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Haptoglobin: Basic and Clinical Aspects

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Abstract

Haptoglobin is an abundant hemoglobin-binding protein present in the plasma. The function of haptoglobin is primarily to determine the fate of hemoglobin released from red blood cells after either intravascular or extravascular hemolysis. There are two common alleles at the Hp genetic locus denoted 1 and 2. There are functional differences between the Hp 1 and Hp 2 protein products in protecting against hemoglobin-driven oxidative stress that appear to have important clinical significance. In particular, individuals with the Hp 2-2 genotype and diabetes mellitus appear to be at significantly higher risk of microvascular and macrovascular complications. A pharmacogenomic strategy of administering high dose antioxidants specifically to Hp 2-2 DM individuals may be clinically effective. *Antioxid. Redox Signal.* 12, 293–304.

Historical Aspects

The presence in serum of a hemoglobin (Hb) binding protein was first described by Polonovski and Jayle in 1938 when they observed that the addition of Hb to serum resulted in an increase in the peroxidase activity of the Hb (45). These authors characterized the protein as an alpha-2 glycoprotein and gave the protein the name haptoglobin (Hp) (46). It soon became evident that measurement of the Hp level had clinical usefulness as a marker of hemolysis. In 1955, Smithies provided a molecular explanation for the apparent heterogeneity in Hp protein molecules purified from different individuals (49). Smithies described the separation by gel electrophoresis of Hp proteins into three distinct patterns which he attributed to a genetic polymorphism (50). In 1957, it was first demonstrated that, since the Hp–Hb complex cannot pass through the glomerular filter, Hp is a major determinant of whether free Hb is secreted and thereby serves to conserve iron (1, 30).

Physiology

Hp is synthesized predominately in the liver by the hepatocyte. Hp is an acute phase protein and its synthesis is rapidly and dramatically increased in response to numerous inflammatory stimuli. This increase in Hp production in inflammation is due a transcriptional activation of the Hp gene (58).

The normal level of plasma Hp varies considerably ranging from 0.3 to 3 mg/ml, but in any given individual the Hp level remains fairly constant and therefore the observation of a marked fall has clinical significance. For example, Hp levels before and after insertion of a cardiac prosthesis have been used clinically to identify red cell destruction, suggesting malfunctioning of the prosthesis (56).

Whenever Hb is released into the circulation, it binds immediately to Hp to form an Hp–Hb complex and this complex is rapidly removed predominately by the monocyte/macrophage CD163 Hp–Hb receptor expressed on Kupfer cells in the liver (23). When tracer amounts of radioactively labeled Hp–Hb complex are injected into humans, the half-life of the complex has been estimated to be only 9 min (21).

When Hp is depleted (25) as a result of hemolysis or in Hp knockout mice (19), Hb is found to accumulate in the kidney and is secreted in the urine. Therefore, a major role of Hp is to prevent renal damage (35) and Hb loss (and thus iron loss) by renal excretion. Mice that lack both Hp and hemopexin (which binds heme with high affinity) display pronounced splenomegaly and liver fibrosis after acute hemolysis (55). An additional role for Hp has been proposed in terms of its ability to stabilize heme iron within Hb and thereby prevent oxidative damage that could be mediated by heme iron. This was first demonstrated by Bunn and Jandl in 1965 who demonstrated that the free exchange of heme from Hb to albumin was prevented by Hp (13). Furthermore, Hp appears to play an essential role in protecting globin itself from destructive oxidative modification that permits the Hp–Hb complex to be efficiently cleared by CD163 (11).

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The Hp–Hb Complex

Each Hp monomeric protein can bind one Hb α-β dimer (29, 42, 44). It has been suggested that Hp is unable to bind to deoxyhemoglobin (25). However, under some conditions, covalently stabilized Hb tetramers (and even larger polymers) can form a complex with Hp that may challenge the concept that only oxygenated Hb dimers can bind to Hb (12). The precise nature of the Hp–Hb complex is not known, as the Hp–Hb complex has never been crystallized but it is not covalent. Once formed, the complex is extremely stable with an estimated K_d of 10^{-15}. Deoxygenated Hb does not bind to Hp. The binding site on Hp for Hb has been mapped to the Hp β-chain. The binding of Hp to Hb results in the formation of a unique epitope that is recognized by the CD163 receptor (23).

The Structure of the Hp Protein

Hp is originally synthesized as a single polypeptide chain which is then cleaved into α and β chains that are linked via disulfide bonds to form an Hp monomer (29). The Hp 1 allele and Hp 2 allele, whose genetic structure will be described below, encode for protein products that differ in their α chain with the α-1 being $\sim$9 kd and the α-2 being $\sim$16 kd. The β chain in both the Hp 1 protein and in the Hp 2 protein is $\sim$45 kd. Polymeric Hp found in serum is comprised of Hp monomers covalently linked by disulfide bridges between α chains. The stoichiometry of these Hp polymers is genetically determined.

Hp Genetic Structure and Definition of the Hp Polymorphism

The Hp gene has been mapped to chromosome 16q22 (29). There exists in man two classes of alleles for Hp (1 and 2) with homozygous (1-1 or 2-2) and heterozygous (2-1) genotypes possible. The human Hp 1 allele is composed of 5 exons and is remarkably similar to the Hp allele found in all animal species. The Hp 2 allele is composed of 7 exons (Fig. 1). The Hp 2 allele, present only in humans, appears to have arisen from the Hp 1 allele by a duplication of exons 3 and 4 of the Hp 1 allele (37). The protein product of the Hp gene (Hp monomer) is found in serum as a polymer of between 2–10 covalently linked monomers. The stoichiometry of the Hp polymer is Hp-genotype-dependent due to differences in the valencies of the Hp 1 (monovalent) and Hp 2 (bivalent) allelic protein products. This is because the Hp multimerization domain is located in exon 3 of the Hp gene that is duplicated in the Hp 2 allele. The net result of these differences, as confirmed by electron microscopy (62), is that Hp is found as a dimer in Hp 1-1 individuals (2 Hp monomers), a linear polymer in Hp 2-1 individuals (2–8 monomers) and a cyclic polymer (3–10 monomers) in Hp 2-2 individuals (Fig. 2). Recently it was demonstrated that deer and cows both have polymeric Hp in their serum as the result of what appears to be convergent evolutionary event (*i.e.*, a distinct mutation from what gave rise to the human polymeric Hp) (28).

The Hp 2 allele is believed to have arisen from the Hp 1 allele $\sim$100,000 years ago early in human evolution in Southeast Asia (29). Subsequently the prevalence of the Hp 2 allele has spread throughout the world, probably as a result of its ability to provide a selective advantage against infectious disease. The Hp 1/2 polymorphism is a very common polymorphism and the prevalence of the three Hp genotypes has been demonstrated to vary dramatically between populations in different geographic areas and ethnic groups (29). In most western countries, the prevalence of the Hp genotypes is 16% Hp 1-1, 36% and 48% Hp 2-1. In Southeast Asia, however, $\sim$90% of all individuals have the Hp 2-2 genotype. In all populations the polymorphism is in Hardy–Weinberg equilibrium (29).

Methods to Determine the Hp Polymorphism

The gold standard for determining the Hp type has been starch or polyacrylamide gel electrophoresis. In this method, 10 microliters of serum is incubated with human Hb and then subjected to nondenaturing gel electrophoresis. A signature banding pattern is produced that is diagnostic for each of the three Hp types (29) (Fig. 3). This method has the advantage that it allows use of serum or plasma that can have been thawed multiple times and is >10 years old. The disadvantage of this method is that it is somewhat time consuming and requires that the Hp concentration in the serum be at least 0.10 mg/ml. A PCR-based assay to determine the Hp polymorphism has been described by several groups (27). While detection of the Hp 2 allele is relatively straightforward by assessment of the presence of the exon 4–exon 3 junction, the identification of the Hp 1 is more problematic due to the need to produce a 1.7 kb fragment that will be diagnostic for the Hp 1 allele. This has led to some apparent inaccuracies in the PCR method which presently is judged to provide a specificity as compared to the gel method of only 85%. A third method to determine the Hp polymorphism is based on ELISA (57). This method takes advantage of the unique polymeric structure of the different Hp types by using capture and detection antibodies of the same binding specificity. Critically in this assay

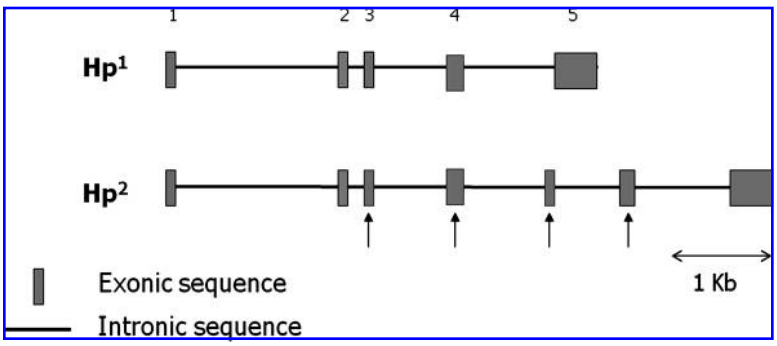

FIG. 1. Gene structure of the Hp 1 and Hp 2 alleles. The Hp 2 allele arose from the Hp 1 allele by duplication of exons 3 and 4.

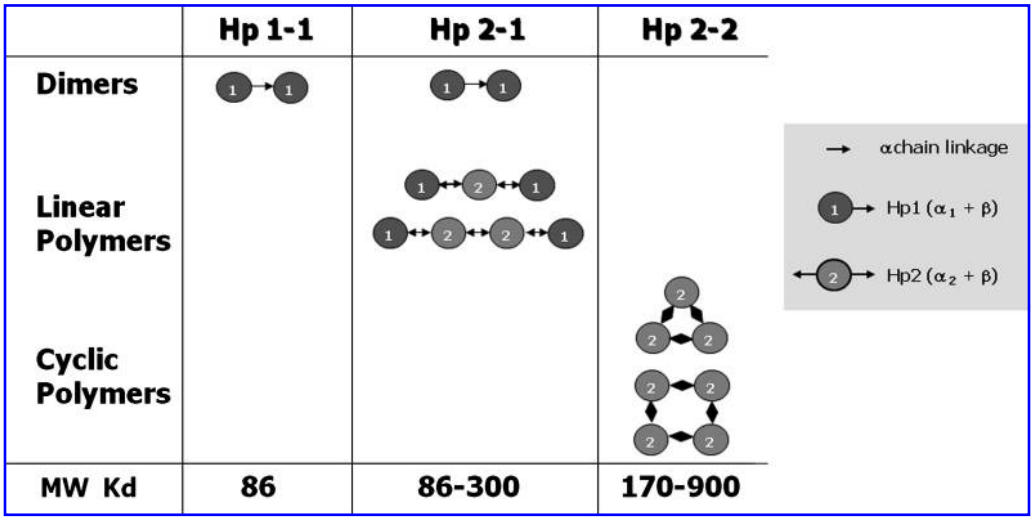

FIG. 2. Polymer structure of Hp in individuals with the different Hp genotypes.

the Hp protein is in vast excess to the amount of capture antibody. This makes the test insensitive to Hp concentration in the blood sample. The specificity of this ELISA has recently been shown to be 99% as compared to the gel electrophoresis method and has the advantage of being extremely simple and allows the Hp typing of >500 samples day by a single operator.

Differences in Function of the Protein Products of the Hp 1 and Hp 2 Alleles: Antioxidant Function

Hb is a biological Fenton agent capable of mediating considerable oxidative damage by generating highly reactive hydroxyl radical species (29). The Hp protein functions as an antioxidant by virtue of its ability to bind to Hb and prevent Hb from mediating oxidative injury. The Hp 1-1 protein has been demonstrated to be superior to the Hp 2-2 protein in preventing Hb-induced oxidation of lipid and protein substrates (20). As the binding of Hp to Hb does not appear to influence the ability of heme iron to redox cycle in the Hp–Hb complex in the presence of H_2O_2 (11), the role of Hp as an antioxidant may be due to its ability to stabilize heme within the heme pocket of Hb and to prevent "spill-over" of toxic and oxidizing radicals to the susceptible environment (such as interacting lipoproteins). The differences in the antioxidant function of Hp do not appear to be due to differences the affinity of the Hp 1 and Hp 2 protein products for Hb (2).

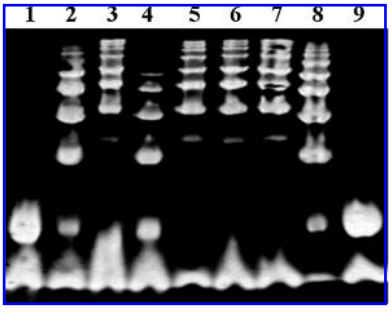

FIG. 3. Hp typing of hemoglobin enriched serum by gel electrophoresis. Each Hp type has a characteristic banding pattern. *Lanes 1* and *9* are Hp 1-1, *lanes 2, 4,* and *8* are Hp 2-1, and the remainder are Hp 2-2.

Clearance of the Hp–Hb Complex

In vitro in CHO cells stably transfected with the Hp–Hb scavenger receptor CD163, the Hp 1-1-Hb complex is endocytosed much more rapidly than the Hp 2-2-Hb complex as demonstrated using rhodamine tagged and ^{125}I-tagged Hp–Hb complexes (2) (Fig. 4). There does not appear to be any difference in the degradation rate of Hp 1-1-Hb and Hp 2-2-Hb complexes once they are internalized (2).

In vivo in mice the half life of ^{125}I-tagged Hp–Hb complexes has also been assessed. The half life of the Hp 1-1–Hb complex was found to be ~20 min in Hp 1-1 mice or Hp 2-2 mice or rats (which are Hp 1-1) but the half life of the Hp 2-2–Hb complex was found to have a half life of ~50 min (5).

These differences in clearance of the Hp–Hb complex are significant for three reasons. First, the Hp 2-2–Hb complex is redox active and can mediate oxidative injury. Impaired clearance of the complex therefore may result in increased oxidative injury in Hp 2-2. Second, the Hp–Hb complex (both Hp 1-1 and Hp 2-2–Hb) can scavenge nitric oxide and mediate its dioxygenation (7). Since the steady state concentration of the Hp 2-2–Hb complex is higher, nitric oxide availability is likely to be decreased in Hp 2-2. Third, the impaired clearance of the Hp 2–Hb complex results in there being a higher state concentration of the complex in the plasma of Hp 2-2 (5). As will be demonstrated below, this allows the Hp–Hb complex to bind to other proteins in serum (notably HDL) and to mediate their structural and functional modification (5).

Binding to High Density Lipoprotein (HDL)

There has been considerable controversy concerning whether Hp is a HDL associated protein. We have recently explained the apparent discrepancy between different research groups by demonstrating that ultracentrifuge purified HDL does not have Hp associated with it but immunopurified HDL does have Hp associated with it (5) (Fig. 5). This most likely

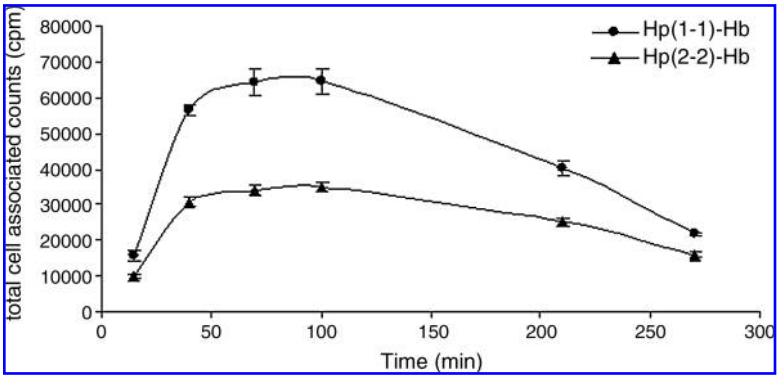

FIG. 4. Receptor mediated endocytosis of Hp-1-1–Hb and Hp 2-2–Hb complexes by CHO cells stably transfected with CD163. The Hp 1-1–Hb complex is taken up more rapidly than the Hp 2-2–Hb complex.

reflects the relatively low affinity of Hp for HDL, an affinity which is of physiological relevance due to the very high levels of Hp in plasma. The binding site for Hp on HDL has been mapped to helix 6 (amino acid residues 141–164) on apolipoprotein A1 (ApoA1) (51). This binding site overlaps with the binding site on ApoA1 for the enzyme LCAT which is critical for the maturation of HDL. Inflammation which results in an increase in Hp concentration has been shown to decrease LCAT activity, possibly thru acting as a competitor for LCAT binding and activation (51). The binding of Hp to ApoA1 may also be significant by virtue of its ability to tether Hb to HDL that may render the HDL proatherogenic and proinflammatory (59). Watanabe and colleagues have suggested that Hb bound to Hp may be increased in the HDL of atherogenic/hyperlipidemic mice and in the HDL of individuals with coronary artery disease (60) and that the amount of Hb bound to HDL correlates with the proinflammatory properties of dysfunctional HDL (60). There exists significantly more Hp in the HDL of Hp 2-2 individuals as compared to Hp 1-1 individuals. This is due to the polymeric nature of Hp 2-2, the binding of single Hp monomer to apoA1 results in the tethering of all the other monomers present to the apoA1 molecule as well (5). As will be discussed below, this may explain why there appears to exist significantly more Hb bound to the HDL of Hp 2-2 mice and humans (5).

Cytokine Expression

The binding of Hp–Hb to CD163 results in both casein kinase 2 (52) dependent and independent (48) production of several cytokines and a noninflammatory mediators. Schaer and colleagues have demonstrated that Hb–Hp induces a antiinflammatory and antioxidative gene expression pattern with the most prominent being induction of inducible heme oxygenase (HO-1) (48). Guetta and colleagues have demonstrated that upon binding to CD163 the Hp 1-1–Hb complex results in a severalfold increase in the anti-inflammatory mediators interleukin-10 and interleukin-6 as compared to the Hp 2-2–Hb complex (24).

Angiogenesis

Hp has been shown to be an angiogenic factor and the angiogenic potency of the Hp 2-2 protein is greater than that of Hp 2-1 and Hp 1-1 (15). In animal models of myocardial ischemia, the Hp protein has been implicated in coronary collateral development (36). In man, Hp type-dependent differences in peripheral arterial collaterals have been suggested (18).

Other Functions

A variety of other biological functions have been attributed to the Hp protein but it is not clear if there are Hp type-dependent differences in these functions (29). These functions include: protease activity (for elastase and collagen); immunosuppression (prevention of cytokine production from monocytes in response to endotoxin challenge), and preventing the denaturation of proteins.

Haptoglobin in Disease

As noted above, Hp is an acute phase protein and as such is increased in a large number of disease states. Whether this increase in concentration is responsible for some of the pathophysiology of these diseases remains to be demonstrated. The Hp genotype has also been shown to be associated with disease. We will review below, for specific disease states, the possible involvement of Hp with a focus on the relationship between Hp genotypes and diabetic cardiovascular disease.

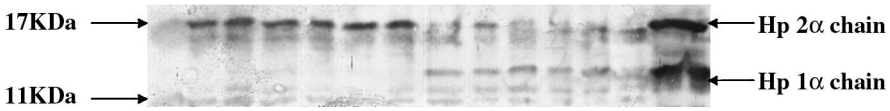

FIG. 5. Western blot for Hp of HDL prepared by immunoprecipitation. Immunoreactive bands correspond to the molecular size of the alpha 1 and alpha 2 subunits of the Hp 1 and Hp 2 proteins, respectively. Hp is present in the HDL of all individuals regardless of Hp type when the HDL is prepared by immunoprecipitation.

Cancer

Several groups have shown that the Hp protein concentration is markedly increased in the serum of individuals with cancer. In addition, the Hp protein may be modified in cancer patients, with evidence of fucosylation being a marker of pancreatic disease burden (40). The anti-inflammatory role of Hp may play a role in failure of the host immune system to recognize the tumor by impairing the tumor surveillance system. The role of the Hp genotype in determining susceptibility to cancer (lymphoma and breast cancer) has been suggested by several groups but has not been consistently confirmed (29).

Infection

Hp is a bacteriostatic agent by restricting access of bacteria to Hb-derived iron that is critical for bacterial growth. As infectious disease was likely the most important selective pressure early in human evolution, the fact that the Hp 2 allele spread so rapidly worldwide is highly supportive of the notion that the Hp 2 allele provides selective resistance to foreign pathogens. One example of how this might work has been demonstrated for the streptococcus bacterium. The T antigen (specifically T4) on the coat of streptococcus binds Hp. Polymeric Hp 2-2 molecule thereby agglutinates and causes clumping of the streptococcus organisms and markedly inhibits their growth as compared to the Hp 1-1 molecule (29). This difference in growth of Streptococcus may explain why individuals with the Hp 1-1 who develop impetigo from Streptococcus have a higher incidence of systemic complications (61). Hp may also play an important role in certain infectious processes associated with hemolysis such as malaria. In particular, it has been suggested that the Hp 2-2 phenotype may be a risk factor for anemia in malaria-endemic areas by virtue of the reduced ability of the Hp 2-2 polymer to scavenge free Hb iron following malaria induced hemolysis (6).

Subarachnoid hemorrhage

Subarachnoid hemorrhage (SAH) due to a ruptured aneurysm is associated with an exceptionally high fatality rate and considerable morbidity. Hemorrhage and specifically Hb released from lysed red cells in the hemorrhage have been implicated as being responsible for much of the pathophysiology of SAH. It has been proposed that Hb is proinflammatory and scavenges nitric oxide, resulting in vasospasm in the hemorrhagic tissue leading to cerebral tissue ischemia. Anti-inflammatory agents and nitric oxide have been shown to attenuate much of the damage mediated by SAH. The Hp genotype may be predictive of clinical sequelae after SAH. This hypothesis has been investigated in transgenic mice with the Hp 2 allele (described below) and in man (9, 14). In transgenic mice, it was demonstrated that after the induction of SAH, mice with the Hp 1-1 genotype had markedly less vasospasm, decreased inflammation, and improved survival as compared to Hp 2-2 mice. In man, individuals with SAH and the Hp 1-1 genotype had significantly less vasospasm as detected by transcranial Doppler and carotid angiography.

Renal disease (nondiabetic)

Hp plays an important role in preventing the spillage of plasma Hb into the kidney and oxidative damage mediated by free Hb. Renal proximal tubule cells contain receptors for Hb (22). In mice, the amount of Hb-derived iron in the proximal tubule has been shown to be increased in Hp 2-2 mice, most likely as the result of an impaired clearance of the Hp 2-2–Hb complex (3). The significance of an increased iron load for the proximal tubule is unclear. The Hp 2-2 genotype may be associated with a more rapid progression to end stage kidney disease requiring dialysis (43). We will discuss diabetic renal disease below separately.

Cardiovascular disease (nondiabetic)

There are conflicting data regarding the role of the Hp genotype in cardiovascular disease (CVD) (29). One reason for this controversy is that almost all of these studies have been cross-sectional in nature, examining prevalent as opposed to incident disease. In the majority of the cross-sectional studies individuals with the Hp 2-2 genotype were found to have more disease (higher incidence of prior MI, earlier age at CABG). However, examination of this question in a longitudinal study examining disease incidence has shown the opposite relationship: specifically increased cardiovascular mortality in Hp 1-1 nondiabetic individuals (17). We suggest that this discrepancy between longitudinal and cross-sectional studies may be due to case ascertainment bias. Further studies will be required to clarify if the Hp type plays any role in nondiabetic CVD.

Hp genotype as a determinant of susceptibility to vascular complications in diabetes mellitus (DM)

Hp type is not a determinant of whether an individual will or will not develop diabetes (Type 1 or 2). However, we will now review data demonstrating that the Hp genotype is a determinant of susceptibility to both microvascular and macrovascular complications.

Epidemiological Data Showing that the Hp 2-2 Genotype is a Major Determinant of Susceptibility to Diabetic Cardiovascular Disease in Man

We have established in multiple independent prospective longitudinal studies of >30,000 individuals that the Hp genotype is an independent risk factor for incident CVD and that this relationship is specific for DM (31, 39, 47, 53). After controlling for all conventional cardiac risk factors and DM characteristics in all of these studies, we have consistently found that there is a two- to fivefold increased risk of CVD in DM individuals with the Hp 2-2 genotype (~40% of all DM individuals) as compared to DM individuals without the Hp 2-2 genotype. The Hp 2-2 genotype therefore appears to account for a large portion of the increased burden of CVD associated with DM that heretofore has been unexplainable. In the Strong Heart Study (31), a population based longitudinal study of CVD in American Indians ($n = 4549$), the Hp 2-2 genotype was associated with a three- to fivefold increased incidence CVD in DM individuals. In the Munich Stent Study (47), a consecutive series of 935 DM patients followed for 1 year after PTCA, the Hp 2-2 genotype was associated with a highly significant increase in the incidence of major adverse cardiac events (MACE). In the Rambam Myocardial Infarction Outcomes in Diabetes Study (53), a prospective study assessing the relationship between the Hp

genotype and 30-day mortality and heart failure in 1437 patients presenting with MI (506 with DM), DM individuals with the Hp 2-2 genotype had significantly larger myocardial infarctions and an eightfold increased incidence of death and heart failure as compared to DM individuals with the Hp 1-1 genotype. In the ICARE (Israel Cardiovascular Vitamin E) study (39), the first prospective study investigating the ability of the Hp genotype to predict major adverse cardiac events (myocardial infarction, stroke, and CV death) in the primary care setting, the Hp 2-2 genotype was associated with a two- to threefold increased risk ($n = 3054$). All of these studies were done with predominately Type II DM cohorts. Costacou *et al*. from the Epidemiology of Diabetic Complications group recently demonstrated that the Hp genotype is also a major determinant of CVD risk in Type I DM with an approximately twofold increased risk in Hp 2-2 Type I DM individuals as compared to Hp 1-1 Type I DM individuals and an intermediate risk in Hp 2-1 Type I DM individuals (16).

Epidemiological Data Examining the Association Between the Hp Genotype and Renal Disease in DM

Three cross-sectional studies have demonstrated an association and two cross-sectional studies have not demonstrated an association between the Hp 2-2 genotype and the prevalence of diabetic kidney disease (albuminuria) (43). Recently in the EDC cohort, Hp 2-2 genotype has been demonstrated to be a powerful predictor of the eventual requirement for dialysis (*i.e.*, ESRD) in DM but not the onset of microalbuminuria, suggesting that the primary affect of the Hp type may not be on disease incidence but rather on the rate of decline of renal function (Costacou T and Orchard T, 2008 European Diabetes Association meeting abstract).

Recapitulation of the Association Between the Hp Genotype and Diabetic Vascular Disease in Hp Transgenic Mice

Creation of mice genetically modified at the Hp locus

To test the hypothesis that there exists a cause-effect relationship between the Hp genotype and diabetic CVD, we created mice genetically manipulated to harbor the same Hp polymorphism found in man. Wild-type C57Bl/6 mice contain only a class 1 Hp allele, which is >90% homologous to the human Hp 1 allele. We created a murine Hp 2(m) allele by engineering a duplication of exons 3 and 4 in the genomic sequence of the murine Hp 1 allele (33). We inserted this murine Hp 2 allele at the endogenous Hp locus using a targeting strategy that specifically selected for a homologous recombination event between the murine Hp 2 allele and the endogenous murine Hp 1 allele. These mice were subsequently backcrossed into a C57Bl/6 background for 10 generations.

We have found that the Hp protein produced from the murine Hp 2 allele has the same stoichiometry and Hb binding properties as the human Hp 2 allele protein product (33). These targeted murine Hp 2 mice have several advantages over mice containing the human Hp 2 mice randomly inserted in the genome. First, the murine Hp 2 allele is present at the same chromosomal locus as the murine Hp 1 allele, allowing for similar regulation of the two genes. This is of critical importance for studies involving mice with the Hp 2-1 genotype. If the transcriptional activity of the Hp 1 and Hp 2 alleles is not equivalent, then the amount of Hp 1 and Hp 2 protein will not be equivalent, resulting in an alteration in the shape and stoichiometry of Hp polymers found in Hp 2-1 mice as compared to Hp 2-1 humans. This issue is demonstrated in individuals with the Hp 2-1m genotype (29). These individuals have a mutation in the promoter for the Hp 2 allele so that the transcription of Hp 2 is depressed and less Hp 2 protein is produced. This results in a change in the type of Hp polymers found in Hp 2-1m individuals; >80% of the Hp protein is found as a dimer (similar to Hp 1-1 and the rest is found as a Hp 2-1 trimer (Hp 1-Hp 2-Hp 1). This is unlike the situation found in Hp 2-1 humans where only 20% of the Hp is found as a dimer and polymers with anywhere from 3 to 10 monomers are found (general stoichiometry $(Hp1)_2{*}(Hp2)_n$, where $n = 0$, 1, 2, 3..10. Furthermore, the plasma Hp concentrations in the Hp 1-1, Hp 2-1, and Hp 2-2 mice are the same and are similar to what is found in man ($\sim$1 mg/ml) (4, 29, 33). Second, the murine Hp 2 model simplifies backcrossing with mice genetically modified at other loci (33).

We found no phenotypic difference between Hp 2 (m) mice and Hp 1 mice that can affect atherosclerosis. No difference was found in blood pressure between wild-type Apo E-/-Hp 1-1 mice and ApoE-/-Hp2-2 mice (33). Likewise, no difference was found in the lipid profile between Hp 1-1 and Hp 2-2 mice (4). In order to produce diabetes in these mice, we have used a low dose streptozotocin (stz) protocol that produces significant hyperglycemia but with low long term mortality.

Mechanistic studies designed to explain the association between the Hp genotype and diabetic CVD

Atherosclerotic lesions in Hp 2-2 mice and humans have more iron, oxidative stress, and inflammation. We have used the Hp transgenic mice described above to assess the role of the Hp 2 allele in the atherosclerotic process. We have demonstrated that Hp 2-2 plaques in mice have more plaque iron, more oxidative stress, and a higher infiltration of macrophages (33). We have recently replicated the relationship between plaque iron and Hp type in human atherosclerotic lesions (41). We have hypothesized that this increased iron in Hp 2-2 DM plaques is due to an impaired clearance of Hp–Hb by the CD163 scavenger receptor pathway in Hp 2-2 DM. The increased inflammation found in Hp 2-2 plaques may be attributed to the increased oxidative stress in these plaques as well as to the pro-inflammatory cytokine profile produced by Hp 2-2-Hb (24).

The ability of HDL to promote cholesterol efflux is impaired in Hp 2-2 DM humans and mice (5)

We have assessed the ability of serum from Type I and Type II DM individuals with the different Hp types to promote efflux of ^{3}H-cholesterol from macrophages *in vitro* (Fig. 6). We observed a significantly decreased cholesterol efflux from macrophages incubated with serum from Hp 2-2 DM individuals, both in Type I and II DM. An intermediate level of efflux was found in Hp 2-1 DM individuals. This effect of the Hp genotype on cholesterol efflux was recapitulated using serum from Hp 2-2 DM mice as compared to Hp 1-1 DM mice. No difference in cholesterol efflux was seen in mice or humans with the different Hp genotypes in the absence of DM (5).

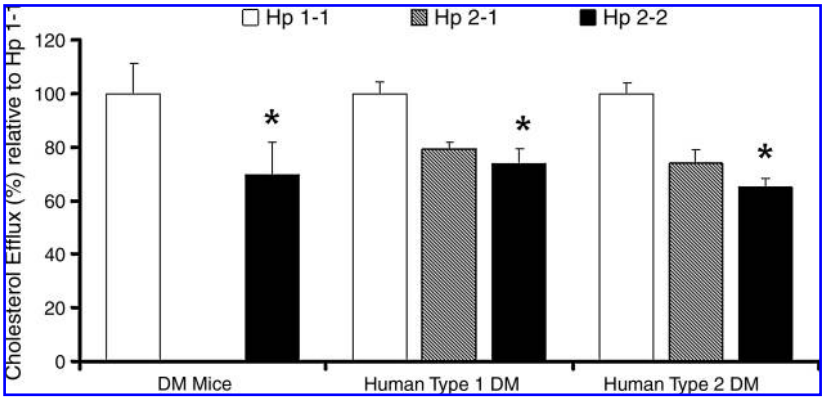

FIG. 6. Cholesterol efflux from cholesterol loaded macrophages stimulated by serum from DM mice and DM humans (Type I and II) with the different Hp genotypes. * indicates that there was significantly less efflux ($p < 0.05$) elicited by Hp 2-2 serum obtained from DM mice, Type I DM, or Type II DM individuals.

We sought to recapitulate this interaction between Hp genotype, DM, and cholesterol efflux using purified components. We evaluated the effect of glycated Hp–Hb (which is increased in DM) on the ability of purified HDL to promote cholesterol efflux from macrophages. We found that purified Hp, or glycated Hp 1-1-Hb, had no effect on efflux elicited by HDL. However, cholesterol efflux elicited by HDL was markedly inhibited by glycated Hp 2-2-Hb (4).

Impaired Reverse Cholesterol Transport *in Vivo* in Hp 2-2 DM mice

We have assessed reverse cholesterol transport (RCT) *in vivo* using a model involving injection of ^{3}H-cholesterol loaded macrophages and monitoring the appearance of the tracer in the plasma, feces, and liver. We found that stz induced DM mice loaded with ^{3}H cholesterol-labeled macrophages had a 40% reduction in ^{3}H-cholesterol in plasma, liver, and feces as compared to non-DM mice. The reduction in RCT was significantly greater in Hp 2-2 DM mice as compared to Hp 1-1 DM mice (54% *vs.* 25% in plasma; 52% *vs.* 27% in liver; 57% *vs.* 32% in feces; $p < 0.03$) (4).

Lecithin Cholesterol Acyl Transferase (LCAT) Activity is Decreased in Hp 2-2 DM Individuals

LCAT is critical for the esterification of HDL cholesterol and the maturation of HDL. We have found that LCAT activity is markedly reduced in the serum of Hp 2-2 DM individuals as compared to Hp 1-1 DM individuals with an intermediate level of activity in Hp 2-1 DM individuals (Fig. 7) (4). LCAT activity was highly correlated with the ability of the serum from DM individuals to promote cholesterol efflux from macrophages *in vitro* ($r = 0.81$, $p = 0.0002$). We did not find any significant correlation between Hp concentration and LCAT activity ($r = 0.19$, $p = 0.50$, $n = 90$).

Increased Lipid Peroxides in the HDL of Hp 2-2 DM Individuals

We assessed the amount of total lipid peroxides in HDL isolated by immunoprecipitation from Hp 1-1 or Hp 2-2 DM individuals (5). We found that lipid peroxides were significantly increased in the HDL of Hp 2-2 DM individuals *versus* Hp 1-1 DM individuals (1.2 ± 0.2 nanomolar *vs.* 1.8 ± 0.2 nanomolar, $n = 20$, $p = 0.04$). This increase in oxidized lipids in Hp 2-2 DM individuals may be attributed to both a decrease in the antioxidant activity of enzymes associated with HDL as well as an increase in the amount of Hb-derived redox active iron associated with the HDL particle. The increase in Hb-derived redox active iron associated with HDL in Hp 2-2 DM is due not only to an increase in the amount of Hb associated with HDL in Hp 2-2 DM but also to the impaired ability of Hp 2-2 to block oxidation by Hb tethered to HDL by Hp (5).

Mechanistic Studies Demonstrating Why There Is Increased Association of Hp–Hb with HDL in Hp 2-2 DM Individuals

The significance of these data is that Hp 2-2-Hb complex associated with HDL in DM may produce oxidative modification of the HDL molecule render it dysfunctional.

The Hp 2-Hb Complex Is Cleared More Slowly than the Hp 1-Hb Complex and These Differences Are Magnified in DM

We assessed the half-life of the Hp–Hb complex *in vivo* by following the clearance of ^{125}I-Hp–Hb, injected by the tail vein, from the serum (5). In non-DM mice, the half-life of the Hp 2-Hb complex during the initial rapid phase of clearance was 2–3 times greater than that of the Hp 1–Hb complex (58 ± 5 *vs.* 23 ± 5 min, $p < 0.0001$). DM resulted in a marked increase in the half-life of the Hp 2–Hb complex. Moreover, the increase in the half-life of the Hp 2–Hb complex in DM mice was significantly greater in Hp 2 DM mice as compared to Hp 1 DM mice (half-life of Hp 2–Hb complex 103 ± 9.5 min in Hp 2 DM mice *vs.* 78.2 ± 8.1 min in Hp 1 DM mice,

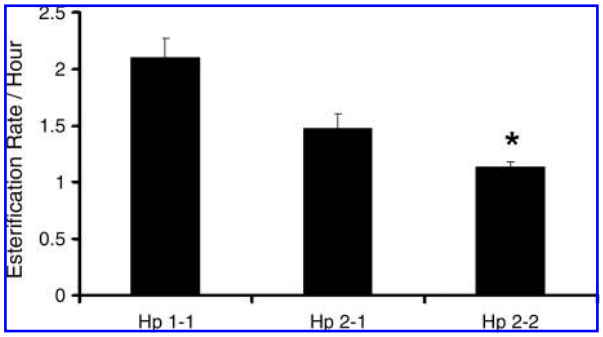

FIG. 7. LCAT activity of serum of DM individuals with the different Hp genotypes. * indicates there was significantly less LCAT activity ($p < 0.05$) in the serum of Hp 2-2 DM individuals.

$p < 0.001$). While intravascular lysis of red blood cells to release Hb into the serum is continuously occurring and would not be expected to be different between Hp 1 and Hp 2 mice, the direct consequence of a slower clearance rate of the Hp 2–Hb complex from the plasmatic compartment is that the steady state serum concentration of the Hp–Hb complex is higher in Hp 2 DM mice. In the next section we show that there is a downregulation of the Hp–Hb scavenger receptor CD163 in Hp 2-2 DM that is very likely to be the cause of the decreased clearance rate of Hp 2-2–Hb in DM.

CD163 Is Downregulated in DM and Hp 2-2 Individuals, and More So in Hp 2-2 DM Individuals

We have demonstrated both by immunohistochemistry and by RT-PCR that in human atherosclerotic diabetic plaques the expression of CD163 is markedly downregulated (34). This is highly remarkable considering that macrophages (the cells expressing CD163) are increased in diabetic plaques. This can be explained by the fact that in non-DM plaques over 70% of macrophages express CD163, while in DM plaques less than 25% of macrophages express CD163. Given that CD163 is associated with an anti-inflammatory macrophage phenotype, this may be reflective of the shift in DM towards a more pro-inflammatory macrophage phenotype. Furthermore, in individuals with DM, with monocyte CD163 surface expression assessed by FACS, we found a marked downregulation of the fraction of monocytes expressing CD163 (2, 34). Finally, in THP-1 monocytes, we have shown that hyperglycemia results in a marked decrease in the expression of CD163 as determined by Western blot (34). The decreased cell associated expression of CD163 appears to be due to both a decrease in CD163 expression (decreased CD163 mRNA) as well as increased shedding of CD163 as evidenced by an increased concentration of a soluble truncated form of CD163 in DM serum (34). Recent work by Boyle and colleagues suggesting the importance of Hp–Hb uptake on the induction of CD163 on macrophages within the atherosclerotic plaque may provide a mechanism for the lower expression of CD163 in Hp 2-2 DM plaques (10).

Taken together, these studies demonstrate that there is decreased clearance of Hp–Hb in Hp 2-2 DM individuals due both to a less efficient clearance of the Hp 2-2–Hb complex by CD163 and to a decrease in the expression of the CD163 receptor in Hp 2-2 DM individuals. As a result of this impaired clearance of Hp–Hb in Hp 2-2 DM individuals, there is a higher concentration of the Hp–Hb complex in Hp 2-2 DM individuals both in the plasmatic compartment as well as in the extravascular compartment which is available to bind to HDL.

Hp and Hb Associate with HDL

We will now review data from humans and transgenic mice showing that more Hp–Hb is associated with HDL in Hp 2-2 DM. We have shown that the binding of Hb to HDL in mice and in man is critically dependent on Hp and that Hb cannot bind alone to HDL.

Hb Is Significantly Increased in the HDL of Hp 2-2 DM Humans

We assessed the presence of Hb by Western blot in HDL immunoprecipitated with apoA1 antiserum from individuals with the Hp 1-1 or Hp 2-2 genotypes with and without DM (5). Quantitative analysis of the amount of Hb identified by Western blots of HDL immunoprecipitates from 15 Hp 1-1 DM and 15 Hp 2-2 DM humans demonstrated an approximately twofold increase in the amount of Hb associated with HDL in Hp 2-2 DM as compared to Hp 1-1 DM. This specific association between Hp genotype, DM, and HDL was recapitulated in mice (5).

The Ability of ^{125}I-Hb to Bind to Human HDL *in Vitro* Is Increased in Hp 2-2

We assessed the ability of ^{125}I-Hb to bind to human HDL in human serum *in vitro* by immunoprecipitation (5). ^{125}I labeled-Hb was incubated with serum from DM individuals with the Hp 1-1 or Hp 2-2 genotypes. We found an increased amount of Hp associated with HDL in the immunoprecipitate from Hp 2 as compared to Hp 1 individuals (3978 ± 171 *vs.* 1984 ± 171 cpm, $p < 0.00005$).

Increased Binding of Hp 2 as Compared to Hp 1 to HDL in Mice Injected with Hp–Hb *in Vivo*

We assessed the ability of Hp to associate with HDL *in vivo* in mice injected with ^{125}I-Hp–Hb (label on the Hp) by measuring the appearance of the label in the HDL immunoprecipitate (5). In Hp 2 mice, 75 min after injection of the complex, a significantly higher percentage of the total plasma Hp 2–Hb complex, as compared to the total plasma Hp 1–Hb complex, consisted of complex bound to HDL ($19.8 \pm 4.4\%$ *vs.* $8.3 \pm 1.3\%$, $p = 0.028$). In Hp 2 DM mice, an even higher percentage of the total plasma Hp 2–Hb complex was found to be bound to HDL as compared to non-DM Hp 2 mice ($46.5 \pm 1.9\%$, $p < 0.0001$ compared to Hp 2–Hb in non-DM mice). Similar results were obtained in Hp 1 mice. Similar results were also obtained in Hp 1 or Hp 2 mice with or without DM when injecting ^{125}Hb (which immediately complexes with Hp *in vivo*). However, when ^{125}Hb was injected into Hp 0 (knockout mice) in whom no Hp–Hb could form, there was no detectable label in the HDL immunoprecipitate (0 cpm in the HDL immunoprecipitate) (5). (As a control in these Hp 0 mice ^{125}Hb–Hp was also injected and the label was found in the HDL immunoprecipitate.) These data provide direct evidence that Hp–Hb can bind to HDL *in vivo* and that Hp is absolutely essential for the association of Hb with HDL *in vivo.*

The Redox Activity of Hemoglobin-Derived Iron Present in the Hp–Hb Complex Is Dependent on the Hp Genotype and DM

We and others have demonstrated significant differences *in vitro* between the Hp 1 and Hp 2 proteins in their ability to block Hb-induced oxidation (20). As opposed to the Hp 1-1 protein, the Hp 2-2 protein is unable to fully stabilize Hb iron in the heme pocket of Hb and therefore the Hp 2-2–Hb complex but not in the Hp 1-1–Hb complex can mediate oxidative reactions (20). *In vitro* the Hp 2-2–Hb complex has been shown to readily oxidize both lipid and protein components of lipoproteins (LDL and HDL) to a markedly greater extent than Hp 1-1–Hb (4, 20). The ability of the Hp 2-2 protein to block Hb-induced oxidation is further impaired in DM, apparently due to the increased glycation of Hb that accelerates

the loss of heme iron from Hb (3). These differences between Hp 1-1 and Hp 2-2 in protecting against Hb-induced oxidation have profound significance for understanding why there is greater oxidative modification of HDL in Hp 2-2 DM. Not only is there more Hb present in the HDL of Hp 2-2 DM but also the Hb which is present in Hp 2-2 DM HDL is more redox active than the Hb which is present in Hp 1-1 DM HDL.

Increase in the Amount of Oxidant Activity and Redox Active Iron Associated with the HDL of Hp 2-2 DM Individuals

We have proposed that Hb-derived iron associated with the HDL in Hp 2-2 DM individuals would result in increased oxidative modification of HDL-associated lipids and proteins. We sought to demonstrate that there was an increase in the amount of redox activity attributable to iron associated with HDL in Hp 2-2 DM individuals. We found that there was significantly more redox active chelatable iron in the HDL of Hp 2-2 DM individuals as compared to Hp 1-1 DM individuals (5). The increased redox active chelatable iron in the HDL of Hp 2-2 DM individuals appears to be responsible for the increased oxidation of HDL-associated lipids and proteins in Hp 2-2 DM HDL. There is an increase in the amount of redox active iron in Hp 2-2 DM HDL not only because there is an increase in the amount of Hb present but also because the Hb present (as a Hp 2-2–Hb complex) is more redox active than the Hb present in HDL of Hp 1-1 DM individuals. Both of these factors must be taken into account in understanding why the oxidative modification and loss of function mediated by Hb on HDL is greater in Hp 2-2 DM individuals.

Taken together, these data provide experimental basis for why there is more Hp 2–Hb associated with HDL in DM and why this Hp 2–Hb associated with HDL can oxidatively modify HDL to a significantly greater degree than Hp 1–Hb. The increased oxidation of Hp 2-2 DM HDL may explain why HDL is dysfunctional in Hp 2-2 DM individuals and why Hp 2-2 DM individuals uniquely derive benefit from antioxidant therapy as demonstrated below.

Antioxidant Therapy Can Decrease HDL Oxidation and Restore Normal HDL Function to Hp 2-2 DM Mice and Individuals

As a result of the combined insult of increased prooxidative activity from Hb and decreased antioxidative activity associated with HDL in Hp 2-2 DM individuals, we hypothesized that there was an increased oxidative modification of HDL in Hp 2-2 DM. Given that oxidative modification of HDL may inhibit its function we hypothesized that antioxidant supplementation to Hp 2-2 DM mice or humans would improve HDL function.

Chronic vitamin E supplementation to Hp 2-2 DM mice decreases HDL oxidation and improves the ability of Hp 2-2 DM serum to promote cholesterol efflux from macrophages in vitro

Hp 1-1 DM or Hp 2-2 DM mice were treated for 28 days with either placebo or vitamin E, alpha-tocophorol acetate at 600 mg/kg/day, beginning 3 weeks after the induction of DM. HDL lipid peroxides and cholesterol efflux from macrophages promoted by the serum of these mice was assessed (5). Vitamin E had no effect on HDL lipid peroxides in Hp 1-1 DM mice but dramatically reduced lipid peroxides associated with HDL in Hp 2-2 mice. Vitamin E significantly improved cholesterol efflux in Hp 2-2 DM mice, but had no effect on HDL function in Hp 1-1 DM mice, thereby demonstrating a pharmacogenomic effect (5). These data are important because (a) they show that changes in HDL oxidation are correlated with changes in HDL function; (b) they show that HDL oxidation can be prevented with vitamin E; (c) they provide biochemical evidence for a pharmacogenomic effect of vitamin E.

Chronic vitamin E supplementation to Hp 2-2 DM humans decreases HDL oxidation and improves the ability of their serum to promote efflux in vitro *(5)*

Vitamin E (400 mg natural source per day) versus placebo was administered to 20 Hp 2-2 DM individuals in a double-blinded crossover study. Half of the patients received vitamin E for the first 2 months of the study and half of the patients received placebo for the first 2 months of the study. Serum was collected at baseline, after the first 2 months of treatment and after the second 2 months of treatment. We found that

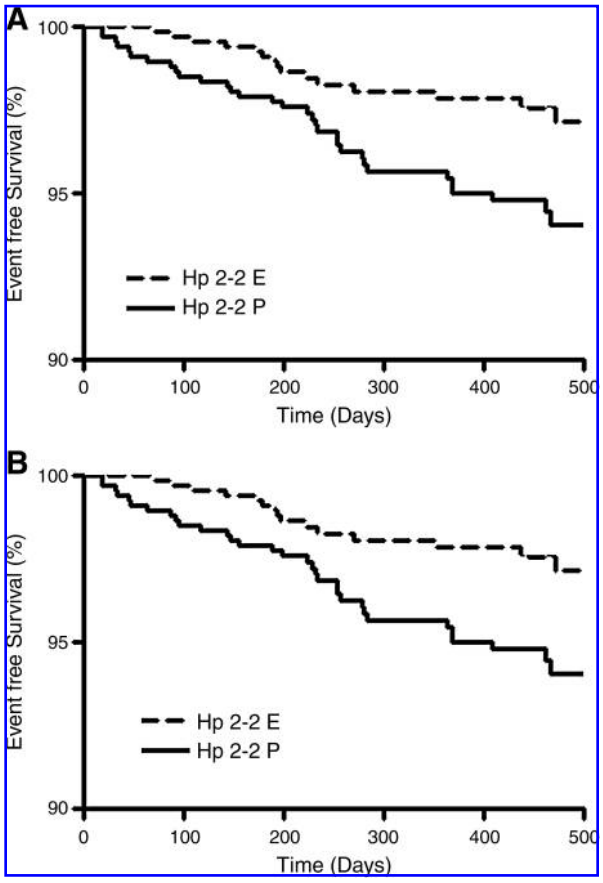

FIG. 8. Kaplan–Meier plot of ICARE results with Hp types determined by polyacrylamide gel electrophoresis (A) or ELISA (B) demonstrating that both methods provide equivalent results. Individuals with the Hp 2-2 genotype who were randomized to placebo (P) had significantly more CVD events (MI, stroke, CV death) as compared to Hp 2-2 individuals who were randomized to vitamin E therapy (E) ($P = 0.01$ by log rank).

vitamin E dramatically decreased HDL lipid peroxides and improved the ability of serum from Hp 2-2 DM individuals to mediate efflux of cholesterol *in vitro* (5). No change in lipid peroxides or efflux was found in the placebo group and importantly, withdrawing vitamin E for as little as 2 months resulted in a loss of the beneficial effect of vitamin E.

Vitamin E Reduced CVD Death and MI in Hp 2-2 DM Individuals in the HOPE Study

Participants in the HOPE study were randomized to natural source vitamin E (400 IU/day) and/or ramipril. No benefit was found from vitamin E supplementation in the entire HOPE cohort or in the diabetic cohort alone (54). We determined the relative risk reduction associated with vitamin E therapy according to Hp type in patients with and without DM. We found that in Hp 2-2, DM participants, vitamin E reduced CV death (relative risk 0.45 95% CI 0.23–0.90) and nonfatal MI (relative risk 0.57 95% CI 0.33–0.97) (32).

Vitamin E Reduced the Primary Composite Endpoint of CVD Death, MI, and Stroke in a Prospective Randomized Double-Blind Clinical Study

Due to the retrospective nature of the HOPE study analysis (32), we recognized the need to validate the HOPE results in a prospective placebo controlled double-blind clinical trial (ICARE) (39). DM individuals with the Hp 2-2 genotype were randomized and treated with either natural source vitamin E (400 IU/day) or placebo. The primary composite outcome was nonfatal myocardial infarction, stroke, and cardiovascular death. The primary composite outcome was significantly reduced in patients receiving vitamin E compared to placebo (2.2% *vs.* 4.7%, Hazard Ratio 0.30, 95% CI 0.27–0.82, $P = 0.01$ by log rank analysis) with equivalent results obtained when the Hp type was determined by polyacrylamide gel electrophoresis (Fig. 8A) or by ELISA (Fig. 8B). Although they were not treated with vitamin E, Hp 1-1 and Hp 2-1 individuals were also followed in the ICARE cohort. Whereas the event rate (unadjusted or adjusted by Cox regression) was increased more than twofold in Hp 2-2 individuals randomized to placebo as compared with Hp 1-1 and Hp 2-1 individuals, the event rate in Hp 2-2 individuals randomized to vitamin E was remarkably similar to that of Hp 1-1 and Hp 2-1 individuals (39).

Significance and Future Directions

Clearly these pharmacogenomic studies will require validation in an additional clinical trial before Hp typing of all DM individuals to determine whether they should or should not take vitamin E can be recommended as a treatment guideline (8). Such a pharmacogenomic approach appears warranted based on the finding in several metanalyses that indiscriminate use of vitamin E supplements in all individuals is associated with a 5–10% increase in overall mortality (38). However, the potential public health and economic benefits from this pharmacogenomic paradigm are enormous. Hp genotyping may allow a personalized approach to medical care enabling identification of individuals who will benefit from vitamin E supplementation dramatically reducing the burden of CVD in this population. DM prevalence is increasing at an alarming rate, primarily due to poor nutrition, among individuals of lower socioeconomic status in the USA and in the developing world as well. Tragically, the medications to prevent diabetes-induced cardiovascular complications (statins, ACE inhibitors) are neither accessible nor affordable to these populations. Vitamin E, however, is so inexpensive that it can be expected to be made available to all populations world wide. Furthermore, the technological requirements for Hp typing (which only needs to be done once in a lifetime) are extremely simple and it is expected that a diagnostic ELISA based kit will become available for Hp typing within the coming year (57).

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Author Disclosure Statement

Dr. Levy serves as a consultant for Synvista Therapeutics which owns a patent that claims to predict susceptibility to diabetic complications based on the Hp genotype.

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Abbreviations Used

ACE = angiotensin converting enzyme
ApoA1 = apolipoprotien A1
ApoE = apolipoprotein E
CABG = coronary artery bypass grafting
CHO = Chinese hamster ovary
CI = confidence interval
CPM = counts per minute
CVD = cardiovascular disease
DHR = dihydrorhodamine
DM = diabetes mellitus
EDC = Epidemiology of Diabetic Complications study
ELISA = enzyme linked immunoabsorbant assay
ESRD = end stage renal disease
FACS = fluorescent activated cell sorter
Hb = hemoglobin
HDL = high density lipoprotein
HO = heme oxygenase
HOPE = Heart Outcomes Prevention Evaluation study
Hp = haptoglobin
Hp–Hb = haptoglobin–hemoglobin complex
ICARE = Israel Cardiovascular Vitamin E study
IU = International units
LCAT = lecithin cholesterol acyl transferase
LDL = low density lipoprotein
MACE = major adverse cardiac events
MI = myocardial infarction
PCR = polymerase chain reaction
PTCA = percutaneous transluminal coronary angioplasty
RCT = reverse cholesterol transport
SAH = subarachnoid hemorrhage
Stz = streptozotocin

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