Haptoglobin phenotypes and plasma haptoglobin levels in patients with abdominal aortic aneurysm

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Objective: **Inflammation is associated with the disruption of the aortic media and appears to play a fundamental role in the progression and development of abdominal aortic aneurysm (AAA). Haptoglobin (Hp) is a genetically determined acute phase protein, the synthesis of which is increased during inflammation. This study was designed to investigate both phenotype and plasma levels of Hp in patients with AAA.**

Methods: **Patients with documented AAA who were admitted for elective open repair operation or endograft stent implantation, and non-AAA subjects admitted for coronary arteriography, but found to have normal or insignificant coronary artery disease, were included in the study. Plasma Hp levels were determined using a standard specific enzyme-linked immunosorbent assay, while Hp phenotype was determined by native polyacrylamide gel electrophoresis. Total cholesterol, high density lipoprotein, low density lipoprotein, and triglyceride levels were analyzed enzymatically, and C-reactive protein was analyzed by immunochemistry.**

Results: **Forty-five patients with AAA and 49 non-AAA subjects were included. The Hp 2-2 phenotype was more predominant in AAA patients compared with non-AAA subjects, but this difference was not significant (67% vs 47%; $P = .141$), while plasma Hp concentrations were significantly higher in AAA patients (237 ± 144 vs 163 ± 86 ng/mL; $P = .024$). Further analysis revealed that plasma Hp concentrations were significantly higher in AAA patients with the 2-2 phenotype compared with corresponding non-AAA subjects (238 ± 144 vs 163 ± 86 ng/mL;$P = .024$).**

Conclusions: **Our findings suggest that plasma Hp concentrations are elevated in patients with AAA, particularly those with the Hp 2-2 phenotype. (J Vasc Surg 2011;53:1189-94.)**

Abdominal aortic aneurysm (AAA) is a documented lethal disorder in older adults, the risk of rupture with which increases with aneurysm size.[1] In normal aortas, the lamellar structure of media is the thickest layer and is an orderly array of collagen, elastin, and smooth muscle cells. Degeneration of this matrix leads to destruction of the media and adventia, which in turn results in gradual aortic dilation.[2,3] Inflammation is associated with disruption of the orderly structure of the aortic media and appears to play a fundamental role in the progression and development of AAA.[4,5]

Haptoglobin (Hp) is the major hemoglobin (Hb)-binding protein in humans and other mammals, and is one of the few acute phase proteins that exhibits increased synthesis during inflammation and is conserved in all vertebrate species studied.[6,7] Hp is an extremely potent antioxidant that directly inhibits low-density lipoprotein oxidation.[8] Structurally, Hp is tetrameric or (β-α-α-β) joined by disulfide linkages between the two α and β chains. Three Hp phenotypes, 1-1, 2-1, and 2-2 share the same two β chains.[9] Functional differences between the Hp phenotypes have been demonstrated; these appear to have important biological and clinical consequences.[10]

Controversy exists with regard to the relationship between Hp phenotypes and the prevalence of cardiovascular disease. Findings from the Framingham offspring cohort suggest that there is decreased prevalence of coronary heart disease (CHD) in diabetic individuals with allele 2 (Hp 2-1 and Hp 2-2).[11] In contrast, an increased prevalence of CHD was found in nondiabetic subjects with the Hp 2-1 phenotype compared with those with the Hp 1-1 phenotype. In another study, it was reported that the risk of cardiovascular disease in patients with sleep apnea aged <55 years with the Hp 2-2 phenotype was 2.32-fold higher than that in corresponding patients with the 2-1 phenotype.[12] Further to this, it has been found that myocardial infarction patients with Hp 2-2 have more frequent left ventricular failure than patients with Hp 1-1 and Hp 2-1.[13] Several other studies have reported relationships between the Hp 2-1 phenotype and the occurrence of AAA.[14,15]

Although Hp phenotypes have been frequently reported to be associated with cardiovascular diseases, human plasma Hp concentrations, which may be associated with diseases and of diagnostic significance, have rarely been reported. This is almost certainly a reflection of the difficult purification procedures necessary for Hp immunoassay,[13,16] although we have previously assayed this protein

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in humans and reported that there is significant between-phenotype variability in plasma Hp levels.[17]

It would be interesting to know more about the relationships between plasma Hp levels, Hp phenotype, and AAA. Hence, the aims of this study were to determine: a) the association (if any) between Hp phenotype and AAA; and b) plasma Hp levels in AAA and non-AAA individuals with different Hp phenotypes.

METHODS

Patients. The study participants were consecutively enrolled between January 2009 and December 2009 in the Divisions of Cardiovascular Surgery and Cardiology of Taipei Veterans General Hospital. The cohort consisted of 45 patients with AAA and 49 non-AAA subjects.

Elective open repair operation or endograft stent implantation was performed whenever informed consent was obtained after consensus indications of surgery were explained to the patients with AAA. During the same period, patients with positive thallium-201 myocardial perfusion scans admitted with suspected coronary artery disease but showing normal or insignificant coronary artery disease (<40% luminal stenosis), normal ventricular diameter (as determined by both coronary arteriography and left ventriculography), and normal abdominal aorta diameter (as determined by aortographic examination) were categorized as non-AAA. Patients with acute or chronic infectious diseases and malignancy were excluded from the study.

Analysis of lipid profiles. Blood samples were obtained (before surgical intervention) from all patients following overnight fasting (12 hours) and mixed with 0.1% ethylenediamine tetraacetic acid. Total cholesterol, high density lipoprotein, low density lipoprotein, and triglyceride levels were analyzed enzymatically using commercial reagents (CHOP-PAP Method; Merck Scientific Corporation, Germany). ApoAI concentration was determined by high sensitivity enzyme-linked immunosorbent assay (AlerCHEK, Inc, Portland, Me).

Measurement of human C-reactive protein (CRP) plasma levels. The IMMAGE Immunochemistry Systems CRPH reagent (Beckman Coulter, Inc, Fullerton, Calif), based on the highly sensitive Near Infrared Particle Immunoassay rate methodology, was used to measure CRP. In this assay, an anti-CRP coated particle binds to CRP in the patient sample, resulting in the formation of insoluble aggregates causing turbidity. The rate of aggregate formation is directly proportional to the concentration of CRP in the sample.[18]

Hp phenotyping. Hb was isolated from lysed red blood cells. Hp phenotyping was conducted by native polyacrylamide gel electrophoresis with Hb-supplemented plasma. This assay has been described in detail in our previous publication.[17]

Hp purification. Hp purification was performed using Ab-affinity chromatography. The affinity column (with a 10 mL bed volume at room temperature) was washed with 50 mL of phosphate buffered saline. The bound materials were further washed with 50 mL of 0.02 M phosphate buffer containing 0.2 M NaCl (pH 7.4), and then eluted with 50 mL of a freshly prepared 0.15 M NaCl solution (pH 11). Five milliliters of each fraction was collected in a tube containing 0.25 mL of 1M Tris-HCl buffer (pH 6.8) to facilitate neutralization of solution.

Pooled fractions containing Hp were then concentrated to a final volume of 1 mL using Centricon tubes (Millipore, Cork, Ireland) and filtered through a 0.45-μm membrane. Finally, the protein was run on a gel-filtration HPLC Superose-12 column (1 × 30 cm; Pharmacia, Uppsala, Sweden). The homogeneity of each isolated Hp type was >95% as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Lyophilized Hp was stored at −80°C until analysis.

Preparation of anti-Hp monoclonal antibodies. Six monoclonal antibodies (8B1-3A, W1-11G, 2-3H, G2D-7G, 12B-1, and 4A2-4H) against human Hp were produced and characterized as previously described.[17] Monoclonal antibody 8B1-3A, which possesses the highest binding affinity for Hp, was selected for preparation of the affinity column. Briefly, 120 mL of cultured medium from the 8B1-3A hybridoma was precipitated in 50% saturated ammonium sulfate. The precipitate was dissolved in 12 mL of phosphate buffered saline (PBS) containing 0.02 M phosphate and 0.15M NaCl (pH 7.4). The solution was then dialyzed exhaustively in PBS to remove the remaining ammonium sulfate. This was followed by dialysis in coupling buffer containing 0.1M $NaHCO_3$ and 0.6M NaCl, (pH 8.3). All monoclonal antibody binding epitopes were directed against the β-chain.

Measurement of human-free Hp plasma levels. Human-free Hp plasma levels were measured using a phenotype-matched standard sandwich enzyme-linked immunosorbent assay, which has been comprehensively described in a previous publication.[17]

Statistical analysis. Normal distributed continuous variables are presented as the mean ± standard deviation. Abnormally distributed continuous variables are presented as the median (interquartile range). Categorical variables are presented as the number in each category and the percentage. Data were compared between groups by independent samples *t* test (normally distributed continuous variables), Mann-Whitney *U* test (abnormally distributed continuous variables) and X^2 or Fisher's exact test (categorical variables). Two-way analysis of variance was performed to examine the relationship between age and Hp concentration with regard to AAA grouping (AAA or non-AAA). Subsequent analysis of covariance was performed due to the lack of a significant interaction effect. All analyses were performed using SAS statistical software (version 9.1.3; SAS Inc, Cary, NC). All tests were two-sided, with differences being considered significant when $P < .05$.

RESULTS

A total of 94 subjects were recruited during the study period. Table I summarizes the characteristics of the 94 subjects with respect to AAA grouping (ie, AAA or non-AAA). There were no between-group differences in terms

Table I. Summary of demographic characteristics, laboratory data, and Hp phenotypes for the 94 subjects included in this study

	Non-AAA (n = 49)	*AAA (n = 45)*	P *value*[e]
Age (years)[a]	74 (72, 79)	76 (73, 80)	.404
Male gender[c]	41 (84)	39 (87)	.684
Body mass index (kg/m^2)[b]	24.8 ± 3.4	23.7 ± 2.9	.106
Total cholesterol (mg/dL)[a]	180.8 ± 32.9	178 ± 45	.771
Triglyceride (mg/dL)[a]	103 (72, 163)	114 (90, 145)	.389
High density lipoprotein cholesterol (mg/dL)[a]	37 (30, 50)	40 (34, 48)	.585
Low density lipoprotein cholesterol (mg/dL)[b]	111 ± 34	110 ± 38	.942
Hp concentration (ng/mL)[b]	186 ± 108	254 ± 158	.017[f]
C-reactive protein concentration (mg/dL)[b]	0.476 ± 1.086	1.107 ± 2.073	.0496
Smoking[c]	24 (50)	31 (69)	.064
Drinking[c]	16 (34)	15 (33)	.943
Diabetes[d]	2 (4)	7 (16)	.084
Hypertension[c]	14 (29)	32 (71)	<.001[f]
Hp phenotype[d]			.141
1-1	4 (8)	2 (4)	
2-1	22 (45)	13 (29)	
2-2	23 (47)	30 (67)	

AAA, Abdominal aortic aneurysm; *Hp*, haptoglobin.
Continuous variables are presented as median (interquartile range) or mean ± standard deviation; categorical are presented as number (%).
[c]*P* values were determined by Mann-Whitney *U* test,[a] independent samples *t* test,[b] X^2 test,[c] or Fisher's exact test.[d]
[f]Indicates a statistically significant between groups difference ($P < .05$).

Table II. Plasma Hp concentrations stratified with respect to Hp phenotype and whether the patient did or did not have AAA

	Plasma Hp concentration (ng/mL)		
Hp phenotype	*Non-AAA (n = 49)*	*AAA (n = 45)*	P *value*[a]
1-1	105 ± 60	276 ± 143	.089
2-1	226 ± 123	288 ± 193	.256
2-2	163 ± 86	238 ± 144	.024[b]

AAA, Abdominal aortic aneurysm; *Hp*, haptoglobin.
[a]Data are presented as mean ± standard deviation and were compared by independent samples *t* tests.
[b]Indicates a statistically significant between groups difference ($P < .05$).

Table III. Plasma Hp concentrations stratified with respect to hypertension and whether the patient did or did not have abdominal aortic aneurism

	Plasma Hp concentration (ng/mL)		
Hypertension	*Non-AAA (n = 49)*	*AAA (n = 45)*	P *value*[a]
No	182 ± 117	333 ± 195	.019[b]
Yes	196 ± 85	220 ± 128	.517

AAA, Abdominal aortic aneurysm; *Hp*, haptoglobin.
[a]Data are presented as mean ± standard deviation and were compared by independent samples *t* tests.
[b]Indicates a statistically significant between groups difference ($P < .05$).

of age, gender distribution, body mass index, smoking or drinking habits, diabetes history, Hp phenotype distribution, or any biochemical measure. Patients with AAA had significantly higher concentrations of Hp than their non-AAA counterparts ($P = .017$), and a significantly higher percentage of these patients also suffered from hypertension ($P < .001$). The concentration of CRP was marginally higher ($P = .049$) in AAA patients.

Table II summarizes plasma Hp concentrations with respect to phenotype and AAA status. Plasma Hp concentrations for the 2-2 phenotype were significantly higher in patients with AAA compared with those who did not have AAA ($P = .024$). Plasma concentrations of Hp in phenotype 1-1 and 2-1 patients were somewhat higher in those with AAA, but this trend did not reach statistical significance.

The effect of hypertension on the association between AAA and plasma Hp concentration is shown in Table III. In patients without hypertension, plasma Hp concentration was significantly higher in the AAA group ($P = .019$), but in patients with hypertension, the plasma Hp concentration was similar in both AAA and non-AAA groups. Hypertension was not associated with Hp levels in the absence of AAA ($P = .069$).

As age has been reported to be related to AAA,[19] the effect of age on plasma Hp levels in the 53 patients with the 2-2 phenotype was examined. There was no significant interaction between age and the occurrence of AAA (ie, the correlation between these variables was similar for both the AAA and non-AAA groups [data not shown]). Subsequent analysis of covariance revealed that AAA status was the only significant factor affecting Hp concentration. For the 2-2 phenotype, plasma Hp concentrations were significantly higher in patients with AAA compared with subjects who did not have AAA ($P = .031$). Age was not significantly associated with plasma Hp level ($P = .380$; Fig).

In the study cohort, aspirin was prescribed in 13 AAA patients and eight non-AAA patients. In the total patient population, no statistically significant difference in Hp con-

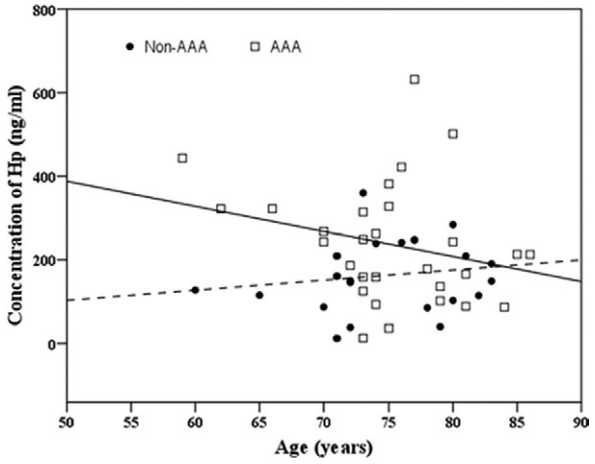

Fig. Scatterplot showing age and plasma haptoglobin (*Hp*) concentration for the 53 2-2 phenotype study participants (abdominal aortic aneurysm [*AAA*] and non-AAA). The *solid line* is the fitted line for the AAA group, while the *dotted line* is the fitted line for the non-AAA group. Two-way analysis of variance revealed that there was no interaction between age and group (AAA and non-AAA). Hence the interaction term was removed, and analysis of covariance was performed. The result of analysis of covariance revealed a significant effect of AAA ($P = .031$) but non-significant effect of age ($P = .380$).

centration was found between patients taking aspirin and those not taking aspirin ($P = .193$). When patients were subgrouped into AAA and control (non-AAA) patients, no difference in Hp concentration was found between control patients taking aspirin and those not taking this drug ($P = .960$). However, AAA patients taking aspirin had lower Hp concentrations than those not taking aspirin ($P = .006$).

DISCUSSION

In this study, we examined the relationships between plasma Hp levels and Hp phenotype in patients who did and did not have AAA. While there were some trends toward differences in phenotypic distribution between the AAA and non-AAA individuals, these did not reach statistical significance. Of note, however, plasma Hp levels were significantly higher in 2-2 phenotype patients with AAA compared with their non-AAA counterparts with the same Hp phenotype. This effect was independent of age. Also, in AAA patients, Hp levels were significantly higher in patients not taking aspirin than in those taking this anti-inflammatory drug. This result indirectly shows that the increase in Hp concentration is closely related to the inflammatory response that leads to AAA pathology.

Hypertension has been documented as a risk factor like other clinical risk factors, including smoking, advanced age, male gender, chronic obstructive pulmonary disease, and family history that predisposes individuals to degenerative diseases in the arterial wall.[20] In our study, a significant interaction between hypertension and AAA was found, but hypertension gave no add-on effect to Hp concentration for AAA diagnosis. Therefore, it seems that Hp is an independent risk factor for AAA.

In humans there exist two classes of alleles for Hp, designated one and two. The Hp polymorphism is a common polymorphism. A geographic ethnic difference in Hp phenotypes has been reported between Western and Asian populations. In Western populations, it has been reported that 16% of individuals have the 1-1 phenotype, 36% the 2-2 and 48% the 2-1.[21] In contrast, the frequency of the 1 to 1 phenotype has been found to be lower in Asian populations, while the frequency of 2 to 2 is higher.[1,10,22] The findings from the current study regarding phenotype distribution are in agreement with those previously reported.

In the present study, there was an increased (but non-significant) tendency for individuals with AAA to have the 2-2 phenotype. This finding contrasts with that reported by Norrgard and colleagues, who found that there was an increased frequency of the 2-1 phenotype in Northern Swedish individuals with AAA.[14] The reason for this inconsistency is unclear, but may be related to ethnicity. It also must be acknowledged that a larger number of patients should be studied to confirm our findings.

Other studies have reported increased incidences of disease in individuals with the Hp 2-2 phenotype. Asleh et al reported that diabetic individuals homozygous for the Hp 2 allele (Hp 2-2) had a five-fold greater risk of cardiovascular disease compared with diabetic individuals homozygous for the Hp 1 allele (Hp 1-1).[23] These researchers also found that hemoglobin within plaque derived from microvascular hemorrhage was cleared more slowly from plaques associated with the Hp 2-2 phenotype as compared with Hp 1-1 plaques.[24] This suggests that the Hp 2-2 phenotype is associated with an increased risk of atherosclerotic cardiovascular disease. In a recently published study of diabetic transgenic mice,[25] it was found that the Hp 2-2 phenotype was associated with increased mortality and more severe cardiac remodeling 30 days after myocardial infarction. In addition, Hp 2-2 has been associated with refractory hypertension,[26] is a predictor of diabetic nephropathy,[27] is associated with a higher risk of mortality in tuberculosis,[28] and a lower hepatitis B level[29] than other phenotypes.

The Hp 2-2 phenotype differs structurally from the other two phenotypes in that these phenotypes are either dimers (Hp 1-1) or form linear polymers (Hp 2-1) while the 2-2 phenotype polymerizes into rosettes of varying complexity. Scavenging of Hb by endocytosis of Hb -Hp complexes has been found to occur via the CD163 receptor.[30] Hp 1-Hb complexes stimulate anti-inflammatory macrophages, while Hp 2-Hb complexes do not. Strauss and Levy reported that casein kinase II activity was increased following the binding of Hp 1-1 Hb to CD163.[31] Reduced clearance of the macrophage-Hp-Hb complex, favoring iron deposition, oxidative stress, and active macrophage accumulation was found in individuals with the Hp 2-2 phenotype.[32] Impaired Hb clearance capacity has

also been found in Hp 2-2 diabetic individuals with plaque rupture propensity.[33]

Increased matrix metalloproteinases (MMP)-9 and MMP-2 activity is associated with destruction of the elastic laminae of arteries and aneurysm formation in humans.[34] Complexes of MMP-9 and Hp have been detected in sera of cows with acute inflammation, but not in clinically normal cows or cows with chronic disease.[35] The interaction between Hp and MMP in aneurysm development has not been fully investigated. A functional study performed by de Kleijn et al revealed that Hp inhibits the activity of MMP-2 by 50% and MMP-9 by 30% in cultured arterial smooth muscle culture cells, but increases the production of gelatin in arterial smooth muscle cells in Hp knockout mice.[36] These findings suggest that Hp may play a role in the regulation of the arterial restructuring.

All of the above observations suggest that individuals with specific Hp phenotypes may respond differently to biological insults such as inflammation and vessel wall degeneration. The synthesis of Hp may reflect a regulatory response to these pathologic processes. In the present study, the finding that plasma Hp concentrations were significantly higher in patients with AAA of the 2-2 phenotype compared to corresponding non-AAA individuals is of interest and agrees with our previous findings demonstrating that significant between-phenotype variability in plasma Hp levels occurs.[17] However, in this case-control observational study, the study cohort was recruited from a hospital-based population, was relatively small in size (with a narrow age range), and although the two study arms had similar demographics, some unknown bias might have occurred because the patients were not randomly selected. If the results of this study are confirmed and extended by a further larger scale, more stringently designed study, Hp phenotype and concentration may be potential biomarkers of abdominal aortic aneurysm development that can be used clinically in association with other biomarkers.

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AUTHOR CONTRIBUTIONS

Conception and design: J-PP, C-CS, SM, S-TL
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Writing the article: J-PP
Critical revision of the article: J-PP, SM
Final approval of the article: J-PP
Statistical analysis: J-PP, S-CChi
Obtained funding: J-PP, C-CS, S-TL
Overall responsibility: J-PP, T-MC, C-CS, S-CChi, S-CCho, SM, S-TL

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