Atherosclerotic progression in diabetes mellitus (DM) is linked with neovascularization and intra-plaque hemorrhage (IPH).1–5 Erythrocyte extravasation releases free hemoglobin (Hb).6 The heme-containing iron in Hb generates free radicals, and induces lipid peroxidation.7 This increased oxidative state may promote macrophage infiltration and neovascularization.8–10 Therefore, effective clearance of free Hb after IPH is a relevant pathway to reduce plaque progression in DM atherosclerosis.

The Haptoglobin (Hp) protein binds to free Hb to form Hb-Hp complex.11,12 Endocytosis of the Hb-Hp complex is mediated by the Hb scavenger receptor-C163, exclusively expressed by the monocye-macrophages.13,14 Within the cell, the heme released from Hb is finally catabolized by heme oxygenase-1 (HO-1).15 Thus, the scavenger receptor CD163 and the enzyme HO-1 are coherently regulated to enhance Hb clearance.16 Impairment in this pathway may increase the expression of the oxidative markers such as myeloperoxidase (MPO), and reduce the anti-inflammatory response involved in the resolution of inflammation.

The Hp gene has 2 alleles, Hp1 and Hp2. In individuals with DM, the Hp2-2 genotype is evolving as a contributor for increased cardiovascular events compared with Hp1-1 or Hp2-1 genotype.17,18 This may be related to a decrease in Hb clearance in DM patients with Hp2-2 genotype. This study tested the hypothesis that DM atheroma from patients with Hp2-2 genotype may have decreased Hb clearance, triggering increased oxidative, inflammatory, and angiogenic patterns.

**Methods**

**Sample Collection**

Forty DM patients with peripheral vascular disease were consented to participate in this study. Blood samples were obtained for...
Hp genotyping. Peripheral plaques (25–100 mg) during atherectomy were obtained in vivo with the Silver hawk atherotome (Fox Hollow Technologies, Redwood City, CA). Multiple plaque fragments obtained from each patient were collectively analyzed, and results presented. Demographic profiles were obtained prospectively. Institutions review board approved the study protocol.

Hp Genotype
To identify Hp genotype, DNA was extracted from blood samples and amplified by polymerase chain reaction using 2 sets of primers specifying Hp alleles Hp1 and Hp2 (GenBank Acc#: AC004682 and M 69197). Oligonucleotide primers A (5′-GAGGGGAGCTTGCCTTTCCATTG-3′) and B (5′-GAGATTTTTGAGCCCTGGCTGT-3′) were used for amplification of a 1757-bp Hp1 allele specific sequence and primers C (5′-CCTGCTCCTGATTAACTGACACAT-3′) and D (5′-CCGATGCTTCCCATAGCCATG-3′) were used to amplify a 349-bp Hp2 allele specific sequence. The polymerase chain reaction products were analyzed by agarose gel electrophoresis and the Hp genotype identified. The genotype of each sample was further confirmed by identifying the Hp phenotype using serum samples by polyacrylamide gel electrophoresis.

Quantification of IPIH and Iron Content
IPIH was assessed semiquantitatively using hematoxylin and eosin stained slides, as previously reported. The amount of extravasated red blood cells and fibrin in the necrotic lipid core of the plaque was graded as grade 0, absent; grade 1, <25%; grade 2, <50%; grade 3, <75%; and grade 4, 100%. Perl’s iron staining was graded as grade 0, absent; grade 1, mild to moderate intensity; and grade 2, severe intensity.

Quantification of Hb Scavenger Receptor CD163 and HO-1 Enzyme
Formalin-fixed paraffin embedded 4 μm thick histologic sections were deparaffinized and used to measure the expression of CD163 and HO-1 proteins. Specific primary mouse monoclonal anti-human CD163 (Lab vision, CA) and rabbit polyclonal anti-human HO-1 (Abcam, MA) antibodies at 1:100 dilutions were used. Immunohistochemistry was performed by adopting avidin-biotin complex method using ABC kit (Vector Lab, CA). Appropriate biotinylated secondary antibodies were used. The protein expression was detected by developing with 3′,3′-diaminobenzidine chromogen and Elite vectastain kit (Vector lab, CA). Appropriate parallel positive and negative controls were included to distinguish nonspecific binding. The protein expression was scored semiquantitatively in 20 random high-power fields per plaque under light microscopy by measuring the intensity of immunostaining in proportion to the total number of cells seen per high-power field. The data were presented as grade 0, absent; grade 1, mild; grade 2, moderate; and grade 3, severe.

Quantification of Oxidative MPO and Anti-Inflammatory IL-10
To evaluate oxidative stress and inflammatory response, MPO and interleukin (IL)-10 were quantified. The protein expression of MPO and IL-10 were measured using specific primary rabbit polyclonal anti-human MPO (Dako, CA) and IL-10 (Abcam, MA) antibodies at 1:100 dilutions as described above by immunohistochemistry. For gene expression, total RNA was isolated from the peripheral plaque tissue, as previously reported. The RNA concentration was quantified using Nanodrop and reverse transcribed to cDNA using TaqMan reverse transcription reagents (Applied Biosystems, CA). The cDNA was used to measure the mRNA expression by quantitative real time polymerase chain reaction with specific primer sequence for MPO, F: 5′-CGTCAACCTGGAGAACCAAGC-3′ and Rev: 5′-CCGCGGATCTT-3′; IL-10, F: 5′-CAGGAGTCGACCATC AAGG-3′ and Rev: 5′-GGGAGAAATCGTGAACGC-3′. The mRNA expression was normalized to the house-keeping gene GAPDH.

Quantification of Inflammation and Neovascularization
Inflammation was quantified using a mouse monoclonal antibody (Dako Corp) against human CD68 for macrophages and CD3 for T-lymphocytes that were linked to a red chromogen. Vascular cell adhesion molecule-1 (VCAM-1) was quantified using mouse monoclonal antibody against human VCAM-1 (Abcam, MA). The inflammatory cells were scored using Olympus BX-45 planimetry microscopy with high-power objective (×40). Score 0, mild inflammation (0–5 inflammatory cells); score 1, moderate inflammation (6–25 inflammatory cells); score 2, severe inflammation (>25 inflammatory cells). Plaque neovessels were quantified using mouse monoclonal antibody against human CD34 (Dako, CA) that was linked to a blue chromogen, and using mouse monoclonal antibody against human CD31 (Dako, CA). Using high-power objective (×40) of the microscope, the neovessels were manually counted numerically and enumerated together as total neovessels per plaque. In addition, neovessel density was calculated by dividing the total number of neovessels by total plaque area occupied. Furthermore, we scored vascular smooth muscle cells using mouse monoclonal α-smooth muscle actin (Enzo Lifesciences Inc, NY) linked to brown chromogen with diaminobenzidine, and graded by immunohistostaining. Appropriate secondary antibodies and positive and negative controls were used as per manufacturer’s instructions.

Statistical Analysis
Data are presented as mean±SEM. For 2-group comparisons, gaussian-distribution samples were compared by the 2-tailed Student t test, preceded by Levene F test for equality of variances. Non-gaussian distribution samples were compared by the Mann-Whitney, non-parametric test. The following variables were used in the analysis: IPIH grade, angina history, heart failure, coronary artery disease, sex, smoking (dichotomous variables); macrophage grade, CD163 grade, HO-1 grade, MPO grade, IL-10 grade, α actin grade, VCAM-1 grade, Fontaine class for ischemia (ordinal variables with values of 0, 1, 2, 3), neovessels density (CD34/CD31) IL-10 gene, MPO gene, HbA1C, glucose, cholesterol, triglycerides, high-density lipoprotein, low-density lipoprotein (continuous variables). SPSS 19.0 software was used for the analysis. Probability values <0.05 were considered significant.

Results
Demographic Profile
Demographic clinical profile shows a marginally decreased age of occurrence in DM Hp2-2 individuals compared with DM control (Hp1-1/Hp2-1). The parameters included in the demographic profile are shown in the table. Serological parameters and Fontaine class severity score for peripheral vascular disease were similar in both groups. Lipid lowering therapy was increased and insulin therapy was decreased in patients with Hp2-2 genotype. All other treatment history shows no significant differences between the groups.

Hp Genotype
The polymerase chain reaction products identified by gel electrophoresis indicate the Hp genotypes (Figure 1). A total of 40 samples were genotyped. Twelve samples had Hp2-2 genotype and 28 Hp1-1 or 2-1 genotype (control). Peripheral atherectomy samples obtained was relatively small (10 mg) in 4 Hp2-2 and 8 controls that made it difficult to perform
Hp Genotype and Diabetic Atherosclerosis

Table. Demographic Clinical Profiles

<table>
<thead>
<tr>
<th>Baseline Clinical Profile</th>
<th>DM Hp2-2 (n=7)</th>
<th>DM Control (n=14)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>63.5±1.6</td>
<td>69.2±2.2</td>
<td>0.05</td>
</tr>
<tr>
<td>HbA1C, %</td>
<td>8.9±1.2</td>
<td>7.3±0.4</td>
<td>0.13</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>180.4±15.5</td>
<td>178.5±16.2</td>
<td>0.94</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>151.8±14.1</td>
<td>148.2±39.9</td>
<td>0.84</td>
</tr>
<tr>
<td>TGL, mg/dL</td>
<td>226.2±57.2</td>
<td>153.2±27.6</td>
<td>0.21</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>66.8±9.0</td>
<td>80.6±8.3</td>
<td>0.28</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>31.8±3.1</td>
<td>38.3±2.5</td>
<td>0.13</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>75</td>
<td>69.2</td>
<td>0.16</td>
</tr>
<tr>
<td>CAD, %</td>
<td>71.4</td>
<td>42.9</td>
<td>0.22</td>
</tr>
<tr>
<td>Angina, %</td>
<td>28.6</td>
<td>14.3</td>
<td>0.43</td>
</tr>
<tr>
<td>Heart failure, %</td>
<td>43</td>
<td>50</td>
<td>0.76</td>
</tr>
<tr>
<td>Angiogram-stenosis, %</td>
<td>84.3±13.9</td>
<td>82.9±14.4</td>
<td>0.83</td>
</tr>
<tr>
<td>Fontaine class grade 1–3, %</td>
<td>14.3</td>
<td>37.5</td>
<td>0.19</td>
</tr>
<tr>
<td>Insulin treatment, %</td>
<td>14.3</td>
<td>64.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Lipid lowering treatment, %</td>
<td>100</td>
<td>50</td>
<td>0.02</td>
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<tr>
<td>β-blocker treatment, %</td>
<td>42.9</td>
<td>57.1</td>
<td>0.54</td>
</tr>
<tr>
<td>ACE-I/ARB treatment, %</td>
<td>57.1</td>
<td>71.4</td>
<td>0.51</td>
</tr>
</tbody>
</table>

DM indicates diabetes mellitus; Hp, haptoglobin; Hb, hemoglobin; CAD, coronary artery disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ACE, angiotensin-converting-enzyme; ARB, angiotensin receptor blockers; TGL, triglycerides.

Demographic clinical profiles show a significant marginally decreased age of occurrence in DM Hp2-2 individual when compared with DM control. All the other parameters show no significant difference between DM Hp2-2 and the DM control group.

Figure 1. Haptoglobin (Hp) genotyping: The polymerase chain reaction products obtained with 2 sets of primers A & B (Hp1) and C & D (Hp2) were identified by gel electrophoresis. Lane 1: Hp2-2, amplification product of 349 bp obtained only with C&D. Lane 2: Hp1-1, amplification product of 1757 bp was obtained only with A & B. Lane 3: Hp2-1, amplification products were obtained with both A & B and C & D. Lane M: molecular weight markers.

Discussion

IPH and Iron Content

Leakage or rupture of the fragile neovessels led to an increase in IPH in DM plaques with Hp2-2 compared with control (0.75±0.16 versus 0.13±0.07; P<0.005) (Figure 2). The rupture of plaque neovessels results in IPH and extravasation of erythrocytes that liberate free Hb. This free Hb may trigger oxidative stress and plaque progression. The Perl’s iron grade was significantly increased in DM peripheral plaques with Hp2-2 genotype compared with control plaques (0.78±0.3 versus 0.20±0.05; P<0.001) (Figure 2).

Hb Scavenger Receptor CD163 and HO-1 Enzyme

The protein expression of the macrophage Hb-binding receptor CD163 measured by immunohistochemistry was significantly decreased in Hp2-2 DM plaques compared with control (0.77±0.15 versus 1.50±0.11; P<0.002) (Figure 2). The downregulation of this key receptor may lead to impaired clearance of Hb-Hp2-2 complex and genotype-dependent plaque progression. HO-1 protein expression was higher in DM plaques with Hp2-2 genotype compared with control (1.94±0.14 versus 0.72±16; P<0.02) (Figure 2).

Oxidative MPO and Anti-Inflammatory IL-10

The oxidation specific marker MPO gene expression was significantly upregulated in DM Hp2-2 plaques when compared with the control groups (0.10±0.02 versus 0.06±0.01; P<0.05). The protein expression measured by immunohistochemistry was also found to be increased in DM Hp2-2 plaques (2.74±0.06 versus 1.33±0.21; P<0.0001) (Figure 3). This may be because of decreased Hb clearance and induction of oxidative stress. The gene expression of anti-inflammatory cytokine IL-10 was downregulated in DM plaques with Hp2-2 genotype (0.03±0.01 versus 0.06±0.01; P<0.04). The protein expression of IL-10 was also reduced in DM Hp2-2 (0.6±0.08 versus 2.09±0.18; P<0.0001) (Figure 3). These results demonstrate defective inflammation resolution in DM Hp2-2 plaques.

Inflammation and Neovascularization

The inflammation score was significantly increased in atherosclerotic plaques obtained from DM patients with Hp2-2 compared with control DM patients without the Hp2-2 genotype (2.5±0.15 versus 0.61±0.09; P<0.0001). In addition, the inflammatory marker VCAM-1 score (2.2±0.20 versus 1.08±0.19; P<0.001) was also increased in plaques derived from Hp2-2 genotype. Neovessels density quantified using CD34 and CD31 were increased in Hp2-2 plaques (56.1±6.21 versus 16.7±2.24; P<0.0001), and (75.25±3.74 versus 25.19±3.89; P<0.0001), respectively (Figure 4). Furthermore, the vascular smooth muscle actin score (2.20±0.20 versus 1.18±0.18; P<0.002) was increased in Hp2-2 genotype.

Discussion

IPH is evolving as a major pathway for atherosclerosis progression in patients with DM.36 The deleterious effects of free Hb are antagonized by Hp, internalized within macrophages by the CD163 scavenger receptor, and finally metabolized by the HO-1 enzyme. Although the Hp1-1 and Hp2-2 protein both bind to extracorporeal Hb with high affinity, several in vitro, transgenic animal, and human studies suggest that Hp1-1 is superior to the Hp2-2 protein in neutralizing free Hb (Figure 5).22-26 Human aortic plaques obtained at autopsy showed a significant downregulation of CD163 protein and mRNA expression in diabetic patients. However, no direct,
in vivo studies have tested this hypothesis prospectively in human atherosclerotic tissue.

The main findings of this study support the concept that the scavenger process of free heme in DM atherosclerosis may be genotype dependent. Specifically, plaques from patients with Hp2-2 genotype had increased IPH and iron deposition. In addition, the scavenger receptor, also known as Hb receptor-CD163 was reduced in Hp2-2 plaques. As a result, free Hb released at the time of IPH may not be efficiently cleared by Hp-CD163-HO-1 pathway in atherosclerotic plaques from DM patients with Hp2-2 genotype.

Previous studies in human atherosclerosis showed increased iron content in Hp2-2 plaques. Increased plaque iron may be associated with increased oxidative stress affecting the stability of carotid plaques. Consistent with these reports, increased plaque iron in this study was associated with increased oxidative and decreased anti-inflammatory pattern, as documented by elevated expression of MPO and a reduced expression of the anti-inflammatory IL-10.

At the cell membrane level, the receptor CD163 may be involved in inflammation resolution and plaque regression. CD163/HO-1 pathway mediates endocytosis of Hb, and final catabolism of free heme. The binding of CD163 to the Hb-Hp1-1 complex as compared with Hb-Hp2-2 results in the release of the anti-inflammatory cytokine IL-10. The downregulation of CD163 demonstrated in this study is indicative of decreased Hb clearance in human atheroma from Hp2-2 patients. Furthermore, previous studies documented a reduced expression of CD163 in peripheral blood monocytes from DM patients with Hp2-2 genotype. Intriguingly, CD163 was found to be significantly upregulated...
in macrophages in vivo under regression conditions only, suggesting an important role for this receptor in atherosclerotic plaque regression.

At the intracellular level, the HO-1 is a fundamental enzyme in the metabolism of free heme. Previous studies have shown significant correlation of HO-1 expression with features of plaque vulnerability in humans, such as macrophage infiltration, lipid accumulation, and matrix metalloproteinase-9, IL-8, and IL-6. The increase in HO-1 protein expression observed in the DM plaques with Hp2-2 genotype
may be related to increased oxidative injury as documented by increased MPO expression in these plaques. MPO has been associated with increased risk of coronary heart disease and dysfunctional high-density lipoprotein, also seen with Hp2-2 diabetic patients.

This study also documented significant downregulation of anti-inflammatory mediator IL-10 gene and protein, an important molecule in the resolution of inflammation. Lower serum IL-10 levels are linked to acute coronary syndromes increasing future atherothrombotic events. Decreased IL-10 was documented with simultaneous increases in macrophage infiltration, VCAM-1, and neovascularization in Hp2-2 plaques. These inflammatory and angiogenic effects may further exacerbate plaque hemorrhage, extracorpuscular Hb deposition, and iron content, contributing to accelerated atherosclerosis observed in DM patients. Hp2-2 genotype may also play a role in high-density lipoprotein function, reducing cholesterol efflux, as previously reported. Finally, the clinical relevance of Hp2-2 genotype in other vascular territories different from peripheral vascular disease is supported by increases in coronary calcification, myocardial infarction, congestive heart failure, and cardiac death. Our results extend these observations by suggesting a potential role for Hp2-2 genotype in the modulation of lower extremity atherosclerosis in the setting of DM. Aggressive anti-oxidative therapy may be considered as a valid option in the prevention of major events in patients with Hp2-2 genotype. However, further multicenter studies are needed to confirm this therapy.

In summary, these data suggest that impaired Hb clearance in Hp2-2 genotype is associated with increased oxidative, inflammatory, and angiogenic modifications, and a simultaneous decrease in anti-inflammatory response in DM patients with peripheral vascular disease. The key mediator CD163 may be at the center of this pathway. Targeting CD163 may have significant relevance for therapeutic modulation and resolution of inflammation in accelerated atherosclerosis observed in diabetic patients with Hp2-2 genotype.

Study Limitations
The sample size was smaller considering that the analysis was done only with diabetic atherectomy plaques with Hp2-2 and compared the control non-Hp2-2 (Hp1-1 and Hp2-2) diabetic samples. Only 1 plaque per individual patient was selected, avoiding multiple sampling clustering, and reducing selection bias. Furthermore, peripheral atherectomy samples were too small, and were used for analysis of immunohistochemistry, and only limited gene expression studies.

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Disclosures
None.

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Vascular cells, hemoglobin, heme, iron, and atherogenesis.


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Genotype-Dependent Impairment of Hemoglobin Clearance Increases Oxidative and Inflammatory Response in Human Diabetic Atherosclerosis

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