Haptoglobin Genotype Is a Determinant of Iron, Lipid Peroxidation, and Macrophage Accumulation in the Atherosclerotic Plaque

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Objective—Intraplaque hemorrhage increases the risk of plaque rupture and thrombosis. The release of hemoglobin (Hb) from extravasated erythrocytes at the site of hemorrhage leads to iron deposition, which may increase oxidation and inflammation in the atherosclerotic plaque. The haptoglobin (Hp) protein is critical for protection against Hb-induced injury. Two common alleles exist at the Hp locus and the Hp 2 allele has been associated with increased risk of myocardial infarction. We have demonstrated decreased anti-oxidative and anti-inflammatory activity for the Hp 2 protein. We tested the hypothesis that the Hp 2-2 genotype is associated with increased oxidative and macrophage accumulation in atherosclerotic plaques.

Methods and Results—The murine Hp gene is a type 1 Hp allele. We created a murine type 2 Hp allele and targeted its insertion to the Hp locus by homologous recombination. Atherosclerotic plaques from C57Bl/6 ApoE−/− Hp 2-2 mice were associated with increased iron (P=0.008), lipid peroxidation (4-hydroxynonenal and ceroid) and macrophage accumulation (P=0.03) as compared with plaques from C57Bl/6 ApoE−/− Hp 1-1 mice.

Conclusions—Increased iron, lipid peroxidation and macrophage accumulation in ApoE−/− Hp 2-2 plaques suggests that the Hp genotype plays a critical role in the oxidative and inflammatory response to intraplaque hemorrhage. (Arterioscler Thromb Vasc Biol. 2007;27:134-140.)

Key Words: atherosclerotic plaque • hemoglobin • inflammation • iron • macrophages

The major cause of acute coronary thrombosis is athero- sclerotic plaque rupture and the precursor lesion has been termed the high-risk plaque.1–6 Pathological features of high-risk plaques include a large lipid necrotic core, thin fibrous cap, inflammatory infiltrate, and intraplaque hemorrhage.1–6 Extracorpuscular hemoglobin (Hb) released from red blood cells after in vitro-plaque hemorrhage represents a potent stimulant to inflammation within the plaque. It is becoming apparent that the frequency of microvascular hemorrhages has been severely underestimated and may occur in up to 40% of all advanced atherosclerotic plaques.7

An important defense mechanism to counteract the effects of in vitro-plaque hemorrhage is mediated by haptoglobin (Hp), an abundant serum protein whose primary function is to bind to extracorpuscular Hb, thereby attenuating its oxidative and inflammatory potential.8 Hp also promotes the clearance of extracorpuscular Hb via the CD163 scavenger receptor present on macrophages.9 This scavenging pathway is the only mechanism that exists for removing free Hb released at extravascular sites, ie, at sites of hemorrhage within the atherosclerotic plaque.

In humans there exist 2 classes of alleles for Hp, designated 1 and 2. The Hp polymorphism is a common polymorphism. In the western world, 16% of the population is Hp 1-1 (homozygous for the Hp 1 allele), 36% is Hp 2-2 (homozygous for the Hp 2 allele), and 48% is Hp 2-1 (heterozygote).8 The Hp 2 allele is found only in humans. All other mammals, including higher primates have only the Hp 1 allele and therefore have the Hp 1-1 genotype. The Hp 2 allele appears to have been generated by an intragenic duplication event of exons 3 and 4 of the Hp 1 allele 100 000 years ago early in human evolution.8

We and others have demonstrated in multiple independent longitudinal and cross-sectional studies from diverse ethnic groups and geographic areas that the Hp 2-2 genotype is associated with an increased risk of atherosclerotic cardiovascular disease and its sequelae such as acute myocardial infarction.10–13 We have recently described in vitro funda-
mental differences in the antioxidant and immunomodulatory properties of the Hp 1-1 and Hp 2-2 proteins that may explain why Hp is a susceptibility gene for cardiovascular disease (CVD). As an antioxidant the Hp 1-1 protein is superior to the Hp 2-2 protein in blocking the oxidative action of Hb. As an immunomodulator, the Hp 1-1–Hb complex stimulates the macrophage to secrete anti-inflammatory cytokines to a markedly greater degree than the Hp 2-2–Hb complex.

Based on these in vitro studies we have proposed that 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. To test this hypothesis we have assessed a variety of oxidative and inflammatory parameters in the atherosclerotic plaques of mice genetically modified at the Hp locus.

**Methods**

**Construction of a Murine Hp 2 Allele**

The rationale and cloning strategy for producing a murine Hp 2 allele and targeting its insertion by homologous recombination are provided in an online supplement. The genomic organization of the human Hp locus is shown in Figure 1A. Figure 1B provides a map of the murine Hp locus before and after gene targeting.

**Care of Mice and Harvesting of Tissues**

These studies were approved by the Animal Care Committee of the Technion. Mice were fed a normal diet and euthanized at 9 months. Total serum cholesterol (Roche), triglycerides (Roche), and high-density lipoprotein (Biosystems, Barcelona) were measured enzymatically. Serum Hp was measured based on the acid stable peroxidase activity of the Hp–Hb complex (Tridelta, Bray, UK).

The aortic arch was fixed in 4% formaldehyde, embedded in paraffin, and sectioned using a Leica RM 2155 microtome. Total plaque area, lipid area, and minimum cap thickness were quantified as previously described.

**Iron Deposition**

Iron deposition in the plaque was identified using Perl’s stain and quantified by measuring the percentage of plaque area staining black.

**Lipid Peroxidation**

Lipid peroxidation was evaluated using 4-hydroxynonenal (4-HNE) and ceroid as described in an online supplement.

**Macrophage Accumulation**

Immunohistochemical localization of macrophages was performed as described in an online supplement.

**Statistical Analysis**

All results, with the exception of total plaque and lipid core area, are reported as the mean ± SEM with differences between groups determined by a 2-tailed t test. Data for total plaque and lipid core area are reported as the 25th/50th/75th percentile with differences between groups determined by the Mann-Whitney test. A value of P ≤ 0.05 was considered significant.

**Results**

**Generation of a Murine Hp 2 Allele**

In an online supplement we have described the strategy used to create a murine Hp 2 allele. The murine Hp 2 allele was engineered to have an intragenic duplication of exons 3 and 4, analogous to that found in the human Hp 2 allele (Figure 1A and 1B). Once generated, we used the murine Hp 2 allele to replace the normal mouse Hp 1 allele by homologous recombination.

**The Shape and Size of the Murine Hp 2 Allele Protein Product Is Similar to the Human Hp 2 Allele Protein Product**

Figure 2A shows schematically the difference as visualized by electron microscopy between the shape and size of Hp polymers found in humans with the Hp 1-1, 2-1, or 2-2 genotypes. Hp is synthesized as a single polypeptide that is proteolytically cleaved to give an α-chain (9 or 16 Kd derived from exons 1 to 4 or 1 to 6 for the 1 or 2 allele, respectively) and a beta chain (45 Kd derived from exon 5 or exon 7 for the 1 or 2 allele, respectively). The Hp α-beta monomer is covalently linked via disulfide bonds with other Hp monomers in an Hp genotype-dependent fashion. This is because the cysteine residues responsible for Hp polymerization are present in the region of the Hp gene duplicated in the Hp 2 allele. An Hp monomer derived from the Hp 1 allele can be cross-linked with only one Hp monomer (it is monovalent) to form an Hp dimer. However, the Hp monomer derived from the Hp 2 allele is cross-linked with 2 Hp monomers (it is bivalent). In individuals with only the Hp 2 protein, the plasma Hp molecules are all cyclic polymers. In heterozygotes, Hp polymers are dimers, trimers, and quaterners that are linear. These different polymeric structures can be easily visualized by taking advantage of the interaction of Hp with Hb and the peroxidase activity of Hb and Hb–Hp complexes.

Electrophoresis on a nondenaturing polyacrylamide gel of Hb-enriched serum followed by immobilization of the gel in 3,3′,5,5′-tetramethylbenzidine (forming a precipitate in the gel at the site of peroxidase activity) produces a signature banding pattern characteristic for each Hp genotype. In such gels, a single rapidly migrating band is seen in serum derived from Hp 1-1 individuals, corresponding to the Hp dimer, whereas more slowly migrating bands are seen in Hp 2-1 or Hp 2-2 individuals corresponding to the higher order linear and cyclic polymers present in these individuals (Figure 2B). The cysteine residues of murine and human Hp are 100% conserved, and therefore the gene duplication event, which we have introduced in the murine Hp allele, would be predicted to result in a similar polymerization profile as the human Hp 2 allele. As demonstrated in Figure 2B, the banding pattern in a nondenaturing polyacrylamide gel of Hb-enriched serum from mice with the Hp 2 allele is remarkably similar to humans with the Hp 2 allele demonstrating that the gene duplication we have produced in the murine Hp 2 allele produces higher-order Hp polymers similar to those seen in humans with the Hp 2 allele (Figure 2B). Furthermore, the serum concentration of Hp protein was similar in mice with Hp 1-1 and Hp 2-2 genotypes (0.92 ± 0.45 versus 1.10 ± 0.37, P = 0.66) and was similar to the Hp concentration reported for human serum.

**Morphometric Measurements of the Atherosclerotic Plaques**

We characterized 18 plaques from 9 C57Bl6/6J ApoE−/− Hp1-1 mice and 15 plaques from 6 C57Bl6/6J ApoE−/− Hp2-2 mice. There was no significant difference between the Hp 1-1
Figure 1. Construction of a murine Hp 2 allele. A, Genomic organization of the Hp locus. The human Hp 1 and Hp 2 alleles are located at chromosomal coordinates 16q22. The murine wild type Hp is a Hp 1 allele and is found on chromosome 8. A murine Hp 2 allele was created as described in this manuscript and inserted by homologous recombination at the wild type Hp locus replacing the murine Hp 1 allele. In the human Hp 2 allele, exons 5 and 6 represent a duplication of exons 3 and 4. The mouse Hp 1 allele has the identical intron–exon boundaries as the human Hp 1 allele and is 90% homologous at the amino acid level. The murine Hp 2 allele, constructed as described in the text, is similar to the human Hp 2 allele in that it has a direct repeat of exons 3 and 4. The exonic organization of the human and murine Hp 2 alleles are identical after RNA splicing has occurred. B, Fine map of the murine Hp locus before and after gene targeting. Top, Genomic organization of the murine Hp 1 allele. B, Bam H1; Bg, Bgl II; E, EcoR1; P, Pvu II. Middle, Genomic organization of the murine Hp 2 allele after successful gene targeting by homologous recombination. A targeting vector was constructed using the pTKLNCL GB 135 vector as a backbone. TKLNCL contains lox P sites (large arrow) bracketing the gene for cytosine deaminase (CD) and the neomycin (Neo) resistance gene. A 5.8-kb E-P fragment of the murine Hp 1 allele was cloned in the KpnI-XhoI site of TKLNCL 5/H11032 to the neo cassette (5/H11032 homology region) and a 3.4 kb BglII fragment of the murine Hp 1 allele was cloned in the Bam H1 site of TKLNCL 3/H11032 to the neo cassette (3/H11032 homology region). Exon 3 of the murine Hp 1 was reconstructed to be exon 343 as described in Methods. The vector was linearized with NotI before transfection. Identification of G418 resistant ES clones that integrated the targeting vector at the Hp locus by homologous recombination was achieved by Southern blot analysis of Bam H1 digested DNA from these clones using a 500-bp BamH1-BglII fragment (in blue) as probe. This probe hybridizes with a 5.8 kb Bam H1 fragment in wild type DNA (Hp 1) and with a 11 kb Bam H1 fragment in successfully targeted clones (Hp 2) (shown in Figure I of online supplement). Bottom, Genomic organization of the murine Hp 2 allele after removal of the Neo and CD cassettes with cre recombinase.
and Hp 2-2 mice with regard to age, weight, total serum cholesterol (432 ± 67 mg/dL versus 353 ± 45 mg/dL, \( P = 0.34 \)), triglycerides (143 ± 20 mg/dL versus 101 ± 12 mg/dL, \( P = 0.15 \)), or high-density lipoprotein cholesterol (22.3 ± 4.6 mg/dL versus 21.5 ± 4.4 mg/dL, \( P = 0.83 \)). Fibrous cap thickness, plaque area, and lipid core area in Hp 1-1 and Hp 2-2 mice are presented in the Table. There was no significant difference in plaque or lipid core area between Hp 1-1 and Hp 2-2 mice. There was a nonsignificant trend showing decreased cap thickness in plaques from Hp 2-2 mice.

**Increased Iron Deposition in Hp 2-2 Plaques**

Our previous in vitro studies have suggested that hemoglobin released from microvascular hemorrhages within the plaque would be cleared more slowly in Hp 2-2 as compared with Hp 1-1 plaques.\(^{16}\) Consistent with this hypothesis, we found significantly increased iron staining, calculated as the percentage of the total plaque area, in Hp 2-2 plaques as compared with Hp 1-1 plaques (2.18 ± 0.26% versus 0.94 ± 0.25%, \( n = 10, P = 0.008 \)) (Figure 3).

**Morphometric Properties of Plaques in Hp 1-1 and Hp 2-2 Mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>( n )</th>
<th>Cap Thickness (μm)</th>
<th>Plaque Area (μm²)</th>
<th>Lipid Core (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoE(^{-/-}) Hp 1-1</td>
<td>18</td>
<td>19.1 ± 2.2</td>
<td>0.019/0.033/0.144</td>
<td>0.006/0.017/0.035</td>
</tr>
<tr>
<td>apoE(^{-/-}) Hp 2-2</td>
<td>15</td>
<td>15.0 ± 1.7</td>
<td>0.027/0.051/0.084</td>
<td>0.008/0.022/0.035</td>
</tr>
</tbody>
</table>

\( n \) indicates total number of plaques analyzed. For cap thickness, the mean ± SEM is shown. For plaque area and lipid core area the quartile values (25th/50th/75th percentiles) are shown. There was no significant difference in cap thickness (\( P = 0.29 \)), plaque area (\( P = 0.76 \)), or lipid core area (\( P = 0.73 \)) between Hp 1-1 and Hp 2-2 mice.
Increased Lipid Peroxidation in Hp 2-2 Plaques
We assessed plaques for 4-HNE, a major end-product of lipid peroxidation, and ceroid, a mixture of autofluorescent oxidized lipid and protein. We found markedly greater 4-HNE (Figure 4A) and ceroid (autofluorescence) (Figure 4B) in the plaques of Hp 2-2 as compared with Hp 1-1 mice.

Increased Macrophage Accumulation in Hp 2-2 Plaques
We found that in the intima and adventitia of atherosclerotic plaques from Hp 2-2 mice there were significantly more macrophages as compared with plaques from Hp 1-1 mice.

Correlation Between Lipid Core Size and Inflammation in Hp 2-2 Plaques but not in Hp 1-1 Plaques
Oxidized lipid within the core of the plaque may act as an inflammatory stimulus. We were intrigued that although there was no significant difference in the lipid core area between Hp 1-1 and Hp 2-2 mice, macrophage accumulation in the Hp 2-2 plaques was significantly greater. We therefore examined the correlation between the lipid area and macrophage accumulation. We found a significant correlation between the size of the lipid core and the number of intimal macrophages in plaques from Hp 2-2 mice (correlation coefficient $r=0.57$, $P=0.01$), whereas finding no correlation between the size of the lipid core and the number of macrophages in plaques from Hp 1-1 mice (correlation coefficient $r=0.08$, $P=0.38$) (Figure 5D).

Discussion
In this study we have provided direct evidence that the Hp genotype contributes to the modulation of the number of macrophages in the atherosclerotic plaque. We have demonstrated that there is significantly greater macrophage accumulation in the intima and adventitia of atherosclerotic plaques of Hp 2-2 as compared with Hp 1-1 mice. We have suggested that this increase in macrophage accumulation in Hp 2-2 plaques may be caused by an increase in intraplaque iron and lipid peroxidation. These data provide a framework linking intraplaque microvascular hemorrhage, the size of the necrotic lipid core, and inflammation in determining plaque vulnerability.

Our prior in vitro studies demonstrating significant differences in the anti-oxidant and anti-inflammatory properties of...
Haptoglobin and Macrophages in Plaques

Figure 5. Increased macrophage accumulation in the plaques of Hp 2-2 mice. Macrophages were identified immunohistochemically as described in methods. Shown in (A) and (B) are representative plaques of similar size but with dramatically greater macrophage accumulation in Hp 2-2 Apo E−/− (A) as compared with Hp 1-1 ApoE−/− (B) mice. C, Histogram of the mean ± SEM of the number of macrophages in the intima and adventitia from all plaques (n = 18 for Hp 1-1 and n = 15 for Hp 2-2). There were significantly more macrophages in the intima (P = 0.03) and adventitia (P = 0.03) of plaques from Hp 2-2 as compared with Hp 1-1 mice. D, Plot of the number of intimal macrophages versus the lipid core area (μm²) in plaques from Hp 1-1 ApoE−/− (n = 18) and Hp 2-2 ApoE−/− (n = 15) mice. There was a statistically significant correlation between the number of macrophages and the lipid core area in plaques from Hp 2-2 mice (correlation coefficient = 0.57, P = 0.01) but not in Hp 1-1 mice (correlation coefficient = 0.08, P = 0.38).

the Hp 1 and Hp 2 allele gene products, provide a mechanistic basis to explain the in vivo observations we have presented here. We have demonstrated in vitro, in cell culture and in transgenic mice that the Hp 1 protein is a superior antioxidant to the Hp 2 protein.14–16 In vitro, we have demonstrated that Hp 2-2-Hb complexes stimulate markedly increased oxidation of low-density lipoprotein (LDL) as compared with Hp 1-1-Hb complexes.14,16 In vivo, we have demonstrated an increase in a panel of oxidation products of arachidonic acid (HETEs) in the myocardium of Hp 2 mice subjected to ischemia-reperfusion injury as measured by ionization tandem mass spectrometry.27 The increased oxidative stress found in Hp 2 mice is attributable not only to a decreased ability of the Hp 2 protein to prevent the mobilization of redox active iron from Hb but also to a decreased ability of the Hp 2 protein to promote the clearance of the redox active Hp 2-2-Hb complex.14–16 Therefore, intra-plaque hemorrhage generates greater iron deposition in mice with the Hp 2-2 genotype, leading to increased oxidation of lipids and other cellular constituents of the plaque. Notably, iron and ceroid have been reported to be colocalized in human atherosclerotic specimens.28

Why is there no difference in the size of the lipid core between Hp 1-1 and Hp 2-2 mice yet there are more macrophages in Hp 2-2 plaques? Cholesterol per se in the lipid core is not inflammatory. The binding of native LDL to the LDL receptor does not stimulate the production of inflammatory cytokines nor promote macrophage infiltration. Oxidized LDL can bind the macrophage scavenger receptor CD36, whose activation results in the release of pro-inflammatory cytokines.26 We suggest that the presence or lack of a correlation between macrophage accumulation and the size of the lipid core in Hp 2-2 and Hp 1-1 mice, respectively, is because of differences in the amount of lipid peroxidation of the core lipids in the plaques of these mice.

An additional explanation for decreased macrophage accumulation in Hp 1-1 plaques may be caused by the ability of the Hp 1-1-Hb complex to stimulate the production of the anti-inflammatory anti-oxidative cytokine IL-10 by macrophages via the CD163 receptor.17–19 IL-10 has been shown to play an important role in reducing inflammatory cell infiltration in atherosclerotic plaques and in modulating plaque progression.29–31 In addition to IL-10, Hp 1-1-Hb has also been shown to stimulate heme oxygenase,17 which also has very potent anti-inflammatory and anti-oxidative activity. However, the Hp 2-2-Hb complex is a very poor ligand for the anti-inflammatory signals generated by CD163 stimulation.19

These findings may have significant relevance for the accelerated atherosclerosis and increased incidence of plaque rupture observed in diabetes,32 which has been associated with increased intraplaque oxidative stress and inflammation.26,33 The hypothesis we have put forth here emphasizing the importance of oxidative stress in the development of plaque instability would appear to be at odds with multiple recent studies showing a clear lack of benefit of antioxidant therapy in preventing cardiovascular disease. However, we have recently demonstrated in a retrospective analysis of the HOPE study that antioxidant therapy provided a significant benefit in preventing death and myocardial infarction in Hp 2-2 diabetic individuals.34 The transgenic model described here, showing Hp genotype dependent differences in plaque macrophage accumulation and oxidation may provide the platform on which this hypothesis can be tested.

Acknowledgments

Dr Andrew Levy dedicates this manuscript to the memory of his sister Dr Joanne Levy, a caring physician and brilliant scientist. Joanne was responsible for developing the strategy for generating the murine Hp 2 allele used in this study. The assistance of Dr Jan Breslow in performing the blastocyst injections is appreciated.

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Methods.

Construction of a murine Hp 2 allele.

One approach to model the Hp polymorphism in mice is to introduce the human Hp 2 allele as a transgene. Human Hp 2 transgenic mice in a Hp knockout background have been used to study mice expressing only the Hp 2 allelic protein product. However, these human Hp 2 allele transgenic mice have several serious shortcomings: (1) insertion of the human Hp 2 allele into the genome is random, i.e., not in the normal location of the murine Hp gene on chromosome 8. Therefore, the cell specific and inducible regulation of the human Hp 2 transgene is different from that of the endogenous murine Hp gene on chromosome 8; (2) it is difficult to study the heterozygote (Hp 2-1) and to differentiate between Hp 2 homozygote and Hp 2 hemizygote mice; (3) it is extremely cumbersome to backcross these mice with other transgenic mice in order to look at the interaction between the Hp genotype and other genes (i.e. Apo E) due to the need to select at three genetic loci; (4) the circulating levels of the protein product from the human Hp 2 allele are different from the levels of the wild type murine Hp 1 allele. This directly affects the polymeric distribution of the circulating Hp polymers found in the serum of these mice. We sought to overcome all of these problems by producing a transgenic mouse with a genetically engineered murine (as opposed to human) Hp 2 allele as described below.

The human genomic locus as well as cDNAs encoding the Hp gene, both for the Hp 1 and Hp 2 alleles have been cloned and sequenced. The Hp 1 allele contains 5 exons and 4 introns. The Hp 2 allele contains 7 exons and 6 introns (Figure 1a of manuscript). The only difference between the two alleles is that the third and fourth exons of the Hp 1 allele have been duplicated in Hp 2 to give rise to exons 5 and 6 as well. Exon 5 in Hp 1 allele and exon 7 in the Hp 2 allele are identical. The reading frame of the duplicated region (exon 3 and 4) is maintained so the primary amino acid sequence produced by this duplicated region is a direct repeat of exons 3 and 4. Furthermore the translated in-frame amino acid sequence of exon 7 is the same as exon 5.

The genomic and cDNA sequence of mouse Hp is known (accession # M96827 C57BL/6J f). The genomic structure of wild type murine Hp is remarkably similar to that of the human Hp 1 allele (Figure 1a of manuscript). There exist 5
exons and 4 introns in murine Hp. The nucleotide sequences at the intron-exon boundaries in mouse Hp and the human Hp 1 allele are 100% conserved. The overall amino acid homology between the murine and human Hp 1 alleles is over 80%. Because the nucleotide sequence at the intron-exon boundaries of the murine Hp 1 allele are conserved, it was possible to create a murine Hp 2 allele by duplicating murine exons 3 and 4. This duplication does not change the reading frame of sequences that come 3′ to the duplicated region allowing the sequence of the final exon (exon 7) to be read in frame unchanged from what occurs in the murine Hp 1 allele.

Genomic mouse Hp DNA from the strain 129Sv obtained from a 129SvJ genomic library was kindly provided by Dr Sai-Kiang Lim and Dr Heinz Baumann. Our strategy to create a duplication of murine exons 3 and 4 was to modify murine exon 3 to become exon 343. In this strategy in the genomic murine Hp 2 allele there is no intron between the extra copy of exon 4 and the extra copy of exon 3. The intron normally occurring after exon 3 in the endogenous murine Hp 1 allele occurs after the 343 exon. The genomic structure of the murine Hp 2 allele is exon 1- intron 1- exon 2- intron 2 -exon3exon 4exon 3- intron 3 -exon 4- intron 4- exon 5 (see Figure 1a, murine Hp 2). The genomic structure of the murine Hp 2 allele is different from the human Hp 2 allele in that there is no intron between the duplicated exons 3 and 4. However, in the mature mRNA (i.e., after the RNA has been spliced and intronic sequences removed) there will be no difference in the genetic organization of the murine and human Hp 2 alleles. The logic we used to generate a duplication and direct repeat of exons 3 and 4 in the murine Hp 1 allele can be explained as follows. Suppose exon 3 has sequence ABCDE and exon 4 has sequence FGHIJ. We cloned into the middle of exon 3 (at a restriction endonuclease site between AB and CDE) the sequence CDEFHJAB (i.e. 2nd half of exon 3, all of exon 4 and the 1st half of exon 3) thereby transforming exon 3 (ABCDE) into exon 343 (ABCDEFGHJABCDE). Using this logic we generated a DNA fragment by RT-PCR of Hp mRNA isolated from the human HepG2 hepatoma cell line with oligonucleotides 343sense (CGGGATCCATGACAGCTGCCCCCCAAGCCCCCAGAGA) and 343 antisense (CGGAATTCCAGCTGTGATCTGGGCTCATATTCGATGTTTCTC). After digesting the fragment with PvuII we cloned it into the PvuII site of exon 3 of the
murine Hp 1 allele to create a modified exon 3 with the sequence of exon3exon4exon3.

Once we replaced the murine 3 exon with a 343 exon, we proceeded to generate a targeting vector for transfection into embryonic stem (ES) cells. In designing targeting vectors for homologous recombination, it is critical that there is at least 2 kb of 100% homology sequences (regions identical between targeting vector and targeted gene) 5’ and 3’ to the targeted region. In our case the targeted region was exon 3 and the homology regions were murine genomic sequences located 5’ (5.6kb) or 3’ (3.4 kb) to exon 3. A second feature of the targeting vector is a selectable marker, which can subsequently be removed. We used the neomycin antibiotic resistance gene (conferring resistance to G418) flanked by two lox P sites (allowing removal of the neo gene with the cre recombinase) for this purpose. We placed a cytosine deaminase (CD) gene casetted and a neo cassette in the intron between exon 2 and exon 343 bounded by 2 lox P sites using the cloning vector pTKLNCL (Thymidine kinase-LoxP-CD-Neo-LoxP) GB 1355 (see manuscript Figure 1b, for schematic picture of this construct after its successful integration showing the relationship between the wild type murine Hp 1 allele, and the targeting DNA after its integration both before (middle panel) and after (bottom panel) removal of the CD and Neo cassettes).

The targeting vector was linearized with Not I, transfected into 129O1a ES cells by electroporation (800 V, 3 uF) and individual clones selected with G418 (150ug/ml). G418 resistant clones undergoing homologous recombination for the transfected sequences were identified by southern blot analysis of BamH1 digested DNA isolated from each clone using as a probe a 265 bp Bgl II-Bam H1 fragment located outside (5’) of the 5’ homology region of the targeting vector. Southern blot using this probe yields a band of 5.8kb in wild type mouse DNA (i.e. wild type murine Hp 1 allele) and 11kb if the targeted Hp gene has undergone homologous recombination with the targeting vector (Figure 1, on-line supplement). Successfully targeted ES clones were then subjected to karyotype analysis and injected into 3.5d post-coitum (dpc) C57BL/6J females to generate several chimeras. The chimeras were mated with C57BL/6J females to produce heterozygous Hp 2 mice that were then intercrossed to produce mice homozygous for the murine 2 allele. The CD and neo gene cassettes were deleted by crossing with EIIaCre mice overexpressing the cre recombinase in all tissues (provided by Heiner Westphal, National Institutes of Health). After the CD and neo gene cassettes was deleted the only difference between
the wild type murine Hp 1 allele and the murine Hp 2 allele which we created, other than exon 3, was in the intron between exons 2 and exons 3. In the murine Hp 1 allele the intron is 250 bp. In the murine Hp 2 gene a Pvu-Bgl fragment (100bp) in the middle of this intron was deleted and additional sequences were inserted (vector sequences from pTKLNCL consisting of the Xho-LoxP and LoxP-Bam) thereby creating an intron between exons 2 and 343 in the murine Hp 2 allele of different length than the intron between exons 2 and 3 in the murine Hp 1 allele. These differences in the size of intron 2 have been exploited for Hp genotyping of the mice by PCR using oligonucleotides that bracket this intron. These oligonucleotides are: exon 2s AGCCCTGGGAGCTGTTGTCAC (located in the coding sequence for exon 2) and 3r (located at the 3' end of the intron between exon 2 and exon 3) TGGGTGCTCCGATGGCTCTCTG. Oligonucleotides 2s and 3r yield a PCR product of 306 bp for the murine Hp 1 allele (83 bp from exon 2 and 223 from the intron) and 406 bp for the murine Hp 2 allele (83 bp from exon 2 and 323 from the intron). Mice having both bands are heterozygotes (haptoglobin 2-1).

Generation of a murine Hp 2 colony and backcrossing with ApoE-/- mice.

C57BL/6J mice containing the murine Hp 2 allele were backcrossed with C57Bl/6J mice for 10 generations. In order to assess the role of the Hp genotype in modulating aspects of atherosclerotic lesions we backcrossed these murine Hp 2 mice with C57Bl/6J ApoE-/- mice to generate C57Bl/6J ApoE-/-Hp2-2 mice. Genotyping at the Hp locus was achieved by analysis of tail DNA by PCR with oligos 2s and 3r as described above. Genotyping at the ApoE locus was performed by PCR based on the methodology recommended by the Jackson Laboratories using oligonucleotides IMR0180 (GCCTAGCCGAGGGAGAGCCG), IMR0181 (TGTGACTTGGAGCTCTGCAGC) and IMP0182 (GCCCGCCCGACTGCATCT). The ApoE wild type allele yields a band of 155bp, the targeted ApoE allele yields a band of 245bp.

Lipid peroxidation.

Lipid peroxidation was evaluated using the 4-hydroxynonenal (4-HNE)\(^6\) and the ceroid content of plaques.\(^7\) As tissue fixation with formaldehyde is itself an oxidative process which may induce lipid peroxidation, this analysis was performed on frozen
sections rather than on formaldehyde fixed tissue. 4-HNE, a lipid aldehyde, is a major end product of lipid peroxidation and is known to be increased in oxidative-stress related disorders. 4-HNE is especially reactive with Cys, His and Lys residues forming 4-HNE-protein adducts which can be identified by immunohistochemistry. Immunohistochemical detection of 4-HNE was performed using a rabbit polyclonal antibody to 4-HNE (Alexis Biochemicals) and a goat anti-rabbit antibody avidin-biotin peroxidase complex (ABC kit, Vector Laboratories) according to manufacturer's instructions. The color reaction product was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with hematoxylin.

Ceroid is an insoluble complex of oxidized lipid and protein frequently identified in human atherosclerotic lesions. Ceroid is autofluorescent and was scored as the percentage of the total plaque area that was autofluorescent. Ceroid was scored by two independent observers who were blinded to the Hp genotype of the specimen.

**Macrophage accumulation.**

Immunohistochemical localization of macrophages was performed using formalin-fixed, paraffin-embedded, 4-µm tissue sections on poly-lysine coated plus glass slides. Tissue sections were deparaffinized and then pretreated with trypsin at 37°C for 15 minutes. Sections were then incubated at 37°C with 2% normal horse serum in Tris buffer to prevent non-specific antigen binding. Endogenous peroxidase activity was blocked with 10% H₂O₂ in methanol. Sections were incubated with mouse monoclonal anti-macrophage antibody clone SPM281 (Spring bioscience) for 60 minutes at 37°C. Secondary antibody avidin-biotin peroxidase complex was obtained from Vector Laboratories (ABC kit). Macrophages were identified using 3’
3’ diaminobenzidine tetrahydrochloride (DAB). Counterstaining was done using hematoxylin. Macrophages were counted manually in all plaques in the intima, media and adventitia.\textsuperscript{9,10}

References

8. Schaur RJ, Zollner H, Esterbauer H. Biological effects of aldehydes with particular attention to 4-hydroxynenal and malondialdehyde. In: Vigo-


**Figure 1. Southern blot of ES transfectants with successful gene targeting.**
Probes correspond to a 265 bp Bgl-Bam fragment located just 5' to the 5' homology region used in the targeting vector. Lane 1: Southern blot of Bam H1 digested genomic DNA from wild-type mice showing a single 5.8 kb band recognized by the probe. Lanes 2-4: Southern blot of Bam H1 digested DNA from three different ES clones that have undergone successful gene targeting at one copy of chromosome 8 at the Hp locus (transgene integrated by homologous recombination at the Hp locus) demonstrating an additional band of 11 kb recognized by the probe.