Review

Heparin Cofactor II as a Novel Vascular Protective Factor Against Atherosclerosis

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Heparin cofactor II (HCII) specifically inhibits thrombin action at the site of vascular wall injury. We encountered a congenital HCII deficiency patient with advanced multiple atherosclerotic lesions. This patient led us to conduct clinical studies to examine the role of HCII against atherosclerosis. We found that the incidence of in-stent restenosis after percutaneous coronary intervention, severity of carotid atherosclerosis and prevalence of peripheral arterial disease are inversely associated with plasma HCII activity. In order to clarify the vascular protective action of HCII, we generated HCII-deficient mice by gene targeting. In contrast to a previous study, HCII−/− mice were embryonically lethal. In HCII−/− mice, accelerated intimal hyperplasia and frequent thrombosis were observed after cuff or wire injury of femoral arteries. The number of protease-activated receptor-1 (PAR-1) -positive cells and the gene expression levels of inflammatory cytokines and chemokines were increased in the thickened vascular walls of HCII−/− mice. The accelerated intimal hyperplasia in HCII−/− mice with vascular injury was attenuated by human HCII administration. Furthermore, HCII deficiency exaggerated aortic plaque formation with increased oxidative stress in apolipoprotein E−/− mice. These results demonstrate that HCII protects against thrombin-induced vascular remodeling in both humans and mice and suggest that HCII is a predictive biomarker and therapeutic target for atherosclerosis.


Key words; Thrombin, Protease-activated receptor-1, Heparin cofactor II, Vascular remodeling, Atherosclerosis

Introduction

Thrombin is a multi-potential serine protease generated at the site of vascular injury. Thrombin transforms fibrinogen into fibrin, accelerates platelet aggregation and elicits many biological effects on a variety of cell types, including endothelial cells, vascular smooth muscle cells (VSMCs), monocytes, lymphocytes and fibroblasts. Cellular effects of thrombin are mediated by protease-activated receptors (PARs), members of the G protein-coupled receptor family.

Among the 4 members of the family of PARs, PAR-1 is known as the most important thrombin receptor in vascular remodeling. Under physiological conditions, PAR-1 is mainly expressed in endothelial cells and participates in the regulation of vascular tone, mostly by inducing endothelium-dependent relaxation. In VSMCs, PAR-1 mediates contraction, migration, proliferation, hypertrophy and production of the extracellular matrix and contributes to the development of vascular lesions such as atherosclerosis. Although approximately half of PAR-1-deficient mice are embryonically lethal, mice that survive manifest diminished neointimal hyperplasia after arterial injury compared with that in wild-type mice. Thrombin has been shown to have numerous associations with not only thrombotic but also nonthrombotic diseases, including coronary heart disease, stroke and peripheral arterial disease. Thus, there is a possibility that inhibi-
tion of thrombin action is effective for treatment and prevention of cardiovascular diseases. Since heparin cofactor II (HCII) is known to be an endogenous thrombin inhibitor in the vascular wall, this review focuses on the role of HCII in protection against vascular remodeling and atherosclerosis via its inhibitory effect on thrombin action in the vascular wall.

**Biochemical Features of Heparin Cofactor II**

Enhanced thrombin action at sites of vascular wall injury is efficiently inhibited by HCII. HCII, as well as antithrombin (AT), is a serine protease inhibitor (serpin) with a molecular weight of 65.6 kDa. HCII is synthesized by hepatocytes and secreted into the blood at a concentration of about 1.0 μmol/L, and it is distributed in the intima and media of atherosclerotic lesions as well as the walls of normal arteries. Although AT targets several coagulation-related proteases, including thrombin, factor Xa and factor IXa, HCII inactivates only thrombin without affecting other proteases involved in the blood coagulation cascade. HCII has the unique ability to inhibit thrombin action by the formation of a bimolecular complex with dermatan sulfate (DS), a glycosaminoglycan, which is synthesized and secreted by vascular smooth muscle cells and fibroblasts and is deposited in the matrix of vascular intima and media. AT inhibits thrombin action and elicits an anti-coagulatory effect at the surface of the vascular wall, whereas HCII can most efficiently act in the intima and media of the vascular wall to protect them from the actions of thrombin on vascular remodeling.

**Advanced Multiple Atherosclerotic Lesions in an Elderly Japanese Female with Congenital HCII Deficiency**

Congenital HCII deficiency is inherited in an autosomal dominant fashion and is classified into quantitative deficiency type (type I) and qualitative deficiency type (type II). Since several families with HCII deficiency associated with thrombosis have been reported, HCII deficiency has been thought to be a low to modest clinical risk factor for venous thrombosis, as is plasminogen deficiency. In 2001, we reported a 66-year-old female with type I congenital HCII deficiency who manifested multiple atherosclerotic lesions, including multiple coronary artery stenosis, bilateral carotid stenosis, right renal artery stenosis and a huge abdominal aortic aneurysm. Although she did not have any other classical cardiovascular risk factors except mild essential hypertension, her plasma HCII activity was 41% and plasma HCII antigen was 44%. Her elder brother, who also had congenital HCII deficiency, had a history of stroke at the age of 70 years. In addition to the above patients with congenital HCII deficiency, Kondo et al. reported a Japanese patient with type I HCII deficiency who suffered from angina pectoris and coronary artery disease. This raised the possibility that because of impaired thrombin inactivation, HCII deficiency causes acceleration of the development of atherosclerosis in elderly individuals who have injured vascular walls.

**Clinical Studies on HCII and Vascular Remodeling**

1. **Coronary Artery Disease**

In order to test the possibility that HCII deficiency accelerates atherosclerotic lesions in the elderly, we first assessed the association between plasma HCII activity and coronary artery disease in Japanese elderly subjects. Sequential coronary arteries (n = 166) with bare-metal stent implantation in 134 patients were evaluated before, immediately after, and at 6 months...
after percutaneous coronary intervention (PCI). Patients with high plasma HCII activity (≥110%) showed low percent diameter stenosis at follow-up compared with that in patients with normal plasma HCII activity (≥80% and <110%) or low plasma HCII activity (<80%). In-stent restenosis rate in the high-HCII group was also lower than that in the low-HCII group, and multivariate analysis demonstrated that a high level of plasma HCII activity is an independent protective factor against angiographic restenosis (Fig. 3).

Huang and colleagues examined the relationship between plasma HCII activity and cardiovascular prognosis in patients with acute myocardial infarction (AMI). In their study, 110 consecutive patients (aged 63±11 years) with AMI were followed up for 42±12 months. The patients were divided into three groups: a high-HCII group (>122%), a normal-HCII group (>98% and ≤122%), and a low-HCII group (≤98%). The high-HCII group had a tendency toward reduced major adverse cardiovascular events (MACE), including rehospitalization because of unstable angina, non-fatal MI, revascularization with either PCI or coronary artery bypass grafting (CABG), ischemic stroke, and

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**Fig. 2.** Multiple atherosclerotic lesions in a female patient with congenital HCII deficiency.


B. MRI angiography. Left panel: left carotid artery. Arrow indicates stenosis of the left internal carotid artery. Right panel: right carotid artery. Arrow indicates stenosis of the right internal carotid artery.

C. Abdominal aortography. Upper arrow indicates stenosis of the right renal artery and lower arrow indicates aneurysm of the abdominal aorta.

**Fig. 3.** Multivariate analysis for risk factors that affect in-stent restenosis after PCI.

Multivariate analysis demonstrated that plasma HCII activity is independently and inversely associated with angiographic in-stent restenosis after PCI. Odds ratio of HCII represents a value of 1% increase in plasma HCII activity. AT: antithrombin, HCII: heparin cofactor II, MLD: minimal lumen diameter Modified from the article by Takamori et al.
cardiovascular death, compared with other groups. Multivariate regression analysis including all patients showed that plasma HCII activity was an independent predictor of future MACE.

2. Carotid Atherosclerosis

Since our HCII-deficient patient manifested carotid atherosclerosis and her elder brother had a history of stroke, we speculated that HCII also has a protective role in carotid atherosclerosis and cerebrovascular events. In order to clarify the relationship between plasma HCII activity and severity of carotid atherosclerosis, we measured plasma HCII activity and performed carotid arterial ultrasonography in 306 Japanese individuals (154 men and 152 women) over 40 years of age (68.9 ± 11.1 years, mean ± SD)\(^25\). In that study, the thickness of carotid plaque was found to be inversely correlated with plasma HCII activity (Fig. 4)\(^25\). Multiple regression analysis revealed that plasma HCII activity and HDL cholesterol independently contributed to the suppression of intima-media thickening and that the antiatherogenic contribution of HCII activity was stronger than that of HDL cholesterol\(^25\).

3. Peripheral Arterial Disease

Patients with impaired blood flow to the extremities as a consequence of peripheral arterial disease (PAD) may present with typical ischemic pain of muscle, atypical pain or no symptoms. The presence of PAD is a strong predictor of future cardiovascular and cerebrovascular events, such as myocardial infarction, stroke and death\(^26-29\). 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\(^30\). Risk factors for development of peripheral arterial atherosclerosis are similar to those for the development of coronary atherosclerosis, such as diabetes mellitus, hyperlipidemia, cigarette smoking and hypertension. Although an endogenous protective factor against the progression of PAD has not been elucidated, we hypothesized that HCII can attenuate the development of PAD. In order to clarify this issue, plasma HCII activity and ankle brachial pressure index (ABI) were determined in 494 Japanese elderly subjects with cardiovascular risk factors\(^31\). Diagnosis of PAD was made by ABI below 0.9, and 62 subjects were diagnosed as having PAD. Multivariate logistic regression analysis showed that age, current smoking and the presence of diabetes mellitus were independent and progressive determinants of PAD (Fig. 5). In contrast, HCII was an independent protective factor against PAD (Fig. 5)\(^31\). In order to elucidate the protective effect of HCII in patients with PAD who underwent femoropopliteal stent implantation after initial failure of plain balloon angioplasty due to significant residual stenosis (>30% lumen diameter reduction) or flow-limiting dissection, Schillinger et al. studied

Fig. 4. Scatter plots between plasma HCII activity and carotid artery maximum intima-media thickness.

An inverse relation between plasma HCII activity and carotid artery Max-IMT was observed. The scatter plots were adjusted for age and sex. Modified from the article by Aihara et al.\(^25\).

Fig. 5. Multivariate regression analysis for determinants of PAD.

Multivariate analysis demonstrated that plasma HCII activity is independently and inversely associated with prevalence of PAD. Odds ratio of HCII represents a value of 1% increase in plasma HCII activity. Modified from the article by Aihara et al.\(^31\).
63 consecutive patients with PAD. Plasma HC activity was measured before stenting and the patients were followed for a median 10 months (interquartile range: 6 to 17 months) for the occurrence of first in-stent restenosis. Cumulative freedom from restenosis at 6 and 12 months in patients with low HC activity (≤100%, lower tertile) was 84% and 35%, respectively, whereas 93% and 72% in patients with high HC activity (>100%, higher tertile). After adjustment for the material of the implanted stents and other cardiovascular risk factors, high HC activity was found to exert a protective effect against in-stent restenosis in the femoropopliteal artery. Taken together, the results indicated that plasma HC activity is a predictor of the development of PAD as well as restenosis after femoropopliteal stent implantation.

4. Endothelial Function and Future Cardiovascular Events

The vasodilator function of the vascular endothelium serves as an indicator of atherosclerotic risk. A healthy endothelium is antiatherogenic through favorable paracrine effects on vasodilation, inhibition of leukocyte adhesion, platelet aggregation/coagulation, and promotion of healing via progenitor cells. Recently, measurement of brachial artery flow-mediated vasodilation (FMD) has become the most widely used method for assessing endothelial function, and evidence of a relationship between FMD and cardiovascular events has accumulated. Huang et al. evaluated the clinical significance of plasma HC activity in relation to FMD and the incidence of cardiovascular events. In that study, 199 patients aged 63 ± 14 years were enrolled and followed up for a median period of 24 months, and endothelial function was assessed using brachial ultrasonography to determine FMD. Cox regression analyses were conducted for the enrolled subjects, with cardiovascular events being defined as MI, PCI, CABG, ischemic stroke, and peripheral artery revascularization. Multivariate analysis showed that age and high-sensitive CRP were inversely correlated with FMD and that plasma HC activity was positively correlated with FMD. Kaplan-Meier and Cox regression analyses demonstrated that reduced plasma HC activity can predict a high incidence of future cardiovascular events.

Basic Studies Using Cultured Cells on HCII and Vascular Remodeling

Hayakawa et al. induced the expression of HCII in cultured human fibroblasts and in vascular smooth muscle cells using adenovirus-mediated gene transfer. The gene transfer-induced expression of HCII inhibited thrombin-induced interleukin-6 (IL-6) release from fibroblasts and from vascular smooth muscle cells. Since IL-6, an inflammatory cytokine, has a pivotal role in extracellular matrix deposition and reorganization leading to atherosclerosis, HCII may...
have a protective role against thrombin-induced vascular remodeling through inhibition of IL-6 production in fibroblasts and vascular smooth muscle cells.

Studies Using HCII-Deficient Mice on Vascular Remodeling

1. Generation and Characterization of HCII-Deficient Mice

Kamp and Ragg showed that the genomic structure of murine and human HCII consists of 5 exons that are interrupted by 4 introns. In 2002, He et al. reported the generation of homozygous HCII-deficient mice with deletion of 2 kb of the murine HCII gene, which encodes the N-terminal half of the protein, including thrombin and dermatan sulfate binding sites. HCII<sup>−/−</sup> offspring were obtained at close to the expected Mendelian frequency by mating male and female HCII<sup>+/−</sup> mice (HCII<sup>+/−</sup> St. Louis mice). Although HCII-deficient mice were indistinguishable from their wild-type littermates in weight and survival and did not have spontaneous thrombosis or other morphological abnormalities, the time to thrombotic occlusion of the carotid artery after photochemically induced endothelial cell injury was significantly shorter in HCII<sup>−/−</sup> mice. The shortened occlusion time in HCII<sup>−/−</sup> mice was corrected by intravenous infusion of purified human HCII protein.

In 2007, we independently generated and reported HCII-deficient mice. Unexpectedly, mice with homozygous deletion of HCII exhibited embryonic lethality after careful backcrossing for 10 generations with C57BL/6J strain. We examined murine karyotypes by chromosome analysis and accuracy of homologous recombination of the targeting vector by Southern blot analysis and FISH analysis in our HCII<sup>−/−</sup> mice. As a result, we confirmed the absence of truncated HCII transcripts and noninterference for mRNA levels of neighboring
genes around the HCII genome after HCII targeting vector introduction in HCII<sup>+/−</sup> Tokushima mice<sup>42</sup>. Since there is a strain difference between ES cells and screened genome libraries in HCII<sup>+/−</sup> St. Louis mice and HCII<sup>+/−</sup> Tokushima mice, the discrepant results appeared to be due to a minor strain-dependent difference in genetic background even after backcrossing for 10 generations.

2. Enhanced Vascular Remodeling in HCII-Deficient Mice

2-1. Acceleration of Mechanical Injury-Induced Vascular Remodeling in HCII-Deficient Mice

Although no morphometrical differences were observed between uninjured femoral arteries in HCII<sup>+/−</sup> and HCII<sup>+/−</sup> Tokushima mice, cuff-injured femoral arteries in HCII<sup>+/−</sup> mice revealed a prominent increase in intimal and adventitial thickness, but not in the thickness of medial area, compared with HCII<sup>+/−</sup> mice<sup>42</sup>. A much greater increase in PCNA and BrdU-stained cells was observed in cuff-injured HCII<sup>+/−</sup> Tokushima mice than in cuff-injured HCII<sup>+/−</sup> mice<sup>42</sup>. In addition, markedly broader PAR-1 expression was observed in cuff-injured HCII<sup>+/−</sup> Tokushima mice than in cuff-injured HCII<sup>+/−</sup> mice along with the phenotype of enhanced vascular remodeling<sup>42</sup>. Moreover, wire-injured femoral arteries in HCII<sup>+/−</sup> Tokushima mice revealed marked neointimal hyperplasia and a high intima-to-media ratio compared with HCII<sup>+/−</sup> mice<sup>42</sup> (Fig. 6). Wire injury also caused a higher incidence (20%) of arterial occlusion due to thrombosis in HCII<sup>+/−</sup> Tokushima mice than in HCII<sup>+/−</sup> mice (10%)<sup>42</sup>. Since we found enhanced platelet aggregation in HCII<sup>+/−</sup> Tokushima mice, activation of PARs due to reduced HCII activity in platelets may be associated with increased thrombogenicity. All of those abnormalities were ameliorated by human purified HCII protein supplementation<sup>42</sup> (Fig. 6). Vicente et al. evaluated neointimal formation in HCII<sup>+/−</sup> St. Louis mice and HCII<sup>+/−</sup> mice 3 weeks after mechanical dilation of the common carotid artery using a beaded probe<sup>16, 43</sup>. Histological findings revealed that intimal hyperplasia was significantly greater in HCII<sup>+/−</sup> St. Louis mice than in HCII<sup>+/−</sup>

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**Fig. 8.** Schematic diagram of HCII action for vascular protection at the site of vascular wall injury.
mice in accordance with the results of our mechanical vascular injury model in HCII\(^{-/-}\) Tokushima mice. They also demonstrated increased thrombin activity in injured carotid arteries of HCII\(^{-/-}\) St. Louis mice\(^{48}\).

2-2. Hypercholesterolemia-Induced Augmentation of Aortic Plaque Formation in HCII-Deficient Mice

In order to examine the role of HCII against lipid disorder-induced atherosclerosis, we generated HCII \(^{-/-}\) (Tokushima) apolipoprotein E (ApoE)\(^{-/-}\) mice and compared double-mutant mice with HCII \(^{+/+}\) ApoE\(^{-/-}\) mice\(^{42}\). Histological findings revealed that the atherosclerotic plaque area was significantly increased in the aortic root of HCII \(^{-/-}\) (Tokushima) ApoE\(^{-/-}\) mice compared to HCII \(^{+/+}\) ApoE\(^{-/-}\) mice (Fig. 7). In addition, lipid deposition, PAR-1-positive cells and superoxide production in the plaques were more prominent in the aortic root of HCII \(^{-/-}\) (Tokushima) ApoE\(^{-/-}\) mice than HCII \(^{+/+}\) ApoE\(^{-/-}\) mice (Fig. 7)\(^{42}\). Vicente et al. also generated mice that were deficient in both HCII and ApoE gene and investigated the vascular phenotypes in these mice\(^{16, 43}\). In the ApoE-null background, atherosclerotic plaque areas in the aortic arch were significantly greater in HCII \(^{-/-}\) (St. Louis) mice than in HCII \(^{+/+}\) mice\(^{43}\).

**Summary and Conclusions**

Clinical studies have shown that plasma HCII activity is inversely associated with the incidence of in-stent restenosis after PCI, severity of carotid atherosclerosis, prevalence of PAD, endothelial dysfunction and future cardiovascular events. Studies using HCII-deficient mice have shown that HCII is required for fetal development in a mouse line and that HCII has protective roles against thrombus formation and vascular remodeling induced by mechanical vascular injury and/or serum lipid abnormality; therefore, HCII is a novel predictive marker and a therapeutic target against atherothrombosis. A conceptual diagram of the antiatherogenic action of HCII at the vascular wall as presented and overviewed in this review is shown in Fig. 8.

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