Heparin Cofactor II is an Independent Protective Factor against Peripheral Arterial Disease in Elderly Subjects with Cardiovascular Risk Factors

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Aim: Heparin cofactor II (HCII) specifically inactivates thrombin action at the injured vascular wall. We have reported that HCII is a protective factor against coronary in-stent restenosis and carotid atherosclerosis; however, it is unclear whether there is any correlation between plasma HCII levels and the development of peripheral arterial disease (PAD).

Methods: Plasma HCII activity and the ankle brachial pressure index (ABI) were determined in 494 elderly subjects with cardiovascular risk factors. PAD was diagnosed by ABI below 0.9, and 62 subjects were diagnosed with PAD. The relationship between factors that affect cardiovascular events and the prevalence of PAD was statistically evaluated.

Results: Mean HCII activity in PAD subjects was significantly lower than in non-PAD subjects (87.5 ± 19.7% vs. 94.6 ± 17.8%, p = 0.009). Multivariate logistic regression analysis showed that age (odds ratio [OR]: 1.062, p = 0.0016), current smoking (OR 3.028, p = 0.002) and diabetes mellitus (OR 2.656, p = 0.008) were independent and progressive determinants of PAD. In contrast, HCII was an independent inhibitory factor of PAD (OR: 0.982, p = 0.048).

Conclusions: Plasma HCII activity is inversely related to the prevalence of PAD. HCII may function as the sole protective factor against PAD in elderly people with cardiovascular risk factors.


Key words: Thrombin, Heparin cofactor II, Peripheral arterial disease

Introduction

Peripheral arterial disease (PAD) not only causes a decline in quality of life due to intermittent claudication but also, more importantly, is a powerful predictor of future cardiovascular and cerebrovascular events, such as myocardial infarction, stroke and death.¹⁻⁴ Because these vascular risks are already increased considerably in patients with asymptomatic PAD and are further increased in PAD patients with intermittent claudication, the clinical importance of early diagnosis during the subclinical stage as well as subsequent management of vascular risks has been noted in recent years.⁵ In fact, it has been reported that 6.9% of subjects aged from 45 to 74 years who underwent ABI examination were diagnosed with PAD and that only 22% were symptomatic,⁶ indicating that history taking and physical examination are not sensitive enough to diagnose PAD.⁷, ⁸ A value of ABI less than 0.90
is 95% sensitive and 99% specific for a diagnosis of PAD\(^9\) and is a powerful and independent predictor of cardiovascular morbidity and mortality\(^1, 2\). Advanced age, cigarette smoking, diabetes mellitus, hyperlipidemia, and hypertension are well-known risk factors for PAD as well as for other atherothrombotic diseases\(^10, 11\). In addition to classical risk factors for PAD, several studies have revealed relationships between the prevalence of PAD and factors such as flow-mediated dilation\(^2\), C-reactive protein\(^13, 14\), lipoprotein (Lp) (a)\(^15\), other serum or plasma biomarkers\(^13, 16, 17\), and renal insufficiency\(^18\). Almost all of these factors promote the development of PAD, while the presence of an endogenous protective factor against the progression of PAD is unknown.

Thrombin has recently been recognized as an important factor for the development of arterial occlusive diseases\(^9\) and thrombin generation in such patients is not interrupted by aspirin administration\(^39\); therefore, the inhibition of thrombin generation and/or inactivation of thrombin activity at the level of thrombin-thrombin receptor interaction may lead to efficient prevention of PAD development.

Heparin cofactor II (HCII) is a plasma glycoprotein and a member of the serine protease inhibitor family (serpin) with a molecular weight of 65.6 kDa\(^21\). HCII inactivates thrombin action by forming a bimolecular complex with dermatan sulfate, and an anti-thrombotic property of HCII has been demonstrated in HCII-deficient mice\(^22, 23\). In addition to the action of HCII in the intravascular lumen, HCII acts at the injured vascular wall where vascular smooth muscle cells, macrophages and fibroblasts are present. Dermatan sulfate has been shown to be deposited in the extracellular matrix of restenotic lesions of human peripheral arteries\(^24\). Thus, there is a possibility that HCII can attenuate the progression of atherosclerosis by inhibiting thrombin action at the injured peripheral arteries. We previously reported an elderly woman with congenital HCII deficiency who manifested multiple atherosclerotic disorders\(^25\), and we have reported the results of clinical studies showing that HCII can reduce in-stent restenosis after percutaneous coronary intervention and reduce plaque formation in the carotid artery\(^26-28\). Moreover, we and Tøllefsen’s group have reported that prominently accelerated vascular remodeling, including atherosclerosis, was observed in HCII-deficient mice compared to HCII wild-type mice\(^29, 30\). These observations suggested a potential role of HCII in countering the development of PAD by inhibiting thrombin action in peripheral arteries; however, it is unclear whether HCII can contribute to reducing the morbidity of PAD. To address this issue, we evaluated the association between plasma HCII activity and the presence of PAD in elderly subjects.

### Methods

#### Immunohistochemistry

With informed consent we obtained surgical specimens of iliac arteries from both normal HCII and heterozygous HCII-deficient patients with infra-renal aortic aneurysms. The removed arterial tissues were fixed in 10% neutral-buffered formalin overnight. The segments of iliac arteries were cut into subserial 5-μm-thick sections with intervals of 200 μm. The sections were then stained with specific antibodies against α smooth muscle cell actin (αSMA; Sigma-Aldrich Corp., Tokyo, Japan), dermatan sulfate (anti-human dermatan sulfate proteoglycan; Seikagaku Corp., Tokyo, Japan) and HCII antigen (monoclonal antibody for HCII; Technoclone, Vienna, Austria) according to the instruction manuals.

#### Subjects for Cross-Sectional Study

Four hundred ninety-four Japanese subjects (274 men and 220 women) over 40 years of age were consecutively recruited from The Tokushima University Hospital and its affiliated hospitals between April 2003 and March 2005.

A standardized interview and physical examination were performed in all subjects. A current smoker was defined as a person who had smoked within the past 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 mmHg and/or diastolic blood pressure > 90 mmHg or those receiving antihypertensive agents. Patients diagnosed with white coat hypertension were not categorized as having hypertension. Hyperlipidemic patients were defined as those with low-density lipoprotein cholesterol (LDL-cholesterol) > 140 mg/dL and/or triglyceride level > 150 mg/dL or those receiving lipid-lowering agents. Patients were classified as diabetics by their use of insulin and/or oral hypoglycemic agents or by glycated hemoglobin A1c (HbA1c) > 6.5%. In this study, the criteria of cardiovascular risk factors included 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 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 assayed immediately for HbA1c and serum lipid parameters, including T-chol., high-density lipoprotein cholesterol (HDL-chol.), triglyceride, low-density lipoprotein cholesterol (LDL-chol.) and lipoprotein (a) (Lp (a)). Serum levels of T-chol., HDL-chol., and triglyceride were measured by the enzymatic method, and the LDL-chol. value was calculated by Friedewald’s formula. Serum Lp (a) was measured by a turbidimetric immunoassay and HbA1c was measured by a latex agglutination assay.

Measurements of Plasma HCII and Antithrombin Activities
Blood was drawn as described above, collected into a tube containing 1/10 volume of 3.8% sodium citrate, and centrifuged at 2,000 g for 20 minutes. Plasma was stored at −80°C until use. Plasma HCII and AT activities were measured as previously described28, 29.

Ankle Brachial Pressure Index Measurement
Diagnosis of PAD was based on an ankle brachial pressure index (ABI) < 0.9 on single or bilateral lower limbs. ABI was determined using a fully automated device (AT-form PWV/ABI; Nippon Colin, Japan).

Statistical Methods
Continuous variables were averaged and values expressed as the mean ± SD or as a percentage for categorical parameters. Male gender and the presence of hypertension, diabetes mellitus, hyperlipidemia, and current smoking were coded as dummy variables. Continuous variables between subjects with and without PAD were compared using the unpaired t test. Differences in categorical variables, including current smoking, hypertension, hyperlipidemia, and diabetes mellitus, were assessed by the χ² test. The 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 determined by univariate and multivariate logistic regression analyses. These analyses were performed on an Apple Macintosh computer using Excel (Microsoft XP) and the Stat View statistical package (Stat View 5.0, SAS Institute Inc.). Statistical significance was defined as p < 0.05.

Results

Reduced HCII Penetration at the Medial Wall of the Iliac Artery in Patients with Congenital HCII Deficiency
Fig. 1 shows the immunohistochemistry of human iliac arteries from surgical specimens. The upper panels showed that HCII in a control subject co-localized with dermatan sulfate around vascular smooth muscles, whereas an HCII deficiency subject, as we previously reported29, showed poor distribution of HCII in the medial area of the vascular wall, where dermatan sulfate is present. These results indicated that the amount of HCII protein penetration into the arterial wall is closely associated with plasma HCII activity.

Characteristics of Subjects for Cross-Sectional Study
Characteristics of subjects enrolled in this study are shown in Table 1. We divided the subjects into two groups: those with and without PAD. Among the 494 enrolled subjects, 62 subjects were diagnosed with PAD by ABI measurement (Table 1). On average, PAD patients were older, had higher values of SBP and HbA1c, a higher prevalence of hypertension and diabetes mellitus, and included a larger percentage of current smokers than non-PAD subjects. In contrast, PAD subjects showed lower BMI and HCII activities than non-PAD subjects.

Prevalence of PAD among Age Groups
Fig. 2 shows the prevalence of PAD stratified by age groups. The prevalence of PAD tended to increase with age, and the rate of PAD markedly increased over 70 years of age.

Correlation between Plasma HCII Activity and Incidence of PAD
As shown in Table 2, univariate logistic regression analysis for all subjects showed that age, SBP, Lp (a), current smoking and diabetes mellitus were positively correlated with the prevalence of PAD (odds ratio [OR] at one year of age increase: 1.044, p = 0.0021, OR: at 1.0 mmHg increase: 1.017, p = 0.0082, OR: at 1.0 mg/dL increase: 1.016, p = 0.0134; OR: 2.394, p = 0.0015 and OR: 2.925, p = 0.0001, respectively). In contrast, BMI and plasma HCII activities were negatively correlated with the prevalence of PAD (OR: 0.885, p = 0.0052 and OR: at 1.0% increase 0.978, p = 0.0044, respectively) (Table 2). Other variables, including LDL-chol, HDL-chol, TG, HbA1c and the presence of hypertension and hyperlipidemia, showed no significant association with PAD.

We entered all univariate baseline parameters
into multivariate logistic regression analysis (Table 3). The results showed that age was the strongest contributor to the prevalence of PAD (OR: 1.062, *p* = 0.0016), followed by diabetes mellitus (OR: 2.656, *p* = 0.0080) and current smoking (OR: 3.028, *p* = 0.0023). In contrast, BMI and plasma HCII activity were shown to be associated with the incidence of PAD (OR: 0.867, *p* = 0.0093 and OR: 0.982, *p* = 0.0482, respectively) (Table 3). Although PAD patients with extremely low values of ABI tended to have lower levels of plasma HCII activity, there was no statistically significant association between the actual ABI value and plasma HCII activity in patients with PAD (data not shown). This inconsistent result may be due to the
small sample size in the present study. Nevertheless, the present results demonstrate that plasma HCII activity is the sole biologically active and protective factor against the development of PAD in elderly subjects even after adjustment by age, sex, and other confounding factors.

### Discussion
The present study showed that measuring plasma HCII activity enables prediction of the incidence of PAD in patients with cardiovascular risk factors. We revealed that HCII is a novel and independent protective factor against PAD, even after adjustment for other confounding cardiovascular risk factors.

Thrombin is generated in the final step of the blood coagulation cascade and converts fibrinogen to fibrin. In addition, it is also known that thrombin activates platelets, vascular endothelial cells, vascular smooth muscle cells, macrophages, and fibroblasts to enhance procoagulation \(^{31}\), chemoattraction \(^{32}\), mitogenesis \(^{33}\), and proliferation \(^{34}\) of these cells. These actions of thrombin are elicited through activation of its specific receptors, known as proteinase-activated receptors (PARs) \(^{35}\). PAR-1 is widely expressed in regions where macrophages, vascular smooth muscle cells, and mesenchymal-appearing intimal cells are present. Thus, PAR-1 activation by thrombin contributes to the development of vascular diseases \(^{36}\). It is well established that thrombin actions are inactivated by two major coagulation modulators, known as serine protease inhibitors AT and HCII. AT is a plasma glycoprotein synthesized by hepatocytes and is a crucial inhibitor of blood coagulation. When human subjects and experimental animals with AT deficiency were subjected to stressful events, such as injury, surgical operation and severe infection, venous thrombosis was often observed \(^{36-38}\). AT effectively inhibits thrombin actions by binding to heparan sulfate or anticoagulatory active heparan sulfate proteoglycans \(^{39}\). HCII is also synthesized by hepatocytes and circulates in plasma at a concentration of 1.0 \(\mu\)mol/L \(^{21, 40, 41}\). HCII inactivates thrombin but not other proteases, a process that involves blood coagulation or fibrinolysis. It has been revealed that HCII exists in the intima

### Table 2. Univariate regression analysis for determinants of PAD

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio</th>
<th>95%CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>1.667</td>
<td>0.953–2.916</td>
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<td>Age</td>
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<td>BMI</td>
<td>0.885</td>
<td>0.812–0.964</td>
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<td>SBP</td>
<td>1.017</td>
<td>1.004–1.030</td>
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<td>LDL-chol.</td>
<td>1.006</td>
<td>0.999–1.014</td>
<td>0.0909</td>
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<td>HDL-chol.</td>
<td>0.999</td>
<td>0.984–1.014</td>
<td>0.9202</td>
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<tr>
<td>TG</td>
<td>1.000</td>
<td>0.996–1.003</td>
<td>0.7772</td>
</tr>
<tr>
<td>Lipoprotein(a)</td>
<td>1.016</td>
<td>1.003–1.029</td>
<td>0.0134</td>
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<tr>
<td>HbA1c</td>
<td>1.136</td>
<td>0.999–1.293</td>
<td>0.0526</td>
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<td>AT activity</td>
<td>0.989</td>
<td>0.973–1.005</td>
<td>0.1610</td>
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<td>HCII activity</td>
<td>0.978</td>
<td>0.963–0.993</td>
<td>0.0044</td>
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<td>Current smoking</td>
<td>2.394</td>
<td>1.396–4.104</td>
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<td>Hypertension</td>
<td>1.794</td>
<td>0.984–3.273</td>
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<td>Hyperlipidemia</td>
<td>1.130</td>
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<tr>
<td>Diabetes mellitus</td>
<td>2.925</td>
<td>1.702–5.026</td>
<td>0.0001</td>
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### Table 3. Multivariate regression analysis for determinants of PAD

<table>
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<tr>
<th>Variables</th>
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<th>p value</th>
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<td>Male</td>
<td>1.416</td>
<td>0.681–2.941</td>
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<tr>
<td>Age</td>
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<td>1.023–1.102</td>
<td>0.0016</td>
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<td>BMI</td>
<td>0.867</td>
<td>0.779–0.965</td>
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<td>SBP</td>
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<td>0.998–1.031</td>
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<td>LDL-chol.</td>
<td>1.008</td>
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<td>HDL-chol.</td>
<td>1.005</td>
<td>0.985–1.025</td>
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<td>TG</td>
<td>1.002</td>
<td>0.998–1.006</td>
<td>0.3600</td>
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<td>Lipoprotein(a)</td>
<td>1.011</td>
<td>0.995–1.027</td>
<td>0.1868</td>
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<td>HbA1c</td>
<td>0.842</td>
<td>0.896–1.144</td>
<td>0.8420</td>
</tr>
<tr>
<td>AT activity</td>
<td>0.998</td>
<td>0.978–1.018</td>
<td>0.8366</td>
</tr>
<tr>
<td>HCII activity</td>
<td>0.982</td>
<td>0.966–0.998</td>
<td>0.0482</td>
</tr>
<tr>
<td>Current smoking</td>
<td>3.028</td>
<td>1.483–6.180</td>
<td>0.0023</td>
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<tr>
<td>Hypertension</td>
<td>1.127</td>
<td>0.520–2.440</td>
<td>0.0762</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>1.109</td>
<td>0.504–2.440</td>
<td>0.7970</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2.656</td>
<td>1.290–5.470</td>
<td>0.0080</td>
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</table>

Fig. 2. Prevalence of PAD stratified by groups in this study.
and media of normal and atherosclerotic human arteries where dermatan sulfate is deposited. The efficacy of thrombin inactivation by HCII is accelerated more than 1,000-fold after binding to dermatan sulfate. Previous studies showed that dermatan sulfate is secreted by smooth muscle cells and fibroblasts and is deposited in the matrix of vascular intima and media. HCII is thought to be able to attenuate the actions of thrombin generated at the injured vascular walls. When the endothelial layer is intact in such young individuals, it is conceivable that the reduction of plasma HCII does not influence the manifestation of apparent cardiovascular disorders, except for rare deep vein thrombosis, although the reduction of plasma HCII activity in elderly patients with cardiovascular risk factors should aggravate vascular damage, leading to atherosclerotic disorders, including ischemic heart disease and cerebral infarction. In fact, we found a 66-year-old Japanese woman with congenital HCII deficiency manifesting as multiple and advanced atherosclerotic lesions. In addition, we and others have recently reported that high plasma HCII activity reduced the restenosis of coronary arteries and femoral arteries after stenting and attenuated carotid plaque formation in elderly patients. In experimental animal studies using HCII-deficient mice, we and Tollefsen’s group have shown accelerated vascular remodeling in HCII-deficient mice with increased inflammatory cytokines and chemokine gene expression and oxidative stress. Taken together, these observations suggest that HCII has a pivotal role in protection against “systemic atherosclerosis” in elderly individuals with atherosclerotic risk factors.

Study Limitations

The results of the present clinical study cannot be extended to the general population because we enrolled only patients with cardiovascular risk factors and because we previously reported that subjects without cardiovascular risk factors had higher levels of plasma HCII activity than subjects with one or more cardiovascular risk factors. Moreover, Giri et al. reported no association between plasma HCII antigen levels and the development of symptomatic coronary heart disease in a large cohort of middle-aged subjects. This apparent discrepancy between the results of their study and ours may be attributable to differences in the study design, the age of subjects, or the method for measuring HCII levels (plasma HCII antigen versus activity).

Dyslipidemia, including high LDL-chol and low HDL-chol, has been recognized as one of the major risk factors for the development of PAD; however, neither high LDL-chol. nor low HDL-chol. was associated with the prevalence of PAD in this study. Dyslipidemia did not influence the prevalence of PAD in this study, possibly because of the small sample size and that a considerable number of hyperlipidemia subjects had been treated with lipid-lowering agents, such as statins or fibrates.

Thus, large-scale investigations are required to assess and clarify the prognostic value of plasma HCII activity in patients with critical limb ischemia and asymptomatic PAD. Measurement of plasma HCII activity might be particularly important in asymptomatic PAD patients, who represent the majority of the PAD population and have latent systemic atherosclerotic disorders, including coronary artery disease and carotid atherosclerosis, which lead to myocardial infarction and stroke. This hypothesis, based on our previous and present results that HCII prevents systemic atherosclerosis, needs to be confirmed by a larger cohort study.

Conclusions

The present study demonstrated that plasma HCII activity is an independent and inverse predictor of the prevalence of PAD in elderly subjects with cardiovascular risk factors. Lower limb ischemia should be evaluated in elderly diabetic individuals with low levels of plasma HCII activity, even if they do not manifest intermittent claudication. Since plasma HCII activity has been shown to be associated with in-stent restenosis and carotid atherosclerosis, HCII is an antiatherogenic factor for not only PAD but also coronary artery disease and stroke; therefore, in addition to predicting the future incidence of PAD, it might be possible to predict the risk of crucial cardiovascular events in patients with PAD by measuring plasma HCII activity.

Acknowledgements

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