Heme Oxygenase-1 Gene Promoter Polymorphism Is Associated With Coronary Artery Disease in Japanese Patients With Coronary Risk Factors

Hideaki Kaneda, Minoru Ohno, Junichi Taguchi, Masako Togo, Hideki Hashimoto, Ken Ogawara, Tadanori Aizawa, Nobukazu Ishizaka, Ryozo Nagai

Objective—Heme oxygenase (HO) is important in the defense against oxidative stress and as a factor in an antiatherogenic mechanism. Compared with long (GT)\textsubscript{n} repeats, short (GT)\textsubscript{n} repeats in the human HO-1 gene promoter were shown to have higher transcriptional activity in response to oxidative stress. There is a strong link between oxidative stress and the pathogenesis of coronary artery disease (CAD).

Methods and Results—We screened the allelic frequencies of (GT)\textsubscript{n} repeats in the HO-1 gene promoter in 577 patients who underwent coronary angiography. Because the distribution of numbers of (GT)\textsubscript{n} repeats was bimodal, we divided the alleles into 2 subclasses: class S included shorter (<27) repeats, and class L included longer (≥27) repeats. Multivariate logistic regression models including standard coronary risk factors revealed that the genotypes were significantly related to CAD status in hypercholesterolemic, diabetic patients or in smokers. In this study, the patients with shorter GT repeats were less likely to have CAD.

Conclusions—Length polymorphism in the HO-1 gene promoter is related to CAD susceptibility in Japanese people who also have coronary risk factors such as hypercholesterolemia, diabetes, and smoking. HO-1 may play an antiatherogenic role in Japanese patients with these coronary risk factors. (Arterioscler Thromb Vasc Biol. 2002;22:1680-1685.)

Key Words: heme oxygenase ■ oxidative stress ■ coronary disease ■ polymorphism ■ risk factors

Heme oxygenase (HO)-1 is upregulated by numerous insults, including oxidative stress, and in these conditions, it is considered to play a protective role.\textsuperscript{1,2} We have previously demonstrated that the induction of HO-1 inhibits neointimal formation after vascular injury.\textsuperscript{3} Ishikawa et al\textsuperscript{4} have shown that the modulation of HO-1 expression in LDL-receptor knockout mice fed a high-fat diet affects atherosclerotic lesion formation in the proximal aorta. The antiatherogenic properties of HO have been attributed to its enzymatic production of carbon monoxide, which is a vasodilator, and bilirubin, which is an antioxidant.\textsuperscript{6,7} Recently, a mild increase in the levels of serum bilirubin has been suggested to have a protective effect; bilirubin may reduce the risk of coronary artery disease (CAD) by acting as an antioxidant.\textsuperscript{6,7}

The (GT)\textsubscript{n} repeat is the most frequent of the simple repeats scattered throughout the human genome, and many of these repeats exhibit length polymorphisms.\textsuperscript{8} This purine-pyrimidine–alternating sequence, possessing Z-conformation potential, negatively affects transcriptional activity in the rat prolactin gene.\textsuperscript{8} A (GT)\textsubscript{n} repeat in the 5′-flanking region of the human HO-1 gene is highly polymorphic\textsuperscript{9} and may modulate gene transcription under thermal stress.\textsuperscript{10} Yamada et al\textsuperscript{11} have shown that in smokers there is a correlation between the length of the (GT)\textsubscript{n} repeat and susceptibility to the development of chronic pulmonary emphysema, an oxidative stress–inducing disease. Furthermore, these authors have shown that H\textsubscript{2}O\textsubscript{2} exposure upregulates the transcriptional activity of the HO-1 promoter/luciferase fusion genes in A549 cells or Hep3B cells transfected with a short (GT)\textsubscript{n} repeat but not with a long (GT)\textsubscript{n} repeat.\textsuperscript{11} They proposed that the large (GT)\textsubscript{n} repeat in the HO-1 gene promoter may reduce the induction of HO-1 by reactive oxygen species present in cigarette smoke, thereby resulting in the development of chronic pulmonary emphysema.\textsuperscript{11}

Therefore, we thought that the microsatellite polymorphism of this gene might be associated with the development of other diseases that are induced by oxidative stress. In the present study, we have focused on CAD because there is a strong link between the incidence of reactive oxygen species in patients and the pathogenesis of CAD. We screened allelic frequencies of the (GT)\textsubscript{n} repeats in the HO-1 gene promoter in patients with and without CAD and examined the association between the development of CAD and the length of the (GT)\textsubscript{n} repeats.
Methods

Clinical Protocol and Characteristics of Patients
At the Cardiovascular Institute in Tokyo, we obtained blood samples from 577 patients who underwent selective coronary angiography because of suspected CAD. All subjects were Japanese and, thus, represented an ethnic isolation. Written informed consent was obtained from each subject after a full explanation of the study to analyze the genes potentially related to cardiovascular disease. The present study was approved by the Committee of the University of Tokyo and the Cardiovascular Institute in Tokyo. Patient data regarding current smoking habits, history of hypercholesterolemia, hypertension, and/or diabetes, and biochemistry (total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride levels) were obtained from medical records.

Patients with hypercholesterolemia were defined as those having a total cholesterol level of >5.70 mmol/L (220 mg/dL) or who were receiving lipid-lowering therapy. Patients considered to have arterial hypertension had a current systolic/diastolic blood pressure of >140/90 mm Hg or were receiving antihypertensive therapy. Diabetes was defined in accordance with the criteria of the American Diabetes Association. Body mass index was calculated as weight (in kilograms) divided by height squared (in meters squared). Films of selective coronary angiography were evaluated for obstructive coronary lesions by experienced angiographers who had no knowledge of selective coronary angiography were evaluated for obstructive coronary lesions by experienced angiographers who had no knowledge of the patients' risk profiles. Stenosis of coronary arteries was categorized according to the reporting system proposed by the American Heart Association. Significant coronary artery stenosis was defined as a major coronary artery with ≥75% luminal obstruction by visual estimation. CAD was defined as the presence of significant stenoses in ≥1 coronary artery.

Analysis of Length Variability of \((GT)_n\) Repeats in HO-1 Gene Promoter
Venous blood samples were collected in tubes containing Na₂EDTA. Genomic DNA was extracted with the use of a QIAamp blood kit (Qiagen) according to the manufacturer's protocol. The HO-1 gene 5'-flanking region containing a poly \((GT)_n\) repeat was amplified by polymerase chain reaction (PCR)\(^{10}\) with a \(^{32}\)P-labeled sense primer (5'-AGAGCTGCAGCTTCTCAGA-3') and an unlabeled antisense primer (5'-ACAAGTCTGGCCATAGGAC-3'), which were designed on the basis of the published sequence.\(^{14}\) The PCR cycle of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds was carried out for a total of 30 cycles. The PCR products were subsequently run on a denaturing polyacrylamide gel (6%, acrylamide:bis-acrylamide 19:1) at 2000 V for 2 hours, followed by autoradiography. Blood samples typically have 2 different sizes of \((GT)_n\) repeats from the 2 alleles. Therefore, we divided the alleles into 2 sub-classes according to the number of \((GT)_n\) repeats: class S (27 GT repeats). The patients were then classified as

Statistical Analysis
The association of CAD status with the allele frequency was assessed with consideration of confounding effects by known coronary risk factors, such as age, sex, diabetes, hypercholesterolemia, hypertension, and smoking habits. After preliminary bivariate analysis using the \(t\) test and \(\chi^2\) test, the multivariate logistic regression method was used to assess the odds ratio of CAD by allele genotypes after controlling for influential confounding factors. Analysis was performed by StatSoft. Continuous variables are expressed as mean±SEM. Values of \(P<0.05\) were taken to be statistically significant. Because all subjects were Japanese, statistical artifacts caused by population stratification, as described by Pritchard and Rosenberg,\(^{15}\) could be ruled out.

Results
Patients with CAD versus those without CAD were older (aged 63±0.5 versus 58±0.7 years, \(P<0.001\)) and had a higher frequency of male sex (84% versus 62%, \(P<0.001\)), hypercholesterolemia (47% versus 18%, \(P<0.001\)), diabetes (52% versus 18%, \(P<0.001\)), smoking (60% versus 45%, \(P<0.001\)), and hypertension (44% versus 29%, \(P=0.002\)). CAD versus non-CAD patients also had higher body mass index (23.7±0.2 versus 22.8±0.2 kg/m², \(P<0.001\)), higher total cholesterol levels (5.21±0.5 versus 5.08±0.05 mmol/L, \(P=0.05\)), higher triglyceride levels (1.88±0.07 versus 1.57±0.07 mmol/L, \(P=0.001\)), lower HDL cholesterol levels (1.22±0.02 versus 1.43±0.03 mmol/L, \(P<0.001\)), and higher LDL cholesterol levels (3.13±0.05 versus 2.90±0.05 mmol/L, \(P<0.001\)).

The numbers of \((GT)_n\) repeats in the human HO-1 gene promoter in the individuals studied ranged from 15 to 37 (Figure 1). The distribution of the \((GT)_n\) repeat numbers was bimodal, with one peak at 22 GT repeats and the other at 27 GT repeats. Therefore, we divided the alleles into 2 subclasses according to the number of \((GT)_n\) repeats: class S included alleles with ≥26 GT repeats; class L included alleles with ≥27 GT repeats. The patients were then classified as having an S/S, S/L, or L/L genotype according to each of their HO-1 alleles. The distribution of genotypes is shown in Table 1.

Initialy, we examined the association between the development of CAD and length of the \((GT)_n\) repeats in all 577 patients. Multivessel disease was found in 49 (28%) of 172 patients with genotype L/L, 81 (26%) of 310 patients with genotype S/L, and 23 (24%) of 95 patients with genotype S/S. Multivariate regression models determined that the genotypes of \((GT)_n\) repeats in the human HO-1 gene promoter were not significantly related to CAD status across all patients (Table 2).

We next determined whether coronary risk factors (male sex, age, hypercholesterolemia, diabetes mellitus, hypertension, and smoking) might influence a relationship between
the genotype of the human HO-1 gene promoter and the development of CAD by evaluating the interaction between risk factors and genotypes by logistic regression analysis. The distribution of the numbers of \((GT)_n\) repeats in the human HO-1 gene promoter in patients with hypercholesterolemia \((n=189)\), diabetes mellitus \((n=205)\), and smokers \((n=305)\) is shown in Figures 2, 3, and 4. The distribution of patient genotypes is shown in Table 1. We found that 3 risk factors have a significant interaction with genotypes: hypercholesterolemia \((P=0.013)\), diabetes mellitus \((P=0.003)\), and smoking \((P=0.023)\). Stratified analysis further showed that genotype S/S is protective against CAD in patients with 1 of the 3 coronary risk factors (Table 2), although the effect was not significant in patients who had none of the risk factors (data not shown). The odds ratio for genotype S/S versus genotype L/L was 0.23 (95% CI 0.064 to 0.65, \(P<0.01\)) in the diabetic patients, and 0.40 (95% CI 0.17 to 0.95, \(P<0.05\)) in the smokers.

**Discussion**

The distribution of the numbers of \((GT)_n\) repeats in the HO-1 gene promoter in the patients enrolled in the present study was consistent with the distribution in patients and controls in previous reports.\(^9,11\) Although we did not observe any differences in allelic frequencies of the \((GT)_n\) repeats in the HO-1 gene promoter in the patients with CAD compared with those without CAD across all patients, we found that length

### Table 1. Distribution of Patient Genotypes

<table>
<thead>
<tr>
<th></th>
<th>Genotype S/S</th>
<th>Genotype S/L</th>
<th>Genotype L/L</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-CAD</td>
<td>48 (17)</td>
<td>145 (52)</td>
<td>86 (31)</td>
<td>279</td>
</tr>
<tr>
<td>CAD</td>
<td>47 (16)</td>
<td>165 (55)</td>
<td>86 (29)</td>
<td>298</td>
</tr>
<tr>
<td>Hypercholesterolemia*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-CAD</td>
<td>14 (29)</td>
<td>28 (57)</td>
<td>7 (14)</td>
<td>49</td>
</tr>
<tr>
<td>CAD</td>
<td>38 (15)</td>
<td>81 (58)</td>
<td>21 (27)</td>
<td>140</td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-CAD</td>
<td>12 (24)</td>
<td>30 (60)</td>
<td>8 (16)</td>
<td>50</td>
</tr>
<tr>
<td>CAD</td>
<td>19 (12)</td>
<td>93 (60)</td>
<td>43 (28)</td>
<td>155</td>
</tr>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-CAD</td>
<td>25 (20)</td>
<td>69 (55)</td>
<td>32 (25)</td>
<td>126</td>
</tr>
<tr>
<td>CAD</td>
<td>23 (13)</td>
<td>103 (57)</td>
<td>53 (30)</td>
<td>179</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-CAD</td>
<td>10 (12)</td>
<td>48 (60)</td>
<td>23 (28)</td>
<td>81</td>
</tr>
<tr>
<td>CAD</td>
<td>15 (11)</td>
<td>78 (60)</td>
<td>38 (29)</td>
<td>131</td>
</tr>
</tbody>
</table>

Values are n (%).
*\(P<0.05\) with the \(\chi^2\) test.

### Table 2. Multivariate Logistic Regression Models for CAD in Patients

<table>
<thead>
<tr>
<th></th>
<th>Total ((n=577))</th>
<th>HL ((n=189))</th>
<th>Diabetes ((n=205))</th>
<th>Smokers ((n=305))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype S/S*</td>
<td>0.65 (0.36–1.2)</td>
<td>0.23 (0.07–0.72)‡</td>
<td>0.23 (0.076–0.71)§</td>
<td>0.40 (0.17–0.95)‡</td>
</tr>
<tr>
<td>Genotype S/L</td>
<td>0.78 (0.50–1.2)</td>
<td>0.42 (0.15–1.15)</td>
<td>0.48 (0.19–1.22)§</td>
<td>0.55 (0.28–1.05)</td>
</tr>
<tr>
<td>Male</td>
<td>3.3 (2.0–5.5)§</td>
<td>3.5 (1.4–9.0)§</td>
<td>2.1 (0.87–5.2)§</td>
<td>3.9 (1.4–11)§</td>
</tr>
<tr>
<td>Age†</td>
<td>1.05 (1.03–1.07)§</td>
<td>1.05 (1.01–1.09)§</td>
<td>1.0 (0.99–1.1)</td>
<td>1.06 (1.03–1.09)§</td>
</tr>
<tr>
<td>HL</td>
<td>3.2 (2.0–5.1)§</td>
<td>–</td>
<td>3.1 (1.5–6.4)§</td>
<td>3.1 (1.7–5.8)§</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3.0 (2.0–4.7)§</td>
<td>3.0 (1.4–6.4)§</td>
<td>–</td>
<td>4.2 (2.4–7.6)§</td>
</tr>
<tr>
<td>Smoker</td>
<td>0.82 (0.54–1.3)</td>
<td>0.70 (0.29–1.7)</td>
<td>1.4 (0.64–3.1)</td>
<td>–</td>
</tr>
<tr>
<td>HT</td>
<td>1.0 (0.67–1.5)</td>
<td>0.85 (0.40–1.8)</td>
<td>0.71 (0.35–1.4)</td>
<td>1.1 (0.59–1.8)</td>
</tr>
<tr>
<td>HDL†</td>
<td>0.28 (0.17–0.48)§</td>
<td>0.20 (0.075–0.54)§</td>
<td>0.34 (0.14–0.82)§</td>
<td>0.22 (0.10–0.49)§</td>
</tr>
</tbody>
</table>

Values are odds ratio (95% CI).
HL indicates hypercholesterolemia; HT, hypertension.
*Genotype L/L was regarded as a reference point.
†Odds ratio per one-unit increase.
‡\(P<0.05\). §\(P<0.01\).
The initial degradation of heme by microsomal HO involves the liberation of iron and carbon monoxide and the formation of biliverdin, which is subsequently reduced to bilirubin by cytosolic biliverdin reductase.\textsuperscript{5} HO-derived carbon monoxide was first reported to function as a neurotransmitter through the activation of guanylyl cyclase.\textsuperscript{16} A few years later, this pathway was reported to play an active vasorelaxing role in the hepatic microcirculation.\textsuperscript{17} This system is now postulated as a ubiquitous defense mechanism against stress.\textsuperscript{1} Recently, it has been postulated to play an antiatherogenic role in the cardiovascular system.\textsuperscript{18} The induction of HO-1, an inducible form of HO, by atherogenic and inflammatory mediators, such as oxidized LDL and cytokines, may ameliorate the insult to cells by restoring the balance of antioxidants and pro-oxidants in the vascular wall.\textsuperscript{5}

It has been suggested that slightly increased levels of serum bilirubin, another product of the HO pathway, act as a protective factor for atherosclerosis.\textsuperscript{6,7} Bilirubin may protect against atherosclerosis through its ability to protect LDL from oxidative modification by a variety of pro-oxidants and to potentiate the actions of vitamins C and E.\textsuperscript{19–22} We have previously shown that balloon injury–induced neointimal formation in the rat carotid artery is markedly inhibited by hemin, an HO-1 inducer.\textsuperscript{3} Recently, compared with control animals, LDL receptor knockout mice treated with the HO-1 inducer, hemin or hemin plus desferrioxamine, exhibited reduced atherosclerotic lesion formation in the proximal aorta.\textsuperscript{4} Opposing results were observed when mice were treated with the HO inhibitor Sn-protoporphyrin IX.\textsuperscript{4} Gene transfer of HO-1 has recently reported to inhibit vascular proliferation.\textsuperscript{23} Taken together, these results indicate that HO-1 may have an antiatherogenic role.

### Coronary Risk Factors

In the present study, we defined CAD as being present if the patient had at least 1 coronary narrowing of >75%, because a 75% cutoff can be found in the large Duke Cardiovascular Database, wherein cardiac survival >10 years in patients with at least one >75% coronary narrowing (62%) was markedly reduced compared with that in patients with only 25% to 50% narrowing (97%) or <25% narrowing (100%).\textsuperscript{24} When we used this cutoff value in the present study, we found that the number of GT repeats in the HO-1 gene promoter was associated with CAD in patients with hypercholesterolemia and diabetes mellitus and also in smokers.

Hypercholesterolemia is reported to be associated with elevated oxidative stress, which causes increased lipid peroxidation\textsuperscript{25} and tends to increase the susceptibility of LDL to oxidation.\textsuperscript{26,27} Oxidized LDL has been found to have various biological effects on vessel walls, including stimulation of cytokine production,\textsuperscript{28,29} inhibition of endothelial cell vasodilator function,\textsuperscript{30} and stimulation of growth factor production.\textsuperscript{31} As well as providing mechanistic links between lipoproteins and the cell biology of atherosclerosis, these observations also raise the more general possibility that abnormalities of the oxidation-reduction state in the vessel wall may be an important pathogenic mechanism in atherosclerosis.
Diabetes mellitus is a major source of morbidity in developed countries, and among its comorbid conditions, atherosclerosis is perhaps the most important. Among the sequelae of hyperglycemia, excessive oxidative stress has received considerable attention as a potential cause of the increased vascular disease seen in diabetic patients. There is considerable controversy regarding the precise mechanism by which hyperglycemia may contribute to the development of CAD in diabetes. Plasma from diabetic subjects contains increased levels of thiobarbituric acid reactive substances and lipid hydroperoxides, 2 markers of lipid peroxidation. The established association between atherosclerosis and lipid peroxidation within the vascular wall has led to a renewed interest in the oxidative stress of hyperglycemia as a potential mechanism for diabetic vascular disease.

Oxidative stress is considered to be the major pathological mechanism that occurs during smoking, leading notably to lipid peroxidation. Several studies have demonstrated that smokers have an increased susceptibility of LDL to oxidation and that there are higher levels of oxidized LDL in smokers. This would provide an important causal mechanism that links smoking to vascular disease given the numerous pathological effects of oxidized LDL. Smoking may enhance oxidative stress not only through the production of reactive oxygen radicals in smoke but also through weakening of the antioxidant defense mechanisms.

There is increasing evidence that hypertension, like hyperlipidemia, induces oxidative stress in the arterial wall. It has even been suggested that superoxide anions might trigger the development of hypertension in some models, presumably by inactivating endothelium-derived NO and thus mitigating this important vasodilator mechanism. Additional data in other models of hypertension support the notion that oxygen-derived free radicals contribute to either the causes or consequences of hypertension.

As mentioned above, these 4 risk factors (hypercholesterolemia, diabetes mellitus, hypertension, and smoking) are related to oxidative stress, which plays a major role in atherogenesis. At present, we do not know precisely why the development of CAD is associated with the genotype of repeats in the human HO-1 gene promoter only in patients with hypercholesterolemia or diabetes mellitus or in smokers. Differences in the way that CAD develops or differences in the HO defense mechanism against oxidative stress among patients with these risk factors may offer reasons and remain to be investigated in future studies.

Study Limitations
Two alternative molecular mechanisms can be proposed to account for the association between CAD and the polymorphism in the 5′-flanking region of the HO-1 gene. First, the actual length of the (GT)n repeat in the HO-1 gene promoter itself may play a role in tissue-specific regulation of HO-1 expression. The small size of the (GT)n repeat in the HO-1 gene promoter may increase the induction of HO-1 by reactive oxygen species in hypercholesterolemic or diabetic patients or in smokers, thereby reducing the risk of CAD. Second, the repeats themselves may have no direct biological function, but the length of the repeat may cause structural interference with other biologically important DNA sequences in HO-1 or in a gene in proximity.

In conclusion, we have shown in the present study that polymorphism in the HO-1 gene promoter is related to CAD susceptibility in Japanese individuals who have hypercholesterolemia or diabetes or who smoke. In addition, HO-1 may play an antiatherogenic role in these same groups of people. Determining the HO-1 genotype in CAD patients may aid in assessing their susceptibility to other risk factors. Because the present study was a small case-control study, however, further cohort studies need to be carried out, examining a larger population and/or people from different ethnic origins.

References


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