Downregulation of the Hemoglobin Scavenger Receptor in Individuals With Diabetes and the Hp 2-2 Genotype

Implications for the Response to Intraplaque Hemorrhage and Plaque Vulnerability


Abstract—In individuals with diabetes mellitus (DM), the haptoglobin (Hp) genotype is a major determinant of susceptibility to myocardial infarction. We have proposed that this is because of DM and Hp genotype–dependent differences in the response to intraplaque hemorrhage. The macrophage hemoglobin scavenging receptor CD163 plays an essential role in the clearance of hemoglobin released from lysed red blood cells after intraplaque hemorrhage. We sought to test the hypothesis that expression of CD163 is DM and Hp genotype–dependent. CD163 was quantified in plaques by immunohistochemistry, on peripheral blood monocytes (PBMs) by FACS, and as soluble CD163 (sCD163) in plasma by ELISA. In DM plaques, despite an increase in macrophage infiltration, CD163 immunoreactivity was lower, resulting in a dramatic reduction in the percentage of macrophages expressing CD163 (27±2% versus 70±2%, P=0.0001). In individuals with DM as compared with individuals without DM, the percentage of PBMs expressing CD163 was reduced (3.7±0.6% versus 7.1±0.9%, P<0.002) whereas soluble plasma CD163 was increased (2.6±1.1 μg/mL versus 1.6±0.8 μg/mL, P<0.0005). Among DM individuals, the Hp 2-2 genotype was associated with a decrease in the percentage of PBMs expressing CD163 (2.3±0.5% versus 5.6±1.3%, P=0.01) and an increase in plasma soluble CD163 (3.0±0.2 μg/mL versus 2.3±0.2 μg/mL, P=0.04). Taken together, these results demonstrate an impaired hemoglobin clearance capacity in Hp 2-2 DM individuals and may provide the key insight explaining the increased incidence of myocardial infarction in this population. (Circ Res. 2007;101:0-0.)

Key Words: diabetes mellitus ■ hemoglobin ■ macrophage ■ intraplaque hemorrhage ■ haptoglobin polymorphism

Diabetes mellitus (DM) atherosclerosis is characterized by increased neovascularization and blood vessel fragility resulting in increased microhemorrhages and extravasation of erythrocytes, and the consequent release of extravascular “free” hemoglobin (Hb) into the atherosclerotic plaque.1–6 Free Hb is highly toxic because of its ability to rapidly destroy nitric oxide by the dioxygenation reaction and to produce reactive oxygen species.7 The Hp protein represents the first line of defense against these toxic effects of free Hb.8 Hp binds to free Hb resulting in the formation of a Hp–Hb complex which may be rapidly cleared by the macrophage CD163 Hp–Hb scavenger receptor.9–11 Clearance of the Hp–Hb complex from the atherosclerotic plaque is mediated exclusively by CD163. In addition to its scavenger function, this receptor is now recognized as an immunomodulator of the atherosclerotic plaque, with pivotal antiinflammatory and antiatherogenic properties leading to the synthesis of interleukin-10, heme oxygenase, and bilirubin.1,12–16 The Hp locus is polymorphic with 2 common alleles denoted 1 and 2.8 The antioxidant function of the Hp protein is Hp genotype– and DM-dependent.17–19 The Hp 2 protein is an inferior antioxidant as compared with the Hp 1 protein with respect to its ability to block Hb-induced oxidative reactions.17,18 In addition, DM markedly reduces the antioxidant function of Hp after it is bound to Hb.19 Taken together, these 2 factors limiting the antioxidant function of Hp generate an increased urgency to clear the Hp 2-2–Hb complex as rapidly as possible in the DM state. Failure in this Hp–Hb scavenger pathway may increase plaque oxidative stress and plaque inflammation resulting in increased plaque vulnerability and acute coronary events. In fact, several recent longitudinal studies have demonstrated an increased
Incidence of myocardial infarction in Hp 2-2 individuals with DM but not in the absence of DM.\textsuperscript{20–22} Accordingly, the mechanism underlying the epidemiological interaction between the Hp 2-2 genotype and DM on the incidence of acute myocardial infarction may be related to a decreased expression of the macrophage Hp–Hb scavenger receptor CD163.

Materials and Methods

Assessment of CD163 Expression on Macrophages in the Atherosclerotic Plaque

Immunohistochemical assessment of macrophages and CD163 expression was performed in 50 formalin-fixed aortic plaques from 6 DM cadavers and compared with 50 formalin-fixed aortic plaques from 9 non-DM cadavers (University of Kentucky Medical Center, Lexington, Ky). Macrophages were identified with the mouse monoclonal CD68 antibody KP1 (DAKO) using an alkaline phosphatase-conjugated secondary antibody for antigen detection. The macrophage scavenger receptor CD163 was identified with the rabbit polyclonal CD163 antibody Ab-1 (Labvision), using an avidin-biotin complex (Vector labs) and 3,3′ Diaminobenzidine (DAB) for antigen detection. Simultaneous CD68 and CD163 staining allows for determination of the fraction of macrophages that express CD163. Double-staining for CD68 and CD163 in the same slide proved technically difficult. As a result, the fraction of macrophages expressing CD163 was assessed using sequential histological sections 4 μm apart. Colocalization analysis was performed using 20 high power fields (HPF) for CD68 with the corresponding 20 HPF for CD163 per plaque, randomly selected in the fibrous cap (10) and the lipid core (10), respectively. A total of 4000 HPF were quantified for analysis.

Quantitation of CD163 mRNA in Atherosclerotic Plaques

RNA was extracted from 24 formalin-fixed paraffin-embedded atherosclerotic plaques (12 diabetic and 12 nondiabetic). These plaques were selected by their immunohistochemistry profile and were considered representative of the 2 groups. Tissue sections (7 μm in thickness) were cut on slides and deparaffinized using xylene. The tissue sections were then scraped into tubes containing Proteinase K and incubated at 37°C for 16 to 20 hours. The total RNA was isolated from the cell extract using the Paradise kit (Arcturus Biosciences) and reverse transcribed to cDNA. The cDNA was then amplified by real-time polymerase chain reaction (PCR) with specific primers for CD163 and β-actin. Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using the threshold value as the point of quantitation for each product. Primer Express software was used to design the primer sequences for CD163: Fwd-5′-CCAGTCCCCAACACTGTCTT-3′; Rev-5′-TTCTGG- AATGGTAG GCCTTG-3′; and for β-actin: Fwd-5′-ATCCCCCA- AAGTCCACA ATG-3′; Rev-5′-GTGGCTTTTAGGATGGCA- AG-3′. The PCR reaction mixture was assembled in a total volume of 25 μL, comprised of SYBR green PCR master mix (Applied Biosystems), forward and reverse primers (final concentration 625 nmol/L), and 2 μL of the cDNA mixture. Gene expression of CD163 was quantified by normalization against the expression of the housekeeping gene β-actin.

Assessment of CD163 on Peripheral Blood Monocytes

CD163 expression on peripheral blood monocytes (PBMs) was performed using fluorescent activated cell sorting (FACS) as previously described.\textsuperscript{18} Blood was taken from DM and non-DM ambulatory individuals at the Rambam medical complex. CD163 analysis by FACS of a subset of these patients has previously been reported.\textsuperscript{18} Red cells were lysed using NH\textsubscript{4}Cl red cell lysis solution (Sigma). Monocytes were identified by FACS using a CD14-FITC–conjugated antibody, and CD163-positive cells were identified using a CD163-biotinylated antibody and a RPE-streptavidin–conjugated antibody. All antibodies were used at a 1:500 dilution, whereas the RPE-streptavidin was used at a 1:1000 dilution as obtained from the manufacturer (DAKO). Fixation was performed with 1% (final) paraformaldehyde. Results were expressed as the percentage of CD14\textsuperscript{+} cells which also expressed CD163.

Measurement of Soluble CD163

Soluble CD163 (sCD163) was measured in human plasma samples by a sandwich ELISA as previously described.\textsuperscript{23} Assessment of CD163 Regulation in Macrophages In Vitro by Glucose

To assess the effects of hyperglycemia on CD163 expression, human macrophage THP-1 cells were treated for 24 hours with dexamethasone\textsuperscript{18} and then cultured for 5 days in either normal RPMI (180 mg glucose/dL) or RPMI supplemented with glucose to a final glucose concentration of 540 mg glucose/dL. THP-1 CD163 expression was then assessed by Western blot analysis.\textsuperscript{18}

Hp Genotyping

Hp typing was performed by polycrylamide electrophoresis of Hb-enriched serum as previously described.\textsuperscript{8} A signature banding pattern is obtained in this methodology which is unique for each of the 3 possible Hp genotypes.\textsuperscript{8}

Statistical Analysis

All results are reported as the mean±SEM. Differences between groups were compared by t test with a probability value of less than 0.05 considered statistically significant.

Results

CD163 Expression Is Decreased in Diabetic Atherosclerotic Plaques

CD163- and CD68-positive cells were identified by immunohistochemistry in atherosclerotic plaques from DM and non-DM individuals (Figure 1). The total number of CD163-positive cells was significantly decreased in DM as compared with non-DM plaques (Figure 2a). However, the total number of CD68-positive cells in the plaque was significantly increased in DM as compared with non-DM plaques (Figure 2b). Accordingly, the percentage of CD68-positive cells expressing CD163 was dramatically lower in DM as compared with non-DM plaques (27±2% versus 70±2%, P<0.0001; Figure 2c).

To provide a more quantitative assessment of changes in CD163 expression in DM as compared with non-DM plaques, we assessed CD163 mRNA in these plaques by quantitative RT-PCR with normalization to β-actin. We found that expression of CD163 was decreased by over 75% in DM plaques (0.3078±0.055 versus 0.0689±0.017 in non-DM versus DM plaques, n=12 plaques for each group, P=0.002).

Expression of CD163 on Peripheral Blood Monocytes Is Reduced and Plasma Soluble CD163 Is Increased in Individuals With DM

The percentage of PBMs expressing CD163 was markedly decreased in individuals with DM as compared with individuals without DM (3.7±0.6 [n=56] versus 7.1±0.9 [n=55], P=0.002) as previously reported (see Figure 5 of reference 18). Plasma soluble CD163 was assessed in a subset of these individuals and was found to be significantly increased in
individuals with DM as compared with individuals without DM (2.6 ± 1.1 [n = 45] µg/mL versus 1.6 ± 0.8 µg/mL [n = 14], P = 0.0005). Baseline characteristics including age, gender, and comorbid conditions were similar between individuals with and without DM.

Macrophage CD163 Expression Is Decreased in Tissue Culture by Hyperglycemia
CD163 expression on human THP-1 macrophages was reduced when the cells were cultured with high (540 mg/dL) as compared with low glucose (180 mg/dL), as assessed by Western blot (Figure 3).

CD163 Expression on Peripheral Blood Monocytes Is Reduced and Plasma Soluble CD163 Is Increased in DM Individuals With the Hp 2-2 Genotype
The percentage of PBMs expressing CD163 was significantly decreased in DM individuals with the Hp 2-2 as compared with the Hp 1-1/Hp 2-1 genotypes (2.3 ± 0.5 versus 5.6 ± 1.3, P = 0.01; Table 1). Furthermore, plasma sCD163 was increased in DM individuals with the Hp 2-2 as compared with the Hp 1-1/Hp 2-1 genotypes (3.0 ± 0.2 µg/mL versus 2.3 ± 0.2, P = 0.04; Table 2). Baseline characteristics including age, gender, glycemic control, and comorbid conditions...
were similar between DM individuals with the Hp 2-2 and Hp 1-1/Hp 2-1 genotypes.

**Discussion**

In this study we have demonstrated that DM and the Hp 2-2 genotype are independently associated with a significant decrease in the expression of the Hp–Hb scavenging receptor CD163. This finding has fundamental implications for how individuals with the Hp 2-2 genotype and DM may respond to intraplaque hemorrhage and why these individuals appear to have an increased plaque vulnerability and myocardial infarction.1–6 Although the Hp 1-1 and Hp 2-2 protein both bind to extracorpuscular Hb with high affinity, the Hp 1-1 is superior to the Hp 2-2 protein in neutralizing the oxidative effects of free Hb.19 The differences in antioxidant protection between the 2 types of Hp are accentuated in the setting of DM.19 As a result, there is a tremendous urgency to clear the Hp 2-2–Hb complex as rapidly as possible in DM patients. However, cell expression of CD163, the only mechanism available to clear extravascular Hb–Hb complexes, is decreased in Hp 2-2 DM plaques and monocytes. This fundamental impairment in the ability to counteract the oxidative effects of extracorpuscular Hb may contribute to the increase in plaque vulnerability and myocardial infarction observed in Hp 2-2 DM patients.

![Figure 3](image120x200to480x667)

**Figure 3.** Decreased expression of CD163 on macrophages cultured with high glucose. Western blot analysis of THP-1 cells treated with (low glucose) 180 mg/dL vs (high glucose) 540 mg/dL of glucose for 5 days. M indicates prestained MW markers.

The decrease in cell-associated CD163 associated with DM appears to be attributable at least in part to increased shedding of CD163 from the monocyte/macrophage. DM was associated with a marked increase in soluble CD163. Soluble CD163 is generated by the proteolytic cleavage of the extracellular domain of transmembrane cell-associated CD163.25 Known activators of this protease include endotoxin via the toll-like receptor 4,26 protein kinase C,25 and oxidative stress.27,28 Both protein kinase C activity and oxidative stress are known to be elevated in DM.29 However, we have shown here that CD163 mRNA is decreased in DM plaques, and therefore DM also appears to be associated with a decrease in the synthesis of CD163. Several proinflammatory cytokines which are increased in DM have been demonstrated to decrease the production of CD163.30

The decrease in cell-associated CD163 expression in individuals with the Hp 2-2 genotype, which appears to be independent of DM, may be mediated by both an increase in CD163 shedding and a decrease in CD163 synthesis. The Hp 2-2 genotype, particularly in the DM state, is associated with an increase in oxidative stress, which would be expected to lead to an increase in shedding of CD163 in Hp 2-2 DM individuals.27,28 This mechanism is supported by the finding presented here of increased soluble CD163 found in the plasma of Hp 2-2 DM individuals. On the other hand, as demonstrated in vitro with purified Hp 1-1–Hb complexes, Hp–Hb appears to increase the synthesis of CD16331 possibly by an autocrine mechanism. We and others have recently demonstrated that binding of Hp 1-1–Hb, as compared with Hp 2-2–Hb, to the CD163 receptor results in the release of significantly more of the antiinflammatory interleukin-10,12,13 which is known to upregulate the CD163 receptor.30,32,33

One important limitation of the analysis of CD163 in the atherosclerotic plaques in this study was that we initially relied mostly on immunohistochemistry for this analysis, which is not a particularly quantitative method. Quantitative retrieval of proteins from formalin fixed tissue, particularly membrane proteins, has proven to be extremely problematic because of the extensive cross-linking induced by the formalin fixation.34,35 Nonetheless, techniques have been developed and successfully applied for the analysis of RNA in formalin fixed tissues and we successfully quantified CD-163 mRNA by RT-PCR in formalin fixed tissue, confirming our immunohistochemistry findings.

In conclusion, atherosclerotic plaques and blood monocytes from DM individuals with the Hp 2-2 genotype have

**TABLE 1. The Percentage of PBMs Expressing CD163 Is Decreased in Hp 2-2 DM Individuals**

<table>
<thead>
<tr>
<th></th>
<th>Hp 1-2/2-1 (n)</th>
<th>Hp 2-2 (n)</th>
<th><strong>P</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DM</strong></td>
<td>5.6±1.3% (22)</td>
<td>2.3±0.5% (25)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>No DM</strong></td>
<td>8.7±1.0% (29)</td>
<td>5.4±1.7% (21)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Results are provided as the mean±SEM of the percentage of CD14+ cells expressing CD163. n indicates No. of individuals for whom analysis was performed. Data from Hp 1-1 and Hp 2-1 individuals are pooled, as there were only 4 Hp 1-1 individuals in both the DM and non-DM cohorts. There was a significant reduction in the percentage of CD14+ cells expressing CD163 in Hp 2-2 individuals with DM.

**TABLE 2. sCD163 Is Increased in the Plasma of Hp 2-2 DM Individuals**

<table>
<thead>
<tr>
<th></th>
<th>Hp 1-2/2-1 (n)</th>
<th>Hp 2-2 (n)</th>
<th><strong>P</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DM</strong></td>
<td>2.3±0.2 (16)</td>
<td>3.0±0.2 (22)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>No DM</strong></td>
<td>1.4±0.1 (6)</td>
<td>1.7±0.4 (8)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Results are provided as the mean±SEM of concentration of plasma CD163 in µg/ml. n indicates No. of individuals for whom analysis was performed. Data from Hp 1-1 and Hp 2-2 individuals are pooled, as there was only 1 Hp 1-1 individual in both the DM and non-DM cohorts for whom this analysis was performed. There was a significant increase in the plasma CD163 in Hp 2-2 individuals with DM.
decreased expression of the macrophage receptor CD163. This observation provides further support for the important role of microvascular hemorrhage in diabetic atherosclerosis and thrombosis. Moreover, it suggests that strategies designed to enhance expression of CD163 may have a cardio-protective role.

Sources of Funding

This study was supported by grants from the Binational Science Foundation, Israel Science Foundation, D Cure Diabetes Care in Israel, and the Russell Berrie Foundation, the Kennedy Leigh Charitable Trust (to P.R.M.) and from the Cardiovascular Institute at the Mount Sinai Medical Center (to P.R.M.).

Disclosures

Dr Andrew Levy is on the scientific advisory board of Alteon Inc.

References

7. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of microvascular hemorrhage and thrombosis. Moreover, it suggests that strategies designed to enhance expression of CD163 may have a cardio-protective role.