Increased Expression of Oxidation-Specific Epitopes and Apoptosis Are Associated With Haptoglobin Genotype

Possible Implications for Plaque Progression in Human Atherosclerosis

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Objectives
The purpose of this study was to test the hypothesis that increased oxidative stress is associated with apoptosis in human plaques with the haptoglobin (Hp) 2-2 genotype.

Background
Intraplaque hemorrhage releases free hemoglobin (Hb). Impaired Hb clearance induces oxidative stress leading to plaque progression. The binding of Hp to Hb attenuates iron-induced oxidative reactions.

Methods
Twenty-six human aortic plaques were Hp genotyped. Hp2-2 plaques (n = 13) were compared with control (Hp1-1/2-1) (n = 13). The iron grade was measured by Perl's staining. Immunostaining was used to detect oxidation-specific epitopes (OSEs) reflecting oxidized phospholipids and malondialdehyde-like epitopes. The percentages of apoptotic cells and apoptotic morphological features were quantified. DNA fragmentation and active caspase-3 were measured by in situ end-labeling and immunohistochemistry, respectively.

Results
In Hp2-2 plaques, iron content was increased (1.22 ± 0.15 vs. 0.54 ± 0.08; p < 0.0001) along with expression of oxidized phospholipid– (78.9 ± 5.8 vs. 38.8 ± 3.8; p < 0.0001), and malondialdehyde-like OSEs (93.9 ± 7.9 vs. 54.7 ± 3.9; p < 0.0001). The total percentages of apoptotic cells (11.9 ± 0.44 vs. 3.5 ± 0.28; p < 0.0001), nuclear fragmentation (11.8 ± 0.50 vs. 3.3 ± 0.26; p < 0.0001), nuclear condensation (10.9 ± 0.58 vs. 3.4 ± 0.20; p < 0.0001), chromatin margination (14.2 ± 0.57 vs. 6.5 ± 0.37; p < 0.0001), cytoplasmic blebs (1.6 ± 0.28 vs. 0.8 ± 0.14; p < 0.002), and eosinophilia (10.8 ± 0.74 vs. 4.2 ± 0.27; p < 0.0001) were increased in Hp2-2 plaques. Furthermore, DNA fragmentation (119.9 ± 1.40 vs. 57.5 ± 0.80; p < 0.001), and active caspase-3 density (84.7 ± 7.62 vs. 50.6 ± 7.49; p < 0.004) were increased in Hp2-2 plaques. Logistic regression analysis identified correlation between the percentage of apoptotic cells and the density of OSEs (r = 0.56; p < 0.003).

Conclusions
These findings provide insights into genetic predisposition to oxidative stress and the relationship between OSEs and macrophage apoptosis that may explain advanced atherosclerosis in human Hp2-2 plaques. (J Am Coll Cardiol 2012;60:112–9) © 2012 by the American College of Cardiology Foundation

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Atherosclerotic plaque progression is associated with increased inflammation, neovascularization, and intraplaque hemorrhage (1–4). Extravasation of erythrocytes into the atherosclerotic plaque releases free hemoglobin (Hb) (5). Impaired Hb clearance leads to increased iron deposition and generation of reactive oxygen species. This may further promote oxidation of lipoproteins (6). As a result, the core of advanced atheromatous plaques is a pro-oxidant milieu, which may promote apoptosis. This pro-oxidant activity of free Hb is antagonized by haptoglobin (Hp) (7), a plasma protein that binds Hb with high affinity. The heme iron in Hb is a very potent oxidant, and the binding of Hp to Hb attenuates the iron-induced oxidative reactions and acts as an antioxidant by promoting clearance of Hb by the macrophage CD163 scavenger receptor (8).

In humans, there is genetic polymorphism at the Hp locus with 2 alleles: 1 and 2. Specifically, the protein product of the Hp2 allele is defective in its ability to block oxidative reactions mediated by iron derived from Hb (6). Impaired Hb clearance and increased iron deposition observed in Hp2–2 plaque may induce oxidative modifications of lipoproteins (7). A variety of oxidation-specific epitopes (OSEs) is generated during oxidative modification of plaque lipoproteins (9–11). These epitopes are not only expressed on oxidized lipoproteins but also on proteins in the extracellular matrix and apoptotic cells (12). Macrophages engulf oxidized low-density lipoprotein (LDL) and oxidized lipids and become foam cells. This leads to increased intracellular stress, evoking a proapoptotic phenomenon in advanced atherosclerotic lesions (13,14). Impairment in the clearance of apoptotic cells induces secondary necrosis, which aggravates plaque destabilization, resulting in atherothrombosis (15). In the present study, we quantified iron deposition, OSE, and morphological and histochemical features of apoptosis in human aortic atherosclerotic plaques stratified by Hp genotype. Specifically, this study was designed to test the hypothesis that human plaques from patients with the Hp2–2 genotype will have increased iron content, oxidation epitopes, and increased apoptosis compared with control human plaques without the Hp2–2 genotype.

**Methods**

**Tissue collection.** Full-thickness aortic wall histological sections from 26 aortas were taken sequentially at autopsy from 26 decedents. A 20-cm aortic segment extending from the lower thoracic aorta into the abdominal aorta above the renal arteries was selected for examination from each case. The aorta from each decedent was dissected, opened longitudinally, and grossly examined after the intima was washed with saline solution. Upon examination, the aortas had diffuse atherosclerotic lesions with variably distanced spaces between plaques. Individual atherosclerotic plaques that rose above the surface with a long axis >0.75 cm were studied (16). A 1.0-cm long × 0.5-cm wide sample with an edge of normal tissue was obtained for each plaque. All samples were obtained within 24 h of death, immediately fixed in 10% buffered formalin, and processed into paraffin blocks, thereby decreasing the likelihood of exposing these tissues to oxidation. Only 1 plaque was randomly selected without knowledge of the Hp genotype and included in the study. Quantification of apoptotic cell morphology was done by an individual totally blinded to the genotype. This study was approved by the investigational review board at Mount Sinai Medical Center, New York, New York.

**Hp genotype.** To identify the Hp genotype, DNA was extracted from liver tissue of the corresponding decedents and amplified by polymerase chain reaction using 2 sets of primers specifying Hp alleles Hp1 and Hp2 (GenBank accession number AC004682 and M69197). Oligonucleotide primers A (5′-GAGGGAGCTTCCTGATCATG-3′) and B (5′-GAGATTCTTGGCCCTGGCCTGTG-3′) were used for amplification of a 1,757-bp Hp1 allele–specific sequence and primers C (5′-CCTGGCTCCTTATTAACCTG-3′) and D (5′-CCGATGTTGCCACCCATAGCCATG-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 (6).

**Plaque characterization and iron grade.** Hematoxylin and eosin–stained plaques were characterized according to the American Heart Association classification (16). Perl’s iron staining was graded as follows: grade 0, absent; grade 1, mild to moderate intensity; and grade 2, severe intensity (17,18).

**Quantification of OSE.** Determination of the presence of OSE was performed by immunostaining using an IgM murine monoclonal EO6 antibody, which is specific for the phosphocholine head group of oxidized phospholipids (OxPLs), and the human Fab monoclonal IK17 antibody, which binds to a unique malondialdehyde (MDA) epitope present on both MDA-LDL and copper-oxidized LDL (19). For EO6 immunostaining, formalin–fixed paraffin sections (5 μm) were incubated overnight at 4°C with primary antibody (1:1,000) and detected using the LSAB2 System-HRP DAB kit (Dako, Carpinteria, California) according to the manufacturer’s protocol. Briefly, endogenous peroxidase activity was quenched by incubating the specimen for 5 min with 3% hydrogen peroxide. The specimen was then sequentially incubated with biotinylated antirabbit immunoglobulin, then peroxidase-labeled streptavidin, followed by incubation with the substrate chromogen 3,3′-diaminobenzidine resulting in a brown-colored precipitate at the antigen site. For IK17 immunostaining, sections were initially incubated with nonimmune
goat anti-human IgG (BA-3000, Vector Laboratories, Burlingame, California) at 1:100 dilution in 2% goat serum to bind to endogenous human immunoglobulins and reduce nonspecific background staining. Then, the IK17 antibody was diluted in 2% goat serum and incubated for 1 h at room temperature. Bound IK17 was then visualized by incubating with alkaline phosphatase–labeled goat anti-human \( \beta \)-H9260 chain secondary antibody (dilution 1:200) (A3813, Sigma Aldrich, St. Louis, Missouri) for 1 h at room temperature and visualized with Vector Red (SK-5100, Vector Laboratories). Individual cell numbers on immunostained slides were quantified with computerized planimetry under a 40× objective. Dako background-reducing buffer was used to reduce the excess background staining in the extracellular matrix. The density of OxPLs (EO6) and MDA-like (IK17) OSEs was calculated by dividing the total number of EO6/IK17-positive cells in the plaque by the total area (mm\(^2\)) counted in 20 high-power fields and expressed as density (i.e., number of positive cells per square millimeter).

**Percentage of apoptosis-positive cells.** The total number of apoptotic cells in plaques, including endothelium, smooth muscle cells, and macrophages, that exhibited all the nuclear and cytoplasmic features of apoptosis were randomly counted by light microscopy using 20 oil-immersion high-power fields.

**Morphological features of apoptosis.** Five morphological features of apoptosis were systematically recorded using hematoxylin and eosin staining as follows: 1) total nuclear fragmentation: discontinuity in nuclear chromatin with breakage in the nucleus, indicating DNA breakage; 2) nuclear condensation: nuclear chromatin with dense basophilic (intense blue) staining; 3) margination of chromatin: chromatin (basophilic) staining with peripheral margination or rimming of the chromatin in the nucleus; 4) cytoplasmic bleb: abnormal vacuolation of the cytoplasm exhibiting protrusions or blebs (bulging); and 5) cytoplasmic eosinophilia: dense eosinophilic (acidophilic; pinkish color) staining of cytoplasmic content, indicating an abnormal increase in protein content.

The total percentage of apoptotic cells and all 5 morphological features of apoptosis were reported as the percentage of the total cells counted in the entire field.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic Features of Aortic Atherosclerotic Plaque Donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hp2-2 Group (n = 13)</td>
</tr>
<tr>
<td>Age, yrs</td>
<td>65.7 ± 3.1</td>
</tr>
<tr>
<td>Female</td>
<td>38</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>47</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>50</td>
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<tr>
<td>Hypertension</td>
<td>43</td>
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</tbody>
</table>

Values are mean ± SD or %.

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**Figure 1** OSE OxPLs (EO6) and (MDA) IK17 Expression

(A and B) Immunostaining for the oxidation-specific epitope (OSE) with murine monoclonal antibody using diaminobenzene brown chromogen labeling showing increased expression of oxidized phospholipids (OxPLs) (EO6) in human aortic atherosclerotic plaque derived from the haptoglobin (Hp)2-2 genotype compared with control plaques (×20 magnification). (C) Bar graph showing the OxPL (EO6) density between the Hp2-2 and control groups. (D and E) Immunostaining for the malondialdehyde (MDA)-like OSEs using human IK17 Fab antibody with red chromogen labeling showing increased expression of MDA-like OSEs in human aortic atherosclerotic plaque derived from the Hp2-2 genotype compared with control plaques (×20 magnification). (F) Bar graph representing MDA-like OSE (IK17) density between the Hp2-2 genotype and control groups.
Terminal deoxyuride-5′-triphosphate biotin nick-end labeling assay. This assay was performed using an in situ apoptosis detection kit (VasoTACS, Trevigen, Inc., Gaithersburg, Maryland) per the manufacturer’s instructions. The in situ end-labeling density was calculated by manually counting the DNA fragments in 20 high-power fields and dividing by the total area counted.

Caspase-3 assay. Immunohistochemistry for active caspase-3 expression was performed using rabbit monoclonal IgG antibody (04-439, Millipore, Billerica, Massachusetts) with 3,3′-diaminobenzidine chromogen and Elite Vectastain kit (Vector Laboratories) using an appropriate secondary biotinylated antibody. The density of active caspase-3 was measured by manually counting the cells expressing active caspase-3 in the intima of plaque in 20 high-power fields and dividing by the total area counted in the plaque.

Immunofluorescence staining. This staining was performed using primary antibodies against the following antigens: active caspase-3 (ab-44976, 1:500 dilution, Abcam, Cambridge, Massachusetts), CD68 (M0814, 1:100 dilution, Dako), and α-smooth muscle actin (SMA) (FITC- F3777, 1:500 dilution, Sigma Aldrich). Additional control slides were routinely stained in parallel by substituting the primary antibody for IgG or specific IgG isotypes from the same species and at the same final concentration as the primary antibody. Secondary antibodies were donkey anti-mouse Alexa Fluor 488 or anti-rabbit Alexa Fluor 594 (A-21202 and A-21207, respectively, Invitrogen, Carlsbad, California) at 1:250 dilutions. Mounting medium containing 4,6-diamino-2-phenylindole (H-1200, Vector Laboratories) was then applied. Quantification of macrophages and smooth muscle cells expressing caspase-3 was performed in a blinded fashion. Three 40× magnification fields, randomly selected by the confocal Leica microscope, were quantified. Images were acquired using Leica TCS SP5 DMI, inverted confocal laser scanning microscope (Leica Microsystems Inc., Buffalo Grove, Illinois) of Mount Sinai’s Shared Resource Facility and analyzed using the Leica LAS AF lite software system.
**Statistical analysis.** Data are presented as mean ± SEM. Comparisons of means in 2 independent groups were performed by the 2-tailed Student *t* test. For nonparametric data, Mann-Whitney rank sum test was used. The categorical variables in Table 1 were compared using the chi-square test, except for age (numerical variable), which was compared using the 2-tailed Student *t* test. For nonparametric data, Mann-Whitney rank sum test was used. The categorical variables in Table 1 were compared using the chi-square test, except for age (numerical variable), which was compared using the 2-tailed Student *t* test. The variables analyzed included OSE (EO6, IK17), apoptosis morphology, in situ end-labeling density, and active caspase-3 density. To evaluate correlations between apoptosis and OSE, linear regression analysis was used. A *p* value <0.05 was used to imply statistical significance. Statistical analyses were performed using IBM SPSS/PASW Statistics 18 (SPSS Inc., Chicago, Illinois).

**Results**

**Demographic data.** Clinical characteristics including mean age, sex, presence of coronary artery disease, and the incidence of hypertension and diabetes mellitus are shown in Table 1. No significant differences in demographic parameters were present between groups (*p* = NS).

**Plaque area and iron grade.** Computerized planimetry showed no difference in plaque area in the Hp2-2 aortic plaques compared with control plaques (5.5 ± 0.49 cm² vs. 4.8 ± 0.41 cm²; *p* = 0.26). The Perl’s iron grade was significantly increased in the Hp2-2 aortic plaques compared with control plaques (1.22 ± 0.15 grade vs. 0.54 ± 0.08 grade; *p* = 0.0001).

**Expression of OSEs.** OxPLs and MDA-like OSEs were increased in Hp2-2 plaques compared with controls (OxPLs: 78.9 ± 5.8 vs. 38.8 ± 3.8; *p* = 0.0001; MDA-like OSEs: 93.9 ± 7.9 vs. 54.7 ± 3.9; *p* = 0.0001) (Figs. 1A to 1F).

**Morphological features of apoptosis.** The total percentage of apoptotic cells (11.9 ± 0.44 vs. 3.5 ± 0.28; *p* = 0.0001) and all 5 morphological features of apoptosis including total nuclear fragmentation (11.8 ± 0.5 vs. 3.3 ± 0.26; *p* = 0.0001), nuclear condensation (10.9 ± 0.58 vs. 3.4 ± 0.2; *p* = 0.0001), chromatin margination (14.2 ± 0.57 vs. 6.5 ± 0.37; *p* = 0.0001), cytoplasmic blebs (1.6 ± 0.28 vs. 0.8 ± 0.14; *p* = 0.002), and eosinophilia (10.8 ± 0.74 vs. 4.2 ± 0.27; *p* = 0.0001) were significantly increased in the plaques derived from the Hp2-2 genotype compared with control plaques (Fig. 2).

**DNA fragmentation and active caspase-3 expression.** DNA fragmentation by the terminal deoxyuride-5'- triphosphate biotin nick-end labeling assay was increased in Hp2-2 plaques (119.9 ± 1.4 vs. 57.5 ± 0.8; *p* = 0.001) (Figs. 3A to 3C). Active caspase-3 was also increased in Hp2-2 plaques when compared to controls (84.7 ± 7.6 vs. 50.6 ± 7.5; *p* = 0.004) (Figs. 3D to 3F). This was localized by immunofluorescence staining, revealing frequent caspase-3 expressing CD68⁺ macrophages in the intimal region, particularly at the fibrous cap (Figs. 4A and 4B). Quantification of macrophages expressing caspase-3 demonstrated an increase in the number of these cells in Hp2-2 plaques versus controls (73.1 ± 6% vs. 44.7 ± 7%; *p* = 0.0001). Furthermore, we found that caspase-3 was expressed by αSMA⁺ smooth muscle.
cells that were localized at the fibrous cap, tunica media, and especially in the few H9251 SMA/H11001 cells within the necrotic core. Quantitation demonstrated an increase in the number of H9251 SMA/H11001 cells expressing caspase-3 in Hp2-2 plaques versus controls (54.9 ± 5% vs. 37.1 ± 2%; p = 0.001). Otherwise, we identified only patchy staining for caspase-3 in the extracellular matrix region (data not shown). As a final analysis, binary logistic regression identified a direct correlation between the density of OSEs (both OxPLs and MDA-like OSEs combined) and the percentage of apoptotic cells (r = 0.56; p = 0.003) (Fig. 5).

**Discussion**

This study documented increased iron deposition and expression of OSEs of plaque lipoproteins in human abdominal aortic-atherosclerotic samples from patients with the Hp2-2 genotype. This increased expression of OSEs was associated with increased apoptosis within these Hp2-2 atherosclerotic samples. Importantly, characterization of apoptosis was systematically documented by a panel of criteria involving cell morphology and both light microscopy and immunohistochemistry features. This increase in oxidative modifications and apoptosis may help to explain...
clinical and epidemiological observations suggesting increased cardiovascular events in patients with the Hp2-2 genotype (20,21).

Several groups have documented increased oxidative stress and inflammation in plaques with intraplaque hemorrhage (16,22,23). The free Hb released from extravascular erythrocytes is highly toxic due to the presence of heme iron, a very potent oxidant. Free heme rapidly intercalates with membranes and causes severe damage. Furthermore, heme-derived iron participates in the Fenton reaction and increases the generation of highly labile reactive oxygen free radicals. The induction of oxidative stress catalyzes the peroxidation of protein and lipids (6,24–26), triggering the recruitment of inflammatory cells (macrophages and T cells). These deleterious effects of free Hb are attenuated by Hp, which binds with high affinity to Hb and acts as an antioxidant by promoting clearance of Hb by the macrophage CD163 scavenger receptor (5).

Our finding of increased iron content in Hp2-2 plaques is consistent with previously reported data showing that Hb-derived iron is redox active, suggesting that plaque iron may induce oxidative stress and lipoprotein modifications (17). Oxidation of LDL has been implicated as a crucial factor in the initiation and progression of atherosclerosis. In this study, we observed increased expression of OxPLs and MDA-like OSEs (9,10,27,28). These epitopes are generated during oxidative modification of plaque lipoproteins, typically in proportion to plaque burden (29,30). These epitopes are also expressed by apoptotic cells (31,32), serving as ligands that trigger recognition and phagocytosis by macrophages. Thus, the increased expression of OSEs in Hp2-2 genotype plaques, as observed in the present study, may be due to increased oxidation of lipoproteins as well as increased expression by apoptotic cells.

Consistent with these findings documenting a relationship between apoptosis and OSEs, it was recently documented that OxPLs, oxidized LDLs, saturated fatty acids, and lipoprotein(a), a carrier of proinflammatory OxPLs (33–35), trigger apoptosis in endoplasmic reticulum-stressed macrophages through a mechanism requiring both CD36 and Toll-like receptor 2 (14). Macrophage apoptosis and necrosis are key mechanisms in the transition of quiescent plaques to unstable plaques. OSEs generated in the vessel wall and, in this case, potentiated by Hp2-2 genotypes, may accelerate macrophage leading to plaque progression. These findings provide insights into the genetic predisposition to oxidative stress and the relationship between OSEs and macrophage apoptosis, which may explain advanced atherosclerosis.

In addition, the Hp2-2 allele is a functionally inferior protein in preventing Hb-induced oxidative stress (36) and formation of reactive oxygen species. As an immunomodulator, the Hp1-1–Hb complex stimulates macrophages to secrete anti-inflammatory cytokines to a markedly greater degree than the Hp2-2–Hb complex (37,38), indicating that Hp2 is a susceptible gene for cardiovascular disease. This may further potentiate the clinical severity in Hp2-2 individuals. Multiple independent longitudinal and cross-sectional studies from diverse ethnic groups and geographic areas have demonstrated that the Hp2-2 genotype is associated with an increased risk of atherosclerotic cardiovascular disease (39,40). As a result, the Hp genotype specifies the nature and intensity of the macrophage response to intraplaque hemorrhage and thereby serves as a determinant of susceptibility to plaque rupture and cardiovascular disease.

Study limitations. The number of cases in our study was limited. Human atherosclerotic plaque samples were obtained from post-mortem derived aorta specimen. Access to fresh tissues for analyses such as quantitation of protein or RNA levels was not possible. Treatment history was not available since the source of the specimen was from the autopsy division and many decedents had investigations/treatments at outside hospitals.

Conclusions
Using human atherosclerotic plaque samples, we identified that plaque iron deposition, oxidation, and apoptosis may be genotype dependent. The further characterization of genotype-related atherosclerosis may be instrumental in the development of strategies for preventive measures against cardiovascular diseases.

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REFERENCES


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