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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 to 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 to 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 to 1 mice.

Conclusions—Increased iron, lipid peroxidation and macrophage accumulation in ApoE$^{-/-}$ Hp 2 to 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;27:000-000.)**

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

The major cause of acute coronary thrombosis is atherosclerotic 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 intra-plaque hemorrhage represents a potent stimulus for 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 intra-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 to 1 (homozygous for the Hp 1 allele), 36% is Hp 2 to 2 (homozygous for the Hp 2 allele), and 48% is Hp 2 to 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 to 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 to 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-

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mental differences in the antioxidant and immunomodulatory properties of the Hp 1 to 1 and Hp 2 to 2 proteins that may explain why Hp is a susceptibility gene for CVD. As an antioxidant the Hp 1 to 1 protein is superior to the Hp 2 to 2 protein in blocking the oxidative action of Hb.[14–16] As an immunomodulator, the Hp 1 to 1–Hb complex stimulates the macrophage to secrete anti-inflammatory cytokines to a markedly greater degree than the Hp 2 to 2–Hb complex.[17–19]

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.[20]

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.[21,22]

Iron Deposition

Iron deposition in the plaque was identified using Perl's stain[15] and quantified by measuring the percentage of plaque area staining black.[15]

Lipid Peroxidation

Lipid peroxidation was evaluated using 4-hydroxynonenal (4-HNE)[23] and ceroid[7] 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 $25^{th}/50^{th}/75^{th}$ percentile with differences between groups determined by the Mann-Whitney test. A value of $P \leq 0.05$ 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 to 1, 2 to 1, or 2 to 2 genotypes.[24] 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 quatermers 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 immersal 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.[8] In such gels, a single rapidly migrating band is seen in serum derived from Hp 1 to 1 individuals, corresponding to the Hp dimer, whereas more slowly migrating bands are seen in Hp 2 to 1 or Hp 2 to 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 to 1 and Hp 2 to 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.[10]

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 to

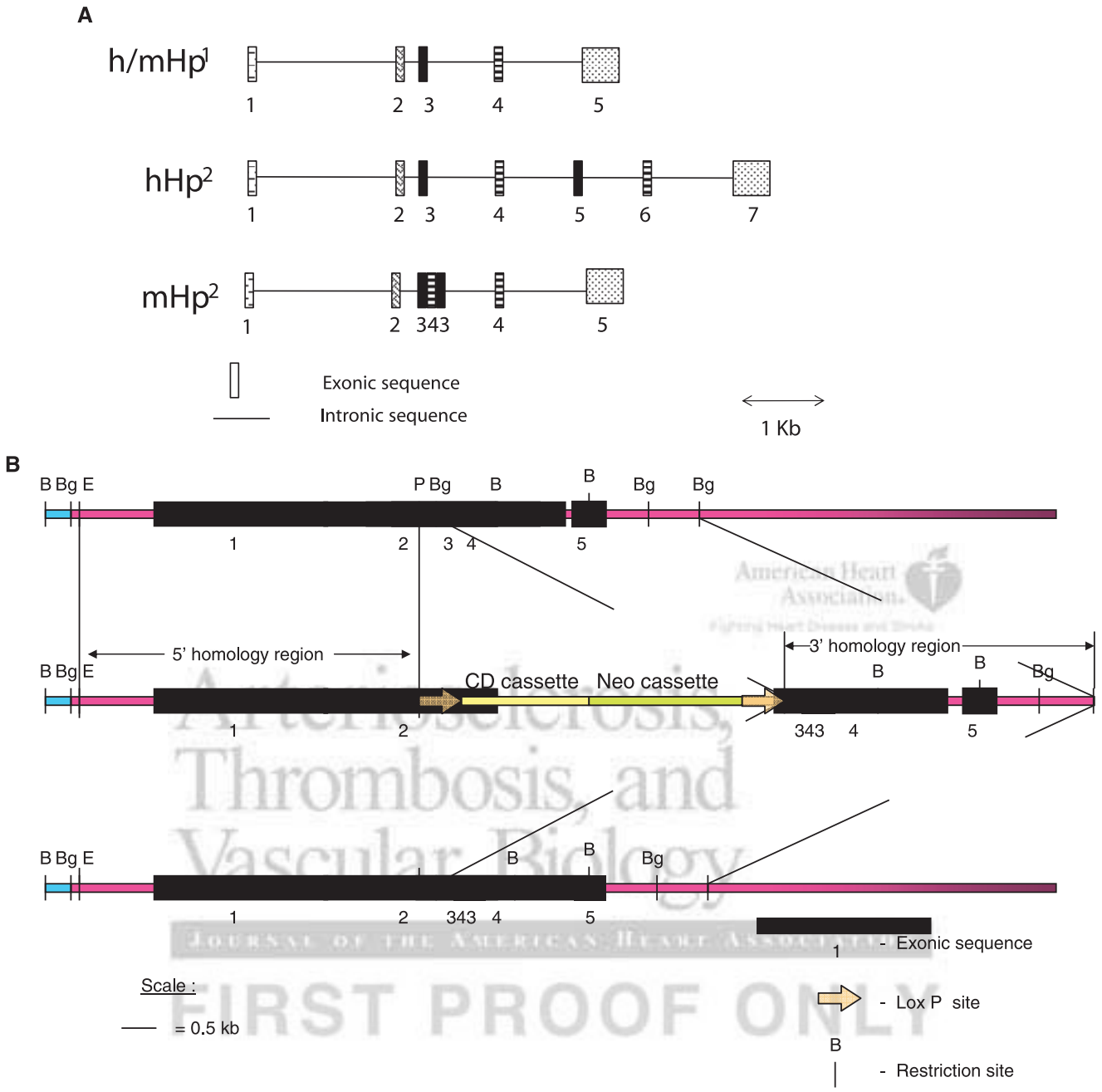


Figure 1. Construction of a murine Hp 2 allele. A, Genomic organization of the Hp locus. The human Hp 1 and Hp 2 alleles are located at chromosomal coordinates 16q22. The murine wild type Hp is a Hp 1 allele and is found on chromosome 8. A murine Hp 2 allele was created as described in this manuscript 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 *Kpn*I-*Xho*I site of TKLNCL 5′ to the neo cassette (5′ homology region) and a 3.4 kb *Bgl*II fragment of the murine Hp 1 allele was cloned in the Bam H1 site of TKLNCL 3′ to the neo cassette (3′ homology region). Exon 3 of the murine Hp 1 was reconstructed to be exon 343 as described in Methods. The vector was linearized with *Not*I 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 300-bp BamH1-*Bgl*II 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.

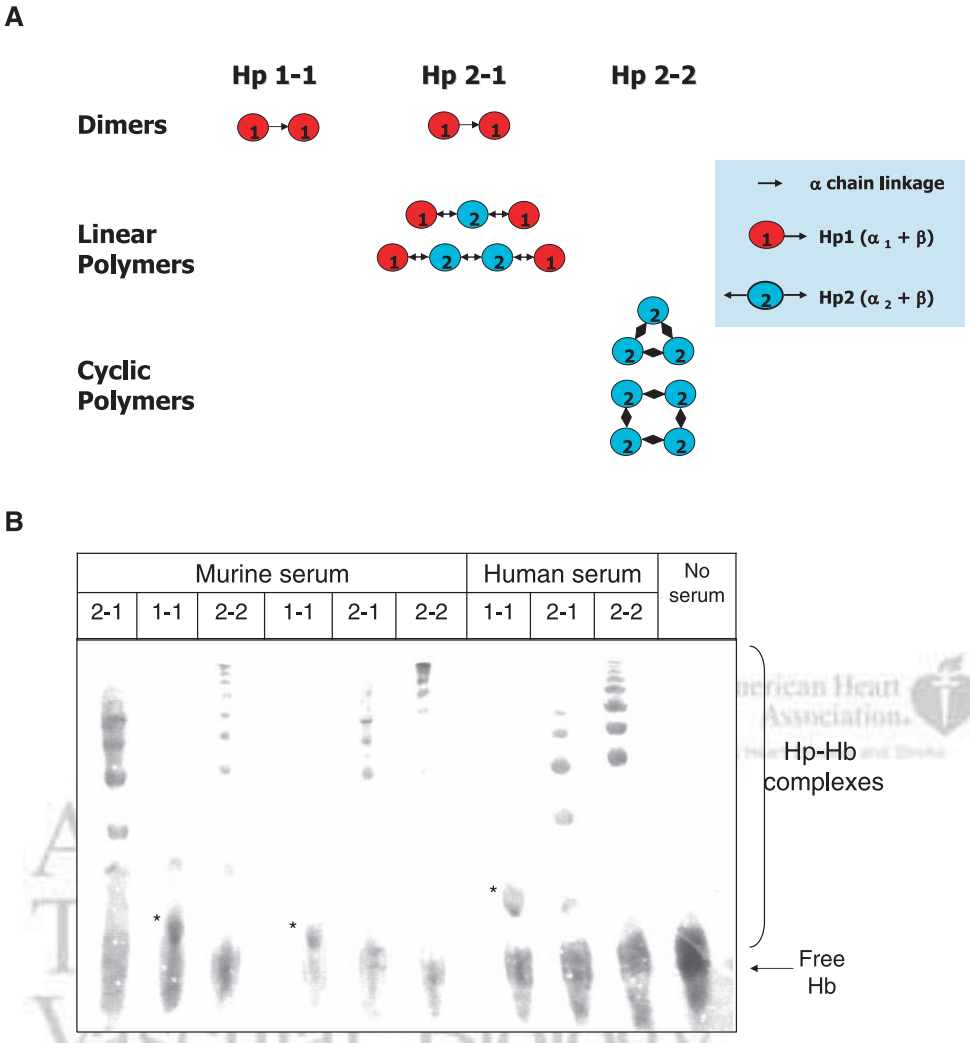


Figure 2. The size and shape of murine Hp 2 polymers are similar to human Hp 2 polymers. A, Schematic illustration of the shape of Hp polymers in humans with the Hp 1 to 1, Hp 2 to 1 or Hp 2 to 2 genotypes. The Hp monomer forms multimers whose stoichiometry is Hp genotype-dependent. Multimerization is mediated by cysteine residue in exon 3 so that the Hp 1 allele protein product can combine with only one other monomer while the Hp 2 allele protein product combines with 2 other monomers. The structures shown have been verified by electron microscopy. B, Demonstration that the polymer distribution in murine Hp 1 to 1, 2 to 1, and 2 to 2 mice is similar to that in humans with Hp 1 to 1, 2 to 1, and 2 to 2. Shown is a polyacrylamide gel of serum samples from humans or mice with the indicated Hp genotypes. Samples were enriched with Hb and then electrophoresed on a nondenaturing polyacrylamide gel. Hp–Hb complexes were identified in the gel using a peroxidase sensitive reagent. A signature banding pattern is present for each Hp genotype. Note that higher molecular Hp–Hb complexes are absent in Hp 1 to 1 mice and that the distribution of the high-molecular-weight complexes in murine Hp 2 to 1 and Hp 2 to 2 mice is quite similar to that in humans with Hp 2 to 1 and Hp 2 to 2. Both the human Hp 1 to 1-Hb complex and the murine Hp 1 to 1-Hb complex are a single species (demarcated with an asterisk*) located just above the free Hb band.

1 and Hp 2 to 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 to 1 and Hp 2 to 2 mice are presented in the Table. There was no significant difference in plaque or lipid core area between Hp 1 to 1 and Hp 2 to 2 mice. There was a nonsignificant trend showing decreased cap thickness in plaques from Hp 2 to 2 mice.

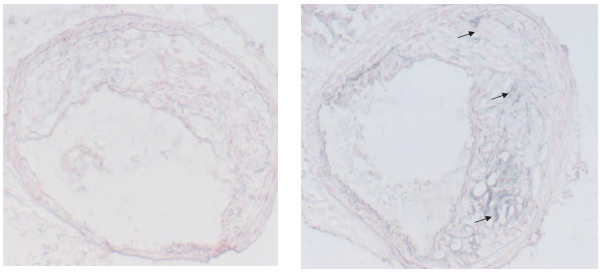
Increased Iron Deposition in Hp 2 to 2 Plaques

Our previous in vitro studies have suggested that hemoglobin released from microvascular hemorrhages within the plaque would be cleared more slowly in Hp 2 to 2 as compared with Hp 1 to 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 to 2 plaques as compared with Hp 1 to 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

Genotype	n	Cap Thickness (um)	Plaque Area (um^2)	Lipid Core (um^2)
apoE$^{-/-}$ Hp 1-1	18	19.1±2.2	0.018/0.033/0.144	0.006/0.017/0.035
apoE$^{-/-}$ Hp 2-2	15	15.0±1.7	0.027/0.051/0.084	0.008/0.022/0.035

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 ($25^{th}/50^{th}/75^{th}$ percentiles) are shown. There was no significant difference in cap thickness ($P=0.25$), plaque area ($P=0.76$), or lipid core area ($P=0.73$) between Hp 1-1 and Hp 2-2 mice.



Hp 1-1 Hp 2-2

Figure 3. Increased iron in plaques from Hp 2 to 2 mice. Intraplaque iron is stained black (representative examples noted with arrows) with Perl's stain. The amount of iron staining in plaques from Hp 2 to 2 ApoE$^{-/-}$ mice was significantly greater than in Hp 1 to 1 ApoE$^{-/-}$ mice when scored as the percentage of the total plaque area (2.18±0.26% vs 0.94±0.25%, n=10, P=0.008).

Increased Lipid Peroxidation in Hp 2 to 2 Plaques

We assessed plaques for 4-HNE[23,25], a major end-product of lipid peroxidation, and ceroid,[7] 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 to 2 as compared with Hp 1 to 1 mice.

Increased Macrophage Accumulation in Hp 2 to 2 Plaques

We found that in the intima and adventitia of atherosclerotic plaques from Hp 2 to 2 mice there were significantly more macrophages as compared with plaques from Hp 1 to 1 mice (Figure 5).

Correlation Between Lipid Core Size and Inflammation in Hp 2 to 2 Plaques but not in Hp 1 to 1 Plaques

Oxidized lipid within the core of the plaque may act as an inflammatory stimulus.[26] We were intrigued that although there was no significant difference in the lipid core area between Hp 1 to 1 and Hp 2 to 2 mice, macrophage accumulation in the Hp 2 to 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 to 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 to 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 to 2 as compared with Hp 1 to 1 mice. We have suggested that this increase in macrophage accumulation in Hp 2 to 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

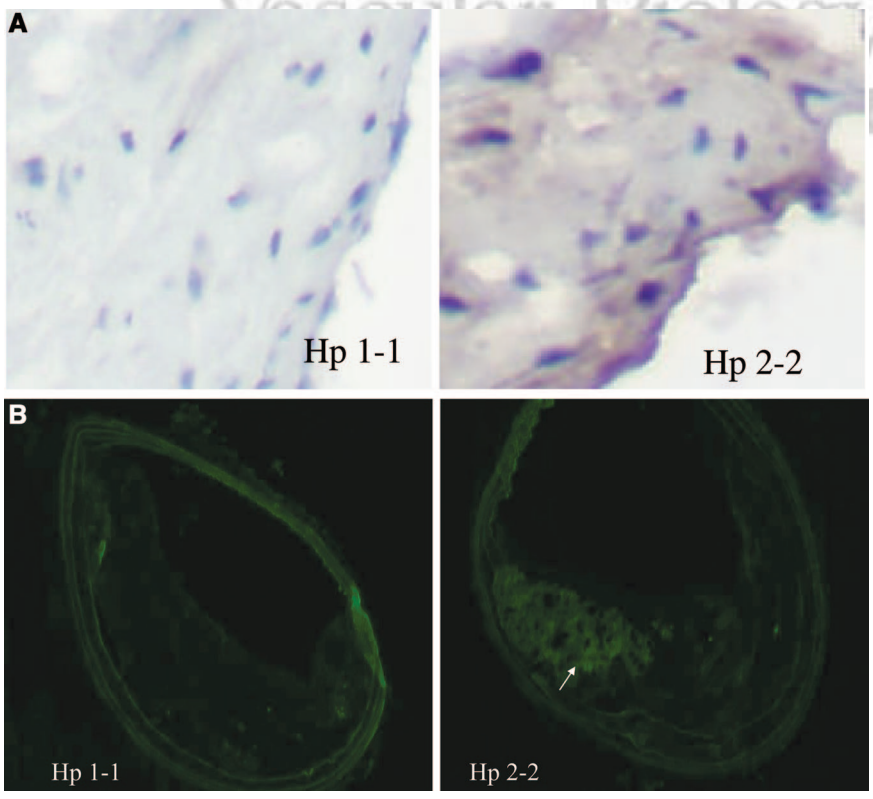


Figure 4. Increased lipid peroxidation in plaques of Hp 2 to 2 mice. A, Increased 4-HNE in plaques of Hp 2 to 2 mice. 4-HNE protein adducts (staining brown) in the plaque were assessed by immunohistochemistry as described in Methods. B, Increased ceroid (autofluorescence) in plaques of Hp 2 to 2 mice. The autofluorescent ceroid pigment (arrow) in the plaque was scored as the percentage of ceroid (autofluorescence) of the total plaque area. There was significantly more ceroid in Hp 2 to 2 plaques as compared with Hp 1 to 1 plaques (10.3±3.9% vs 2.6±0.5% of total plaque area, n=8, P=0.05).

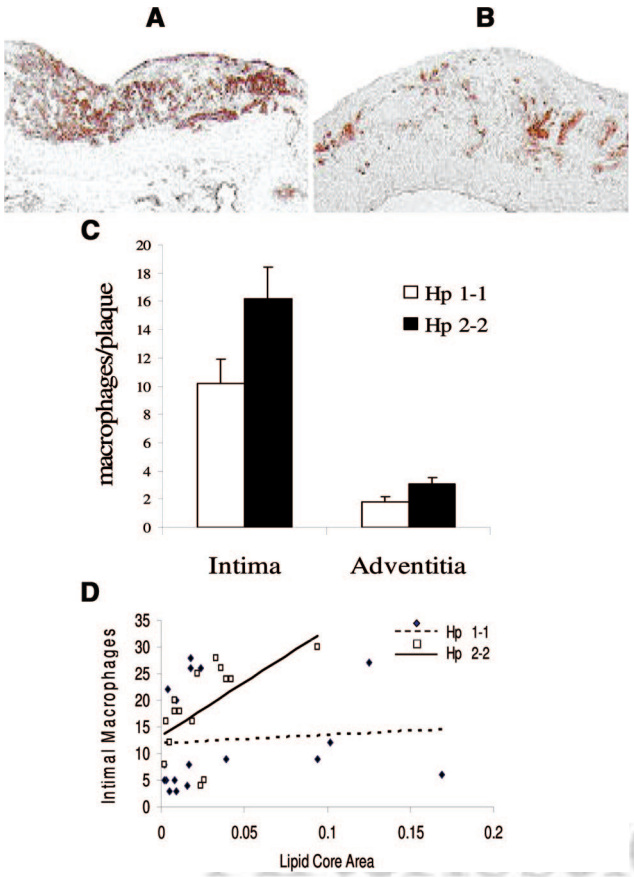


Figure 5. Increased macrophage accumulation in the plaques of Hp 2 to 2 mice. Macrophages were identified immunohistochemically as described in methods. Shown in (A) and (B) are representative plaques of similar size but with dramatically greater macrophage accumulation in Hp 2 to 2 Apo $E^{-/-}$ (A) as compared with Hp 1 to 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 to 1 and n=15 for Hp 2 to 2). There were significantly more macrophages in the intima (P=0.03) and adventitia (P=0.03) of plaques from Hp 2 to 2 as compared with Hp 1 to 1 mice. D, Plot of the number of intimal macrophages versus the lipid core area (um^2) in plaques from Hp 1 to 1 $ApoE^{-/-}$ (n=18) and Hp 2 to 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 to 2 mice (correlation coefficient=0.57, P=0.01) but not in Hp 1 to 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 to 2-Hb complexes stimulate markedly increased oxidation of low-density lipoprotein (LDL) as compared with Hp 1 to 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 to 2-Hb complex.[14–16] Therefore, intra-plaque hemorrhage generates greater iron deposition in mice with the Hp 2 to 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 to 1 and Hp 2 to 2 mice yet there are more macrophages in Hp 2 to 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 to 2 and Hp 1 to 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 to 1 plaques may be caused by the ability of the Hp 1 to 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 to 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 to 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 to 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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Disclosures

None.

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