activity were negatively correlated with MPT ($r = -0.117$, $P < 0.05$ and $r = -0.202$, $P < 0.0005$, respectively) (Table 2 and Figure 2). Other variables, including T-chol, LDL-chol, TG, lipid peroxide, or presence of hypertension, hyperlipidemia, or current smoking exhibited no significant association with MPT.

We entered all univariate baseline parameters into a multiple regression analysis (Table 3). The results showed that age was the strongest contributor for the increase of MPT ($P < 0.0001$), followed by presence of diabetes mellitus ($P < 0.005$), male gender ($P < 0.05$), and Lp(a) ($P < 0.05$). In contrast, in addition to HDL-chol as a well-known protective factor against atherosclerosis, plasma HCII activity was shown to act protectively against the increase in MPT (Table 3). Furthermore, plasma HCII activity ($P < 0.001$) was more protective than HDL-chol ($P < 0.05$) against the increase in MPT. These results demonstrate that plasma HCII activity is a novel protective factor against the development of carotid plaque even after adjustment by age, sex, and other confounding factors.

### Discussion

With the use of ultrasound imaging of the carotid artery, the present study demonstrated that plasma HCII activity negatively correlates with the severity of carotid atherosclerosis and is identified as a novel independent protective factor against carotid atherosclerosis. Although HDL-chol has been known as a sole antiatherogenic factor, our results demonstrated that the preventive contribution of HCII against carotid atherosclerosis is stronger than that of HDL-chol (Table 3).

Many attempts to analyze clinical effects of interventions or risk factors on the development of atherosclerosis have been hampered by heterogeneity in clinical background as well as problems in the use of various surrogates as end points. To solve these problems, we used noninvasive B-mode ultrasound scanning as a powerful tool to monitor the presence and severity of early atherosclerotic lesions of the carotid artery. Direct assessment of atherosclerotic changes by this method has yielded strong association of IMT/MPT of the carotid artery with both main cardiovascular risk factors and/or carotid plaque and coronary atherosclerosis has been confirmed by autopsy studies. Moreover, a close correlation between carotid IMT and/or carotid plaque and coronary atherosclerosis has been confirmed by autopsy studies. Thus, ultrasound imaging has contributed not only to evaluate the effects of antihypertensive and lipid-lowering therapies but also to identify new risk factors for atherosclerosis. In view of the strong association of the assessment of carotid IMT/MPT with development of atherosclerotic disorders reported thus far, it is plausible to conclude from the present observations that plasma HCII activity should be considered as a useful negative predictor against atherosclerosis.

Previous studies have demonstrated that the AT/heparan sulfate complex can inhibit the actions of soluble thrombin but not surface-bound thrombin, whereas the HCII/dermatan sulfate complex can inhibit actions of both forms of thrombin. A study with HCII gene knockout mice showed that the time to thrombotic occlusion of the carotid artery after photochemical damage to the carotid endothelium was shorter in HCII-deficient mice, suggesting that HCII is capable of inhibiting both atherosclerosis and thrombosis after endothelial damage in vivo.

Previous reports also have shown that endothelial cells, smooth muscle cells, and macrophages express tissue factor in human atherosclerotic plaque, and, after injury of the vascular...
endothelium, expression of tissue factor on vascular smooth muscle cells, macrophages, and fibroblasts in both intima and media is enhanced, which leads to overproduction of surface-bound thrombin. Moreover, secretion of dermatan sulfate from vascular smooth muscle cells and fibroblasts is also enhanced at atherosclerotic lesions. Because binding of HCII to dermatan sulfate greatly enhances its antithrombin actions, the protective role of HCII against thrombin can be exerted most effectively at arterial walls injured by cardiovascular risk factors. Thus, higher plasma HCII level is expected to give more access of HCII to dermatan sulfate in insulted arterial walls, which enables formation of more HCII/dermatan sulfate complexes to inactivate thrombin action in the process of atherosclerotic lesion formation. In the present study, there was no significant relation between plasma AT activity and the severity of carotid atherosclerosis by multiple regression analysis (Table 3). These results suggest that between the two major antithrombin factors, AT and HCII, only HCII can counteract the progress of atherosclerosis in vascular walls.

Recently, Prandoni and colleagues have reported that the prevalence of carotid plaque examined by ultrasonography was significantly higher in patients with spontaneous venous thrombosis than those with secondary venous thrombosis or control subjects. They suggested that there is an association between atherosclerosis and spontaneous venous thrombosis and that the two conditions may share unknown common risk factors. Based on the results presented herein, low plasma HCII activity can be a good candidate to link arterial plaque formation and venous thrombosis.

Thus, the present observations are consistent with the notion that plasma HCII acts as a novel protective factor against carotid atherosclerosis in elderly individuals. These observations warrant further investigation into the possible role of HCII as a therapeutic target against the development and/or progression of atherosclerosis.

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