Clinical Restenosis after Coronary Stent Implantation Is Associated with the Heme Oxygenase-1 Gene Promoter Polymorphism and the Heme Oxygenase-1 +99G/C Variant

Talin Gulesserian,1,2† Catharina Wenzel,4† Georg Endler,1 Raute Sunder-Plassmann,1 Claudia Marsik,1 Christine Mannhalter,1 Nelly Iordanova,4 Mariann Gyöngyösi,3 Johann Wojta,3 Stefan Mustafa,1 Oswald Wagner,1* and Kurt Huber4

Background: Vascular remodeling after percutaneous coronary stent implantation frequently leads to restenosis. Heme oxygenase 1 (HO-1) is involved in the generation of the endogenous antioxidant bilirubin and carbon monoxide, both of which exert anti-inflammatory and antiproliferative effects. The aim of the present study was to evaluate the influence of genetic risk factors combined with the conventional risk factors on the development of coronary restenosis after percutaneous coronary intervention (PCI) with stent implantation.

Methods: The HO-1 gene GT dinucleotide repeat promoter polymorphism and HO-1/H1154599G/C variant were evaluated in 199 patients with coronary artery disease after coronary stent implantation and control angiography at 6 months after the intervention. Coronary restenosis was confirmed by quantitative angiography.

Results: Carriers of the long allele of the HO-1 gene promoter (>29 repeats) had a significantly higher risk of developing restenosis after PCI than noncarriers [odds ratio (OR) = 1.9; 95% confidence interval (95% CI), 1.0–3.4; P = 0.04]. Interestingly, the allele longer than 29 repeats conferred a significantly higher risk of developing restenosis (OR = 3.4; 95% CI, 1.2–9.1; P = 0.017) in nonsmokers than in smokers (OR = 2.0; 95% CI, 0.7–5.2; P = 0.18).

Conclusions: The long allele of the HO-1 gene promoter (>29 repeats) polymorphism, which leads to low HO-1 inducibility, may represent an independent prognostic marker for restenosis after PCI and stent implantation. The effect of the >29 repeat allele is attenuated in smokers, who have chronic exogenous CO exposure.

Atherosclerotic vascular disease, including coronary artery disease, and its complication myocardial infarction (MI)5 are the major causes of morbidity and mortality in industrialized countries. Endogenous and exogenous risk factors, such as smoking, hyperlipidemia, diabetes mellitus (DM), and hypertension (HT), significantly increase the individual risk for MI. Although new treatment strategies such as percutaneous coronary intervention (PCI) followed by stent implantation of acute and chronic coronary vessel occlusions have significantly reduced morbidity and mortality, in a considerable number of patients, in-stent restenosis after stent implantation significantly diminishes the success of revascularization.

Over the past decade, several experimental approaches have been used to study the cardioprotective role of endogenous specific stress proteins, including heme oxygenase (HO), which is part of the physiologic vascular...
response to oxidative stress to prevent further cell damage. HO degrades heme into biliverdin, releasing free iron and CO (2, 3), a potent mediator of antiapoptotic and antiproliferative activity. The reduction of biliverdin by biliverdin reductase leads to the generation of bilirubin, a powerful endogenous antioxidant. Interestingly, the inducible isoform of the enzyme HO, heme oxygenase-1 (HO-1), is expressed in atherosclerotic lesions (4) and is increased in endothelial and smooth muscle cells in response to oxidized LDL (5). Thus, up-regulation of HO-1 and increased concentrations of antioxidants in atherosclerotic lesions may present a physiologic mechanism to attenuate the progression of inflammatory processes in the vessel wall.

Individual HO-1 expression increases in response to oxidative stress, cytokines, heavy metals, hypoxia, or heme and is influenced by genetic variability. The up-regulation of HO-1 by numerous insults, including oxidative stress, is considered to play a protective role (2) against atherosclerotic progression.

Yamada et al. (6) observed that a frequent dinucleotide (GT) repeat polymorphism in the promoter region of the HO-1 gene modulates individual HO-1 responses to exogenous stimuli. Carriers of long HO-1 alleles (>29 GT repeats) have significantly lower HO-1 induction after exposure of exogenous stimuli. We also analyzed another recently described G-to-C transversion located at cDNA position +99 (rs2071747; http://www.ncbi.nlm.nih.gov/entrez, 2005) in the first exon of the HO-1 gene (HMOX1 +99G/C). The polymorphism causes a change of aspartic acid to histidine at amino acid position 7 of the HMOX1 protein, but whether the change is of clinical relevance has yet to be investigated.

We evaluated the influence of polymorphisms in the HO-1 gene on the risk of restenosis after percutaneous coronary stent implantation in a cross-sectional study. We also studied interactions of the HO-1 genotype with smoking, which is known to lead to chronic CO exposure.

Patients and Methods

Our study population included 199 consecutive patients (142 male and 57 female; median age, 61 years; interquartile range, 55–70 years) referred to the Department of Cardiology, Medical University of Vienna and who underwent PCI and stent implantation. Follow-up coronary angiography was performed at 6–9 months after PCI and stent implantation because of renewed onset of chest pain, a pathologic exercise test, or other clinical indications of increased risk for restenosis. Angiographically significant coronary restenosis was considered present if clinical symptoms persisted and coronary angiography showed lumen narrowing ≥50%, as measured by quantitative coronary angiography (Quanctor; Siemens).

Clinical histories were obtained and physical examination performed on all patients to establish cardiovascular risk factors, including DM, smoking (>20 cigarettes/day for >5 years), hypertension (systolic blood pressure >140 mmHg or diastolic blood pressure >80 mmHg at repeated measurements or a known history of hypertension requiring treatment with antihypertensive drugs), hyperlipidemia, body mass index, and family history of cardiovascular disease. Hyperlipidemia was defined as baseline cholesterol concentrations >5.18 mmol/L (200 mg/dL), serum LDL-cholesterol concentrations >3.37 mmol/L (130 mg/dL), or triglyceride concentrations >2.03 mmol/L (180 mg/dL) after overnight fasting and was considered to be present in all patients receiving lipid-lowering medication. DM was considered present in patients with a known history of diabetes and in patients with a fasting glucose >7 mmol/L (126 mg/dL) according to American Diabetes Association criteria (7). Age and sex were also documented. The study was approved by the local ethics committee, and all individuals participating in the study gave written informed consent for DNA analysis.

Genomic DNA was isolated from whole blood by use of the Puregene DNA Isolation Kit (Gentra Systems). PCR amplifications of the HO-1 (GT)n repeat length polymorphism were performed as described elsewhere (8, 9). We divided allelic repeats into 2 subclasses according to a classification based on transfection studies and clinical studies with low and high GT repeats: short repeats, with (GT)n ≤29, were designated as allele class S (short), and long repeats, with (GT)n ≥29, as allele class L (long) (6). Homozygous and heterozygous class L carriers were grouped together and compared with homozygous class S carriers.

Patient DNA samples were genotyped for the HMOX1 +99(G/C) polymorphism by the 5′ nuclease assay for allelic differentiation. Primers and probes were designed with Primer Express (Ver. 2.0) software (Applied Biosystems) and synthesized and supplied by Applied Biosystems UK. The reporter dyes chosen were VIC© (G-specific probe) and 6-carboxyfluorescein (FAM; C-specific probe). All PCR reactions (25 μL) contained 20 ng of DNA, 0.32 μM primers (forward primer, 5′-GGAGCCAACGACGACAGAACGA-3′; reverse primer, 5′-CACAAGGGTGCTTGGAGGGA-3′), and 0.08 μM probes (G-specific probe, 5′-FAM-TCCGACAACCCTAG-3′; C-specific probe, 5′-VIC-TCCGACACCCTGA-3′) and were performed in 96-well plates according to the Allelic Discrimination PCR protocol on the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems UK). Several water controls were run in parallel with the patient DNA samples. SDS software (Ver. 2.0; Applied Biosystems) was used to analyze end-point fluorescence.

For statistical analyses, we used the SPSS 12.0 software package (SPSS Inc.). Continuous data are presented as the mean (SD), and discrete data are given as counts and percentages. Univariate analysis was performed with a nonparametric Kruskal–Wallis test for continuous variables and χ² tests for dichotomous variables. Multivariate logistic regression analysis was applied to assess the association of the HO-1 genotype, smoking, hyperlipid-
em, DM, hypertension, age, and sex with the restenosis rate, to adjust for potentially confounding variables, and to test for potential interactions. Regression analyses were performed according to standard recommendations: the logit assumption was checked for continuous variables, an analysis of residuals was performed, global goodness-of-fit testing was performed with the Hosmer–Lemeshow test, and interactions were evaluated by use of multiplicative interaction terms and log likelihood ratio $\chi^2$ tests. Results of the logistic regression models were given as the odds ratio (OR) and the 95% confidence interval (95% CI). A 2-sided $P$ value $<0.05$ was considered statistically significant.

**Results**

Of 199 consecutive patients with coronary artery disease who underwent coronary stent implantation and repeat angiography at least 6 months after the intervention, 102 (51%) developed an angiographically confirmed restenosis. As shown in Table 1, the HO-1 $99G/C$ allele was present in 21 of the 199 (11%) patients investigated: 7 of the 97 (7%) who did not experience restenosis and 14 of the 102 (14%) who did ($P = 0.08$; OR = 2.4; 95% CI, 0.9–6.3). The HO-1 class L allele was found in 49 of the 199 (25%) patients investigated: 32 of the 102 (31%) patients who developed in-stent restenosis, but only 17 of the 97 (18%) who did not (OR = 1.9; 95% CI, 1.0–3.4; $P = 0.04$). Interestingly, all individuals carrying the HO-1 $+99G$ allele also cosegregated for the HO-1 L allele; however, when we integrated both variants in a multivariable logistic regression model, we found no significant gain of model fit compared with presence of the class L allele alone. Although the HO-1 $+99G/C$ variant causes an amino acid change from aspartic acid to histidine at amino acid position 7 of the HO-1 protein, the biological relevance of this variant seems to be limited in the development of restenosis. All further analyses were therefore performed with only data for the HO-1 GT repeat.

We tested the HO-1 class L allele for possible interactions with other conventional risk factors, including male sex, hypertension, hyperlipidemia, DM, and smoking, in a logistic regression analysis applying the respective interaction terms. Interestingly, we observed a statistically significant interaction between the class L allele and smoking (Table 2). Nonsmokers carrying the class L allele had a 3.4-fold (95% CI, 1.2–9.1; $P = 0.017$) increased risk of suffering restenosis, whereas the effect of the class L allele was significantly lower in current smokers (OR = 2.0; 95% CI, 0.7–5.2; $P = 0.18$).

None of the other cardiovascular risk factors showed any significant interrelation with the HO genotype. Apart from the HO-1 class L allele, only DM contributed significantly to the risk of suffering restenosis (OR = 2.0; 95% CI, 1.0–3.9; $P = 0.04$), an observation that has been described previously (10).

**Discussion**

HO and its inducible isoform, HO-1, are part of the physiologic response to oxidative stress to prevent further cell damage. The majority of these antiinflammatory and antiproliferative effects are mediated by products of heme catabolism (11–14). The HO reaction mediates the conversion of prooxidant heme to biliverdin, CO, and free iron (3). The elimination of prooxidant heme and the production of these metabolites are supposed to provide cytoprotection against different pathophysiologic stresses in the vascular wall (15, 16).

Additionally, by eliminating free hemoglobin from the circulation, HO-1 has been shown to significantly lower LDL oxidation, one of the key players in atherogenesis (5). CO in particular has been associated with a profound inhibition of graft leukocyte infiltration/activation as well as with the inhibition of smooth muscle cell proliferation (17). The antinflammatory and antiproliferative effects of CO have been successfully applied in experimental mouse models to prevent acute and long-term transplant rejection and lipopolysaccharide-induced shock or development of arteriosclerotic lesions after transplantation. In light of these results, our at first glance paradoxical results seem explainable: genetically impaired HO-1 up-regulation, which is determined by presence of the L allele, represents an independent major risk factor for restenosis. These results are in concordance with previously reported

**Table 1. Patient characteristics and HO-1 genotype distribution, reported as HO-1 class L allele and HMOX1 + 99(G/C).**

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Restenosis present (n = 102)</th>
<th>Restenosis absent (n = 97)</th>
<th>Total (n = 199)</th>
<th>$P^a$</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, n (%)</td>
<td>72 (71)</td>
<td>70 (72)</td>
<td>142 (71)</td>
<td>0.8</td>
<td>0.9 (0.5–1.8)</td>
</tr>
<tr>
<td>Age (IQR), b years</td>
<td>63 (56–70)</td>
<td>60 (54–70)</td>
<td>61 (55–70)</td>
<td>0.5</td>
<td>1.0 (0.9–1.0)</td>
</tr>
<tr>
<td>DM, n (%)</td>
<td>35 (34)</td>
<td>21 (22)</td>
<td>56 (28)</td>
<td>0.04</td>
<td>2.0 (1.0–3.9)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>71 (70)</td>
<td>75 (77)</td>
<td>146 (73)</td>
<td>0.2</td>
<td>0.7 (0.3–1.3)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>43 (42)</td>
<td>41 (42)</td>
<td>84 (42)</td>
<td>0.9</td>
<td>1.0 (0.5–1.8)</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>83 (81)</td>
<td>86 (89)</td>
<td>169 (85)</td>
<td>0.4</td>
<td>0.7 (0.3–1.6)</td>
</tr>
<tr>
<td>HO-1 class L allele, n (%)</td>
<td>32 (31)</td>
<td>17 (18)</td>
<td>49 (25)</td>
<td>0.04</td>
<td>1.9 (1.0–3.4)</td>
</tr>
<tr>
<td>HMOX1 + 99(G/C), n (%)</td>
<td>14 (14)</td>
<td>7 (7)</td>
<td>21 (11)</td>
<td>0.08</td>
<td>2.4 (0.9–6.3)</td>
</tr>
</tbody>
</table>

$^a$ P values and ORs were calculated in a multivariate binary logistic regression model adjusting for sex, age, DM, hypertension, hyperlipidemia, and smoking.

$^b$ IQR, interquartile range.
results by Chen et al. (18) and Schillinger et al. (19). However, this effect is largely restricted to nonsmokers. Smoking is known to lead to chronic CO exposure (20) and has recently been shown to decrease the risk of restenosis (10). Schillinger et al. (21) already described this effect in patients who underwent percutaneous transluminal angioplasty; they showed that patients who smoked more than 10 cigarettes daily had a lower rate of intermediate-term restenosis after lower-limb endovascular intervention. Smoking indeed seems to influence vascular remodeling and neointimal hyperplasia after endovascular or angiographic treatment. This protective effect is mediated by an interaction with wound healing and vascular smooth muscle cell proliferation. Smokers are known to have an increased concentration of carboxyhemoglobin and increased blood CO concentrations (12, 22, 23). Increased blood CO concentrations may lessen vascular inflammation and could inhibit vascular smooth muscle cell proliferation in the treated segment. Our findings are corroborated by these studies. Interestingly, this effect is more pronounced in carriers of the L allele, who have a genetically determined low HO-1 inducibility, whereas this effect was not present in homozygous carriers of the S allele, who have high HO-1 inducibility. Nevertheless, it should be considered that smoking itself presents one of the major risk factors for MI and the need for coronary artery stent implantation, and our results should not encourage patients to continue smoking after coronary intervention. Rather, our results could encourage further studies with CO-releasing compounds as potential drug targets to prevent restenosis after stent implantation, as has been described in animal models for MI or ischemia-induced renal failure (24, 25).

We are aware of several limitations applying to our study. Our analysis does not involve patients who developed acute MI or died within 6 months after coronary stenting. Thus, our findings cannot be applied completely for all patients receiving coronary stents. Nevertheless, the composite occurrence of acute MI and death after coronary stenting in patients with stable angina pectoris usually does not exceed 3%-5%; thus, it occurs only in a minority of this patient population. Another limiting factor is that we did not analyze the endogenous risk factors for different coronary stents that might cause different restenosis rates. Similarly, because only non-drug-releasing stents were implanted in our study, we have no data on the combined influence of drug-releasing stents and HO-1 allele on restenosis. Additionally, in our observational study, we evaluated smoking status by questionnaire and patient history only. Objective measurements of cigarette consumption (cotinine and carboxyhemoglobin concentrations) are therefore not available. Thus, we have no direct evidence supporting our hypothesis that smoking in L-allele carriers acts via CO and not via other, as yet undetermined, pathways. However, in spite of these potentially confounding variables, the HO-1 gene promoter polymorphism and DM remained independent risk factors for restenosis. Thus, both the HO-1 polymorphism and DM might reflect path mechanisms for restenosis independent of the applied type of stent and clinical protocol.

In conclusion, the HO-1 gene promoter polymorphism and DM are independent prognostic risk factors for patients who develop a restenosis after PCI with stent implantation, and the effect of the HO-1 class L allele as a risk factor for restenosis is significantly attenuated in smokers.

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References

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Table 2. Relative risk of restenosis in current smokers and nonsmokers carrying the HO-1 class L allele.

<table>
<thead>
<tr>
<th></th>
<th>P</th>
<th>OR (95% CI)</th>
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<tbody>
<tr>
<td>Nonsmokers, L allele absent</td>
<td>0.58</td>
<td>1.0 (0.6–1.4)</td>
</tr>
<tr>
<td>Nonsmokers, L allele present</td>
<td>0.80</td>
<td>0.8 (0.6–1.0)</td>
</tr>
<tr>
<td>Smokers, L allele absent</td>
<td>0.017</td>
<td>3.4 (1.2–9.1)</td>
</tr>
<tr>
<td>Smokers, L allele present</td>
<td>0.72</td>
<td>1.1 (0.6–2.3)</td>
</tr>
</tbody>
</table>

*b P values and ORs were calculated in a multivariate binary logistic regression model adjusting for sex, age, DM, hypertension, and hyperlipidemia.


