

Immunochemical property of human haptoglobin phenotypes: Determination of plasma haptoglobin using type-matched standards

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Received 20 December 2006; received in revised form 2 April 2007; accepted 10 April 2007

Available online 24 May 2007

Abstract

Objectives: Haptoglobin (Hp) phenotypes 1-1, 2-1, and 2-2 are associated with inflammatory diseases. Since their biochemical structures are rather heterogeneous, it is necessary to accurately determine the plasma Hp levels.

Design and methods: Immunodiffusion, immunoturbidimetric, and noncompetitive ELISA were conducted to determine the differences in immunoreactivity among Hp phenotypes and to verify that such difference may significantly affect the outcome of Hp determinations. A novel ELISA using phenotype-matched calibrators was performed to compared with a commercial GenWay ELISA kit using a single calibrator in normal healthy males.

Results: In immunodiffusion and immunoturbidimetric assays, the immunoreactivity of Hp 1-1 was markedly higher than 2-1 and 2-2, while an opposite result was observed using an ELISA. The latter was primarily due to the repeated antigenic epitopes in polymeric 2-1 and 2-2. Thus, Hp levels could be significantly over- or underestimated depending on the method. An accurate ELISA could be achieved when using each type-specific Hp calibrator matched to each type subject. We show the mean levels of Hp 1-1 subjects ($n=16$; 184 ± 42 mg/dL) to be significantly and differentially greater than 2-1 ($n=28$; 153 ± 55 mg/dL) ($p<0.05$) and 2-2 ($n=24$; 93 ± 54 mg/dL) ($p<0.01$) subjects.

Conclusions: Due to the diverse immunochemical structure among the Hp types, phenotyping should be performed in all the patients and a type-matched Hp calibrator should be used in clinical Hp determination.

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Keywords: ELISA; Immunoreactivity; Haptoglobin phenotypes; Plasma concentration; $\alpha_1\beta$ and $\alpha_2\beta$ chain expression; HepG2

Introduction

Similar to blood type, Hp has been classified into three phenotypes: Hp 1-1, 2-1, and 2-2. The structural and functional relationship has been reviewed previously [1,2]. As shown in

Fig. 1, Hp 1-1 represents the simplest combination with dimeric $\alpha_1\beta$ chains or $(\alpha_1\beta)_2$. Hp 2-2 is heterogeneous in size, composed of trimeric $\alpha_2\beta$ or $(\alpha_2\beta)_3$ and other cyclic polymers. Hp 2-1 is also heterogeneous, but composed of a simple dimer $(\alpha_1\beta)_2$, trimeric $\alpha\beta$ or $(\alpha\beta)_3$, and other linear polymers; where α represents a mixture of α_1 and α_2 chains. The specific sequence of Hp 1 and Hp 2 is contained in the EMBL/GenBank Data Libraries under access numbers CAA25267 and AAA52685. α_1 is a polypeptide containing 83 amino-acids. Interestingly, α_2 is identical to α_1 , but with an insertion of 3/4 repeat of α_1 (residues 12-70 or 59 amino acids). Therefore, α_2 is comprised of 142 amino acid in sequence. Due to an extra

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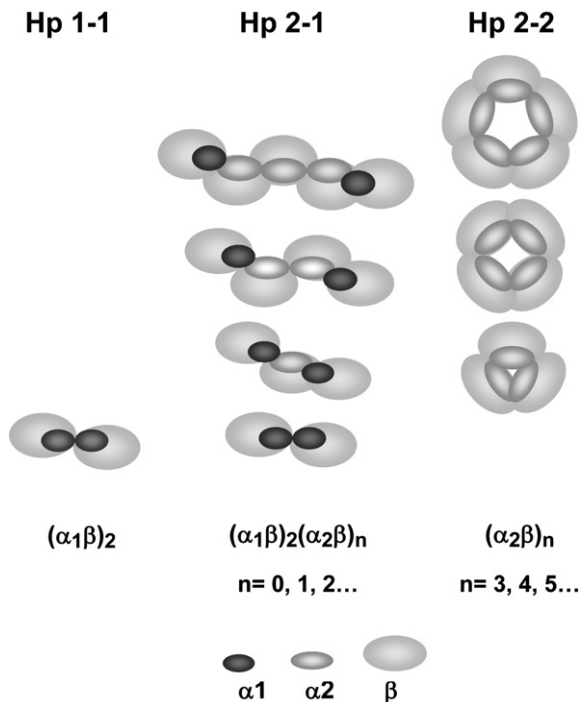


Fig. 1. A schematic model illustrating the structure of human Hp phenotypes. Hp 1-1 represents a homodimer, the simplest combination of dimeric $\alpha_1\beta$ chains $(\alpha_1\beta)_2$. Hp 2-2 is heterogeneous in size, starting with cyclic trimeric $\alpha_2\beta$ chains $(\alpha_2\beta)_3$ and other cyclic polymers. Hp 2-1 is also heterogeneous, but composed of simple homodimer $(\alpha_1\beta)_2$, a linear trimeric $\alpha\beta$ chain $(\alpha\beta)_3$, and other linear polymers; where α represents a mixture of α_1 and α_2 chains. All types share a common structure of the β chain.

insertion of Cys-74 in α_2 , Hp 2-1 and 2-2 form complicated polymers (Fig. 1).

Recently, we and the others have shown that Hp is an antioxidant directly involved in preventing LDL from Cu^{2+} -induced oxidation [3,4]. Its activity is extremely potent and markedly higher than (about 10 folds) that of probucol: one of the most potent antioxidants used in antioxidant therapy [5]. Transfection of Hp cDNA into Chinese hamster ovary (CHO) cells protects the cells against oxidative stress [4]. Epidemiological studies indicated that patients with polymeric forms of Hp 2-1 or 2-2 were associated with the complications of myocardial infarction [6], kidney failure [7], and coronary artery diseases in diabetics [8,9]. Presumably, this was due to the markedly complicated arrangement of Hp 2-1 and 2-2 in which the biologically functional groups are not fully expressed on the surface.

Although Hp phenotypes have been frequently reported to be associated with the diseases, determination of human plasma Hp concentrations, which may be associated with the diseases and diagnosis, is rarely reported. This is probably due to the difficult purification procedures for Hp used in the calibration of immunoassays [10,11]. We have recently described simple procedures, in which each Hp phenotype can be isolated using a monoclonal antibody (mAb) affinity column [11,12]. Limited studies [13–15] have indicated that the plasma concentration of Hp 1-1 is differentially higher than that of 2-1 and 2-2, but other reports are varied and inconsistent [16–19]. In the present study,

we hypothesized that the inconsistency of reported Hp values could be due to the differences in surface antigenic structures among Hp 1-1, 2-1, and 2-2. Using antibody immobilized immunodiffusion and solution immunotubidimetric assays, we demonstrated that the immunoreactivity of Hp 1-1 is differentially greater than that of 2-1 and 2-2. Whereas, using captured noncompetitive ELISA, the “immunoreactivity” of Hp 2-2 was dramatically higher than 2-1 and 1-1. Nevertheless, plasma Hp levels in Hp 1-1 subjects may be either over- or underestimated depending on the method of choice.

To overcome this discrimination, we proposed a novel method using specific Hp type for constructing the calibration curves in the determination of plasma Hp. The Hp phenotype of each subject must be pre-determined to assure an accurate assay. Accordingly, we show that the Hp levels of 1-1 subjects were significantly and differentially higher than 2-1 and 2-2. We also provide a possible mechanism by which Hp 1-1 subjects possess higher plasma Hp levels than 2-1 and 2-2 from the gene expression of HepG2 cells. A hypothesis elucidating the heterogeneous structure of Hp 2-1 was also proposed and discussed in this study.

Materials and methods

Study subjects

Healthy male subjects (genetically unrelated) with normal plasma lipid levels enrolled between years 1999 and 2004 to the Cardiology Division of Taipei Veterans General Hospital were chosen for this study. Mean \pm standard deviation (S.D.) age of these subjects were 61.4 ± 12.3 years. Patients with acute or chronic infectious diseases or malignancy were excluded. Informed written consents were obtained from all patients before blood drawing.

Haptoglobin phenotyping

Hemoglobin was isolated from lysed red blood cells as that described previously [10]. Hp phenotyping was conducted using a native polyacrylamide gel electrophoresis (PAGE) with hemoglobin-supplemented plasma. Briefly, tested sample containing 7 μL plasma and 5 μL of 8 mg/mL hemoglobin was premixed and equilibrated with 3 μL of sample buffer (containing 0.625 mol/L Tris-base, pH 6.8, 50% glycerol (v/v), and 0.125 mg/L bromophenol blue). The mixture was ran on a 7% native polyacrylamide gel (pH 8.8), while a 5.5% polyacrylamide (26.5:1; acrylamide: bis-acrylamide) was employed as a top stacking gel (pH 6.8). Electrophoresis was conducted at an initial voltage of 120 V and increased up to 150 V when the dye front reached the separating gel. After electrophoresis, the Hp-hemoglobin complexes were visualized by shaking the gel in freshly prepared peroxidase substrate [final 0.05% 3,3'-Diaminobenzidine (w/v) and 0.07% hydrogen peroxide (v/v) in phosphate-buffered saline]. The method was further confirmed by a Western blot using an α -chain specific monoclonal antibody [11].

Purification of Hp 1-1, 2-1, and 2-2

The plasma of each Hp phenotype was chromatographed on a mAb-based affinity column followed by a HPLC procedure as described previously [11]. Briefly, two mL of filtered human plasma of each Hp-phenotype was loaded onto the antibody affinity-column (10 mL in bed volume) at room temperature. The column was then washed further with 50 mL of 20 mmol/L phosphate buffer containing 0.2 mol/L NaCl, pH 7.4, and then eluted with 50 mL of freshly prepared 0.15 mol/L NaCl solution with pH 11 adjusted by ammonia [20]. Five milliliters of each fraction was collected in a tube containing 0.25 mL of 1 mol/L Tris-HCl buffer, pH 6.8, to immediately neutralize the base. 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 μ m membrane. Finally, the protein was rechromatographed on a gel-filtration HPLC Superose-12 column (1 \times 30 cm) (Pharmacia, Uppsala, Sweden). The homogeneity of each isolated Hp type was greater than 95% as judged by sodium dodecyl sulfate (SDS)-PAGE.

Preparation of monoclonal antibodies

Hp 2-1 was used for the immunization of mice ($n=3$). mAb were produced according to the standard procedures previously described [21–23]. In brief, Hp 2-1 in PBS was premixed and homogenized with an equal volume of complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) using a 3-way stopcock. Each animal was initially given a total emulsion of 500 μ L containing 100 μ g of Hp including 6 subcutaneous injections onto the back and an intraperitoneal injection. After 10 days, 500 μ L of sterile PBS solution containing 200 μ g of Hp without adjuvant was boosted by 2 intramuscular injections. Seven days following the final booster, blood was collected in 0.1% EDTA and plasma was obtained. Typically, the titers of mouse antiserum reached greater than 1 to 10,000 dilutions before the hybridoma fusion [24]. Myeloma cell line (FO) was fused with spleen cells from immunized BALB/c mice at a ratio of 1:5. The culture medium (between days 14 and 21 after fusion) was assayed for the production of specific antibodies by an enzyme-linked immunosorbent assay (ELISA) using Hp 2-1 as an antigen. Each monoclonal was established by limiting dilutions at least two times.

SDS-PAGE

Electrophoresis was carried out on a 0.75-mm-thick slab gel, using the discontinuous system described by Laemmli with some modifications [25]. In general, gel containing 15% (for reducing samples) or 8% polyacrylamide (for non-reducing samples) was used with a top stacking gel of 5% polyacrylamide. About 5 μ g of purified Hp was preheated at 100 $^{\circ}$ C for 10 min in 5 \times concentrated loading buffer [12 mmol/L Tris-HCl, pH 6.8, 0.4% (w/v) SDS, 5% (v/v) glycerol, 0.02% (w/v) bromophenol blue] with or without 2.88 mmol/L 2-mercaptoethanol. The samples were run for about 1.5 h at 100 V (for

reducing samples) or run for about 4 h at 120 V (for non-reducing samples).

Immunodiffusion

For each well of immunodiffusion plate, 2 μ g of Hp 1-1, 2-1, or 2-2 in 2 μ L was applied onto a 1.5% agarose gel (w/v) containing 1% of rabbit anti-human Hp antiserum (v/v). The precipitation ring was allowed to develop for at least 48 h at room temperature and photographed without staining [26].

Immunoturbidimetry

To 50 μ L of goat anti-human Hp (1:20), 150 μ L of tested human plasma (1:80) was added in a microtiter plate and incubated at 24 $^{\circ}$ C for 15 min with gentle shaking. Turbidity resulting from the antigen-antibody complex was read at 415 nm by a Molecular Devices/spectra MAX 190 (Molecular Devices; Sunnyvale, CA). A standard curve was constructed using purified Hp 1-1, 2-1, or 2-2 spiked into an ahaptoglobulinemic plasma (described below) as a separate set.

ELISA

A conventional noncompetitive sandwich ELISA was conducted according to the method similar to that previously described by us [26]. Initially, 1 μ g of the IgG fraction of goat anti-human Hp in 50 μ L of PBS was immobilized onto a 96-well ELISA plate at room temperature for 1 h, followed by an addition of 300 μ L blocking buffer (PBS containing 1% of bovine serum albumin). After washes, 100 μ L of Hp 1-1, 2-1, 2-2 (ranging from 2.5 to 20 ng/mL), or tested plasma (1:160,000 dilution) in washing buffer (PBS containing 0.1% BSA and 0.05% Tween-20) was then added to each well and incubated at 24 $^{\circ}$ C for 90 min to construct a standard curve. One hundred microliters of 1 to 160,000 diluted human plasma in washing buffer was used for the Hp determination. Following washes, 100 μ L of appropriately diluted mAb specific to human Hp β -chain (typically G2D in excess as a primary antibody) was added and incubated at 24 $^{\circ}$ C for 1 h. Finally, after incubation of a horseradish peroxidase labeled goat anti-mouse IgG antibody and washes, the plate was developed by the addition of 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and read at 415 nm by an ELISA reader. All the determinations were conducted in triplicates. The protein concentrations of Hp used for the assay or elsewhere were determined by the Lowry method [27] with bovine serum albumin as a standard.

Analytical recovery

Hp phenotyping was carried out in a total of 1322 human subjects. Among those, 7 subjects were found to be ahaptoglobulinemia without formation of Hp-hemoglobin complex. It was further confirmed by the lack of immunoreactive Hp in ELISA and Western blot analysis. Those plasma samples were then pooled and used as a basal line for the study of analytical recovery. In brief, two concentrations of each purified Hp 1-1,

2-1, or 2-2 were spiked into the aphaetoglobulinemic plasma to a final concentration of 120 and 240 mg/dL. The plasma was then diluted to 1:160,000 prior to the recovery study. The theoretical concentration of Hp spiked sample was determined in triplicates.

ELISA of a commercial kit

A GenWay human Hp ELISA quantitation kit (40-288-20080F) was purchased from GenWay Biotech (San Diego, CA), in which one human Hp calibrator, affinity purified chicken anti-Hp (primary antibody), and HRP-conjugated chicken anti-Hp (secondary antibody) are provided. Other reagents and plates are not included. The noncompetitive ELISA was conducted according to the procedures provided by the manufacturer and was relatively similar to our routine ELISA described above. In brief, 100 μ L of primary anti-Hp diluted to 1:100 in PBS was coated onto a 96-well ELISA plate, followed by the addition of diluted human plasma (100 μ L in 1:20,000) (or Hp standards) and HRP-conjugated anti-Hp. The plate was then developed by the addition of 150 μ L of tetramethylbenzidine (TMB) and read at 650 nm. The entire assay including immobilization, washes, and antibody incubation was completed within 4–5 h.

Cell culture

Human HepG2 cells (ATCC, Rockville, MD) were grown in a minimal essential medium (MEM)- α (GIBCO-Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated bovine calf serum (GIBCO-Invitrogen) (v/v) containing 100 units/mL penicillin G, 100 μ g/mL streptomycin (GIBCO, Carlsbad, CA), 2 mmol/L L-glutamine (GIBCO), and nonessential amino acids (GIBCO) at 37 °C in a 95% humidified incubator containing 5% CO₂. HepG2 cells were seeded in a 24-well tissue culture plate (1×10^5 cell/well) and cultured for 24 h, followed by starvation for 8 h before the conditional treatment. To induce the Hp expression, cells were treated with LDL (100 μ g/mL) in a MEM- α medium containing no bovine calf serum for 0–24 h at 37 °C.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was first extracted from HepG2 cells using Trizol reagent (Invitrogen, Carlsbad, CA). The total RNA (0.1 μ g) was used for RT-PCR. The sequence of primers used for amplification of Hp-forward was 5'-ATG GTG GAC TCA GGC AAT GAT-3' and Hp-reverse was 5'-GTT CTC AGC TAT GGT CTT CT-3'. β -actin was amplified as an internal control using the following primers: forward, 5'-GGC TGT GCT ATC CCT GTA CG-3', reverse, 5'-CAC ATC TGC TGG AAG GTC GAC-3'. The amplification of the Hp and β -actin was performed using Taq polymerase (Violet BioScience, Taipei, Taiwan) with the following thermocycle condition: 5 min at 94 °C and 30 cycles of 30 s at 94 °C, 30 s at 53 °C, 2 min at 72 °C, and final extension of 10 min at 72 °C, followed by a final incubation at 4 °C. RT-PCR amplified the Hp mRNA corresponding to the full length of matured $\alpha_2\beta$ of Hp 2-2 containing 1164 bp and

$\alpha_1\beta$ of Hp 1-1 containing 988 bp. The β -actin was amplified to 657 bp. The RT-PCR products from Hp were further confirmed by the DNA sequence in addition to the analysis by 1.8% agarose gel electrophoresis.

Statistical analysis

Data are reported as the mean \pm S.D. The difference between the groups was calculated by *t*-test. All *P*-values are based on a two-tailed comparison. *p* < 0.05 was considered as significant. Coefficients of variations of inter- and intra-assay were calculated by S.D./mean \times 100%.

Results

Homogeneity of purified Hp 1-1, 2-1, and 2-2

Fig. 2A shows a typical profile of purified Hp 1-1, 2-1, and 2-2 when analyzed on a 7% native-PAGE analysis. It reveals that the heterogeneous forms exist in Hp 2-1 and 2-2, but not in 1-1. By Western blot analysis using a native gel, each phenotype of isolated Hp was almost identical to that native form in plasma (data not shown) [10,11]. Following reduction by 2-mercaptoethanol on a 15% SDS-PAGE, each Hp phenotype possessed its own unique α_1 , α_2 , or a combination of both subunits (Fig. 2B). Homogeneity of each isolated Hp phenotype was greater than 95% (Fig. 2B). These proteins were then used for immunochemical studies and immunoassays.

Immunoreactivity of Hp phenotypes

To address whether the structural difference among the Hp phenotype may affect its immunoreactivity, a radial immu-

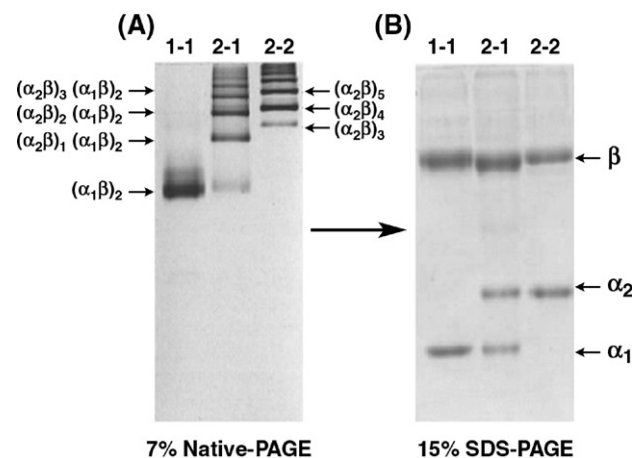
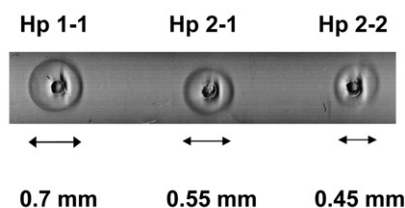


Fig. 2. Profile of purified Hp phenotypes using native- and SDS-PAGE. Isolation of Hp was achieved using plasma of each phenotype which was chromatographed on a mAb-based affinity column followed by a gel-filtration HPLC column (Superose-12; 1×30 cm). The pattern reveals the heterogeneity in Hp 2-1 and 2-2, but not in 1-1 (A). Following reduction by 2-mercaptoethanol, each Hp phenotype possesses a unique arrangement of α_1 , α_2 , or both as shown on a 15% SDS-PAGE (B). The homogeneity of each isolated Hp type was greater than 95%. The heterogeneity in (A) is similar to that in plasma as verified by a Western blot analysis [11].

nodiffusion assay was conducted in which rabbit Hp polyclonal antibody was immobilized in an agarose gel. Fig. 3A shows that, with an equal concentration of each respective Hp phenotype (100 mg/dL), Hp 1-1 gave a significantly larger immunoprecipitated ring than Hp 2-1 and 2-2. The diameter was 0.7, 0.5, and 0.45 cm for Hp 1-1, 2-1, and 2-2, respectively. The data indicate that with equal plasma Hp concentrations of different type, Hp could be overestimated in 1-1 subjects because only the ring size has been taken into account. In terms of immunoreactivity, it seems difficult to interpret which type possesses higher reactivity. One reason is that the molecular mass of Hp 1-1 (β - α - α - β) (~100 kDa including carbohydrate moiety) is less than that of 2-1 (~100–700 kDa) and 2-2 (~150–800 kDa). The diffusion or penetration rate of Hp 1-1 might be higher in agarose gel. In the next experiment, we tested the interaction between Hp and its polyclonal antibody in solution using an immunoturbidimetric assay. The reactivity of each Hp type with the antibody was monitored by the resulting turbidity at 415 nm. Fig. 3B shows that the formation of antigen–antibody complex of Hp 1-1 was superior to 2-1 and 2-2. Thus, it suggests that the immunoreactivity of Hp 1-1 in solution is markedly greater than 2-1 and 2-2. One proposed explanation is that the surface of Hp 1-1 is well exposed, providing more available antigenic epitopes than structurally hindered polymers 2-1 and 2-2 (Fig. 1).

(A) Immunodiffusion



(B) Immunoturbidimetry

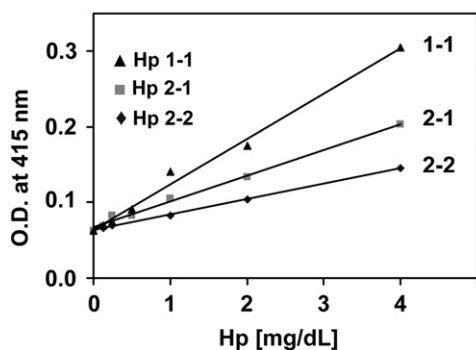


Fig. 3. Antigen–antibody interaction on radial immunodiffusion and in solution. For immunodiffusion, each precipitation ring was developed for 48 h at room temperature and photographed without staining (A). Hp 1-1 gave a larger immunoprecipitated ring than did 2-1 and 2-2. The antigen–antibody interaction in solution was monitored by an immunoturbidimetric assay (B). The latter data suggest that the immunoreactivity of Hp 1-1 in solution is greater than 2-1 and 2-2.

Immunoreactivity of Hp among the phenotypes using noncompetitive ELISA

In an attempt to develop an ELISA, we examined the differences in immunoreactivity among the three Hp types using a conventional noncompetitive ELISA [26]. Polyclonal antibody was immobilized first onto an ELISA plate. Following the capture of each type of Hp, a secondary mouse mAb prepared against human Hp was added. The degree of mAb binding was then determined by an HRP-conjugated anti-mouse IgG. A typical example of the test is shown in Fig. 4A using mAb prepared against Hp β -chain (2-3H). Surprisingly, the “immunoreactivity” of Hp 1-1 was markedly and differentially less than that of 2-1 and 2-2. To ascertain that this was not due to the specificity of the mAb used, the same ELISA was conducted using a mAb prepared against Hp α -chain (3H8) or others prepared against β -chain (G2D and 8B1). As shown in Figs. 4B–D, similar results were observed using these mAb. We proposed that these results were not due to the low immunoreactivity of Hp 1-1. A schematic drawing explaining a different mode of binding for each Hp phenotype is depicted in Fig. 5. With an equal amount of Hp polyclonal antibody immobilized, one captured Hp 1-1 molecule [$(\alpha\beta)_2$ or $\alpha\beta$ homodimer] possesses only 2 copies of a given epitope that is available for the binding of secondary anti-Hp mAb (assuming each copy is not pre-occupied by the immobilized antibody). Whereas, one Hp 2-1 or 2-2 captured is polymeric containing up to 6 or more repeated epitopes, it drastically amplifies the final binding of HRP-conjugated antibodies yielding high but false immunoreactivity.

Overestimation using ELISA and underestimation using immunoturbidimetric method

To show a direct effect of the assay method chosen, we spiked an equal amount of isolated Hp 1-1, 2-1, or 2-2 to an ahaptoglobinemic plasma to yield a final concentration of 100 mg/dL. Using Hp 1-1 as a calibrator, the level of Hp 2-2 and 2-1 in spiked plasma was markedly overestimated by ELISA and underestimated by immunoturbidimetric assay (Fig. 6A), which is consistent with our proposed hypothesis (Figs. 3B and 5). While using Hp 2-2 as a calibrator, Hp 1-1 and 2-1 were underestimated by ELISA, but overestimated by immunoturbidimetric assay (Fig. 6C). Showing a relative trend, the concentrations of Hp were Hp 2-2>2-1>1-1 in an ELISA and Hp 1-1>2-1>2-2 in an immunoturbidimetric assay without using a type-matched calibrator. Choosing either of the phenotypes as a standard did not give a plausible value for overall Hp determinations. However, such false values can be “normalized” using a type-specific calibrator that matches each phenotype samples (Fig. 6D). In other words, the spiked Hp level could be accurately determined using type-matched calibrators in either ELISA or immunoturbidimetric method.

Normalized ELISA using Hp type-matched calibrator

Taken together, we demonstrated that the structural difference among the Hp phenotypes determined the difference in “immu-

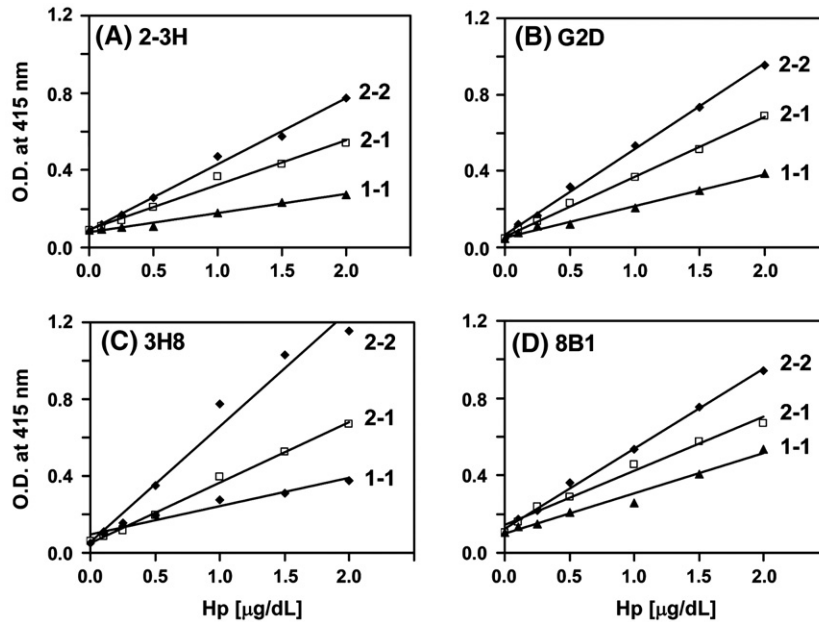


Fig. 4. Binding of Hp 1-1, 2-1, and 2-2 by mAb in ELISA. Goat anti-human Hp polyclonal antibody was first immobilized onto an ELISA plate. Following the capture of each type of Hp, mAb prepared against Hp was added and washed, HRP-labeled anti-mouse IgG was then added. (A) mAb (2-3H) specific to Hp β -chain; (B) mAb (3H8) specific to Hp α -chain; (C and D) mAb (G2D and 8B1) specific to Hp β -chain. Hp 1-1 seems to bind differentially less to immobilized Hp polyclonal antibody than does Hp 2-1 and 2-2 (see Fig. 5 for more detail).

noreactivity” of each phenotype. This difference leads to either over- or underestimation of plasma Hp. To overcome the differential immunoreactivity among the Hp phenotypes, we proposed that an independent type-matched Hp should be used for constructing a calibration curve. The phenotype of assessed plasma samples needs to be pre-classified prior to the Hp immunoassay. A typical example for the determination of plasma Hp levels in each phenotype group using matched standard for ELISA is shown in Fig. 7A. The mean levels of Hp 1-1 subjects ($n=16$; 184 ± 42 mg/dL) were significantly and differentially greater than Hp 2-1 ($n=28$; 153 ± 55 mg/dL) ($p<0.05$) and 2-2 ($n=24$; 93 ± 54 mg/dL) ($p<0.01$) subjects. Of remarkable interest, such discrepancy was not observed while using Hp 1-1 (Fig. 7B), 2-1 (Fig. 7C), or 2-2 (Fig. 7D) as a sole calibrator.

Comparison between ELISA and immunoturbidimetric method using Hp type-matched calibrator

As described above, for Hp determination without using type-matched standards in the assay, ELISA and immunoturbidimetric method could result in underestimating and overestimating, respectively. Fig. 8 shows that when a type-matched calibrator was employed, these two methods were correlated well in Hp 1-1, 2-1, and 2-2 subjects with a correlation coefficient ranging from 0.927 to 0.970. It revealed that Hp levels in Hp 1-1 subjects were differentially higher than that in 2-1 and 2-2 using both methods.

Comparison between our in-house ELISA and commercially prepared GenWay ELISA kit method

First, to confirm that the “false” high-immunoreactivity of Hp 2-2 or 2-1 in ELISA was due to the amplification of repeated

epitopes (Fig. 5), immobilized chicken anti-human Hp and HRP-conjugated secondary anti-human Hp provided from the GenWay kit were used to carry out this study. Fig. 9A reveals that “immunoreactivity” of Hp 2-2 was also consistently greater than 2-1 and 1-1 using our in-house prepared Hp standards. Interestingly, the single pure Hp calibrator provided by the kit was fit right between our Hp 2-2 and 1-1, almost superimposable to our 2-1. This result strongly supports the notion that Hp calibrator with each respective Hp 1-1, 2-1, and 2-2 should be provided in the kit.

Next, we determined the plasma Hp concentrations by the GenWay method in normal healthy subjects with three phenotypes. Table 1 clearly demonstrates that, using the sole “Hp 2-1” calibrator provided from the kit, it over- and underestimated Hp concentrations in Hp 2-2 and 1-1 subjects, respectively. However, when using our type-matched calibrators, the mean plasma Hp concentrations in Hp 1-1 (186 ± 53 mg/dL) determined by the GenWay method were differentially higher than 2-1 (152 ± 50 mg/dL) and 2-2 (91 ± 55 mg/dL). These values were almost identical to our in-house ELISA. Interestingly, the overall Hp determination between the two methods is well correlated with a correlation coefficient value (r) greater than 0.95 (Figs. 9B–E).

Sensitivity and analytical recovery

The sensitivity of our in-house ELISA for Hp ranged from 0.5 to 2 μ g/dL, usually giving an optical density less than 1.0 at 415 nm with 2, 2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acids] (ABTS) as a chromogen for horseradish peroxidase (HRP)-conjugated secondary antibody (Fig. 4). The upper linear range could be maintained with the standard up to 3 μ g/dL of Hp.

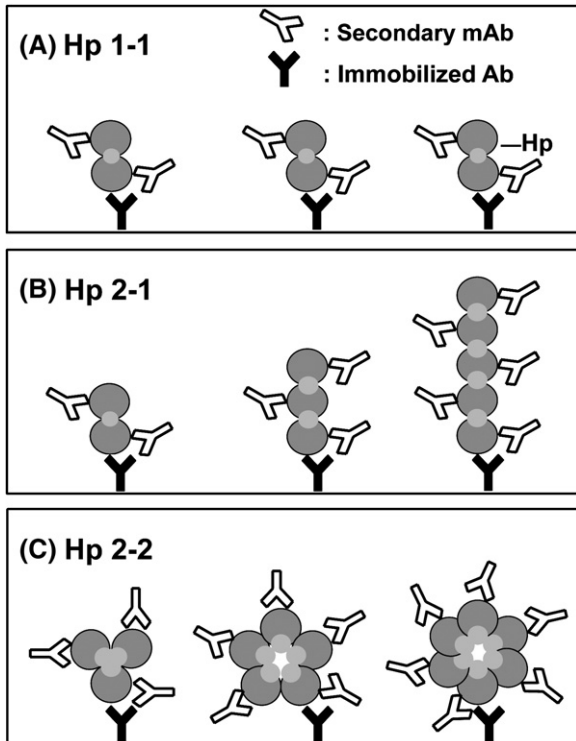


Fig. 5. Schematic drawing for differential binding of mAb to Hp 1-1, 2-1, and 2-2 on ELISA plate. With an equal amount of Hp antibody immobilized, each captured Hp 1-1 antigen possesses only 2 copies of a given epitope ($\alpha\beta$ - $\beta\alpha$ homodimer) for secondary mouse mAb binding (A). On the other hand, Hp 2-1 (B) or 2-2 (C) is polymeric, each containing up to 6 or more repeated copies of a given epitope for mAb binding. It amplifies the final binding for secondary mAb yielding a high reading of optical density as a result. HRP-conjugated antibody against mouse IgG is not shown.

Since the Hp content in plasma is abundant and usually greater than 100 mg/dL, we normally need to dilute the tested plasma to 1:160,000 for the assay. The lower detection limit was approximately between 0.2 and 0.5 $\mu\text{g/dL}$. Spiking the purified Hp 1-1, 2-1, or 2-2 into an ahaptoglobinemic sample (see Materials and methods) gave a recovery ranging from 95.3% to 104% (Table 2).

To evaluate intra-assay variations, three different type plasma samples were assessed in 10 replications at the same time. The mean variation on average was approximately of 2.0, 3.4, and 4.4% for Hp 1-1, 2-1, and 2-2, respectively. The inter-assay variations in 5 independent assays (once a week) were 4.8, 5.9, and 4.2% for Hp 1-1, 2-1, and 2-2, respectively (data not shown).

Expression of $\alpha_1\beta$ and $\alpha_2\beta$ mRNA in HepG2 Cells

To address a possible mechanism for the phenomenon where Hp 1-1 levels are significantly higher than 2-1 and 2-2, we used a human HepG2 cell line for studying the molecular expression of Hp. HepG2 cell lines were chosen because the liver is one of the major sites for the biosynthesis of plasma Hp [1]. Using RT-PCR, we have identified this HepG2 cell line to be of a Hp 2-1 genotype. It expresses two linear forms of Hp mRNA, namely $\alpha_1\beta$ and $\alpha_2\beta$ with 988 and 1164 base pairs (without leading

signal sequence), respectively. Each of the sequences of the RT-PCR products was identical to the reported nucleotide sequence (data not shown). As depicted in Fig. 10, using human LDL as an acute reactant [28], Hp mRNA expression was dramatically induced in a time-dependent fashion, while the expression of the house keeping gene, β -actin, was almost unaffected. The rate of induced $\alpha_1\beta$ mRNA was significantly greater than that of $\alpha_2\beta$. The data suggest that the *Hp 1* allele was predominant over *Hp 2* in producing Hp mRNA. We speculate that this may be the cause for Hp 1-1 subjects to possess higher levels of plasma Hp (Fig. 7).

Discussion

Due to the existence of two different Hp alleles (*Hp 1* and *Hp 2*) in chromosome 16q22, three main Hp phenotypes are ex-

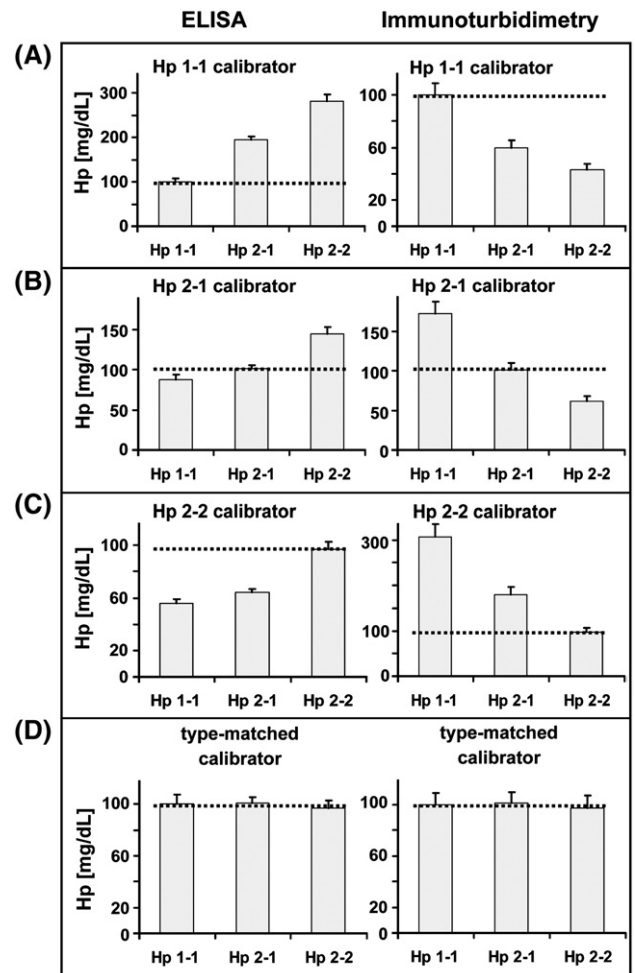


Fig. 6. Over- and underestimation of Hp in ELISA and immunoturbidimetric assay. Equal amounts of Hp 1-1, 2-1, or 2-2 were spiked into an ahaptoglobinemic plasma to give a final Hp concentration of 100 mg/dL. The spiked Hp level was then determined by an ELISA (left) and immunoturbidimetric assay (right) using a type-specific Hp calibrator. Values determined using Hp 1-1 (A), Hp 2-1 (B), or Hp 2-2 (C) of non-matched standard are shown. Dotted lines represent the theoretical Hp values. The data demonstrate that Hp could be either over- or underestimated depending on the Hp phenotype used for calibration. The value, however, was accurately determined when using each matched calibrator (D). Each bar represents the mean \pm S.D.

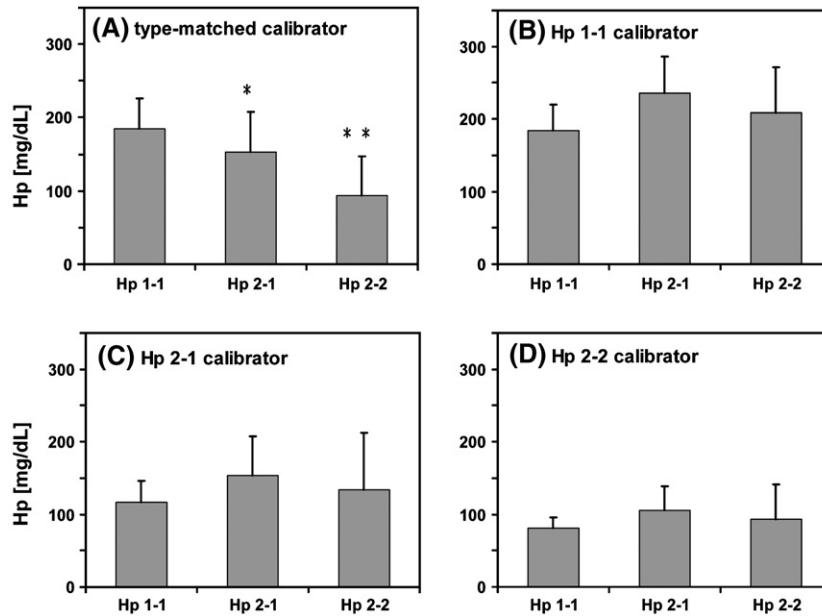


Fig. 7. Determination of plasma Hp levels using ELISA with type-specific calibrators. (A) Hp levels were determined in subjects using type matched Hp calibrator. (B, C, and D) Hp levels were determined in subjects using Hp 1-1, 2-1, and 2-2 as a sole calibrator, respectively. Number of human subjects assessed was 16, 28, and 24 for Hp 1-1, 2-1, and 2-2, respectively. Each bar represents the mean \pm S.D. * $p < 0.05$; ** $p < 0.01$.

pressed as Hp 1-1, Hp 2-1, and Hp 2-2 [29]. A homozygote 1-1 or 2-2 contains a pair of *Hp 1* or *Hp 2*, respectively, while a heterozygote 2-1 contains both *Hp 1* and *Hp 2*. The main physiologic function of Hp is to bind and clear free hemoglobin from the plasma via a rapid uptake by hepatocyte receptors [30]. The high binding affinity between Hp and hemoglobin has consequently resulted in preventing the hemoglobin-mediated

free radicals from “leakage” [31,32]. We have recently shown that Hp also acts as an extremely potent antioxidant by directly inhibiting Cu^{2+} -induced LDL oxidation [4].

The structural difference among various Hp types may drastically affect its biological function and therefore contribute to its clinical outcomes. Some reports indicate that patients with Hp 2-1 and 2-2 are often associated with kidney failure and

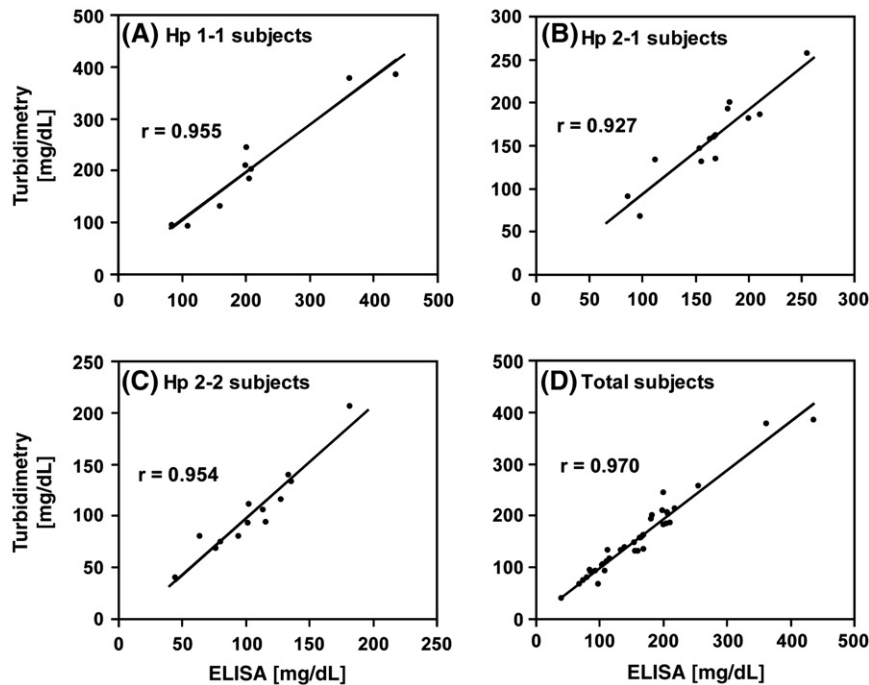


Fig. 8. Correlation of plasma Hp levels measured between ELISA and immunoturbidimetric method. Each Hp type subject was determined using a type matched calibrator. The number of subjects assessed was 10, 15, and 14 for Hp 1-1, 2-1, and 2-2, respectively.

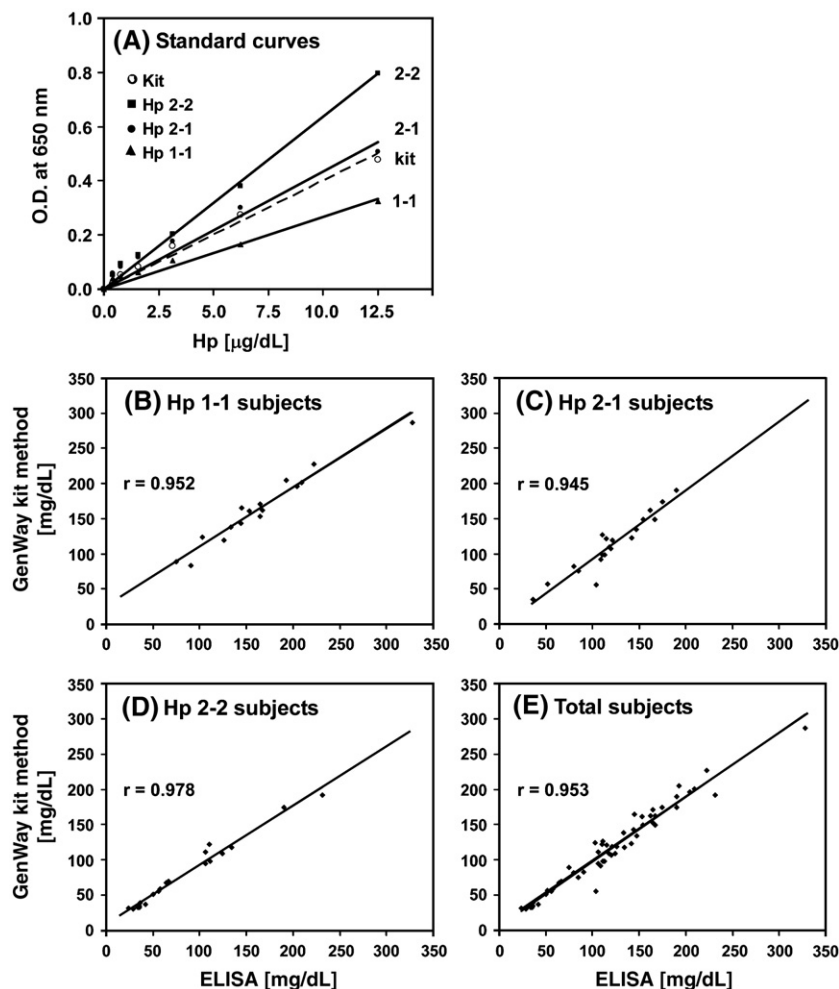


Fig. 9. Immunoreactivity of Hp 1-1, 2-1, and 2-2 evaluated by GenWay ELISA kit and correlation of plasma Hp levels determined between our in-house and GenWay ELISA. (A) Immunoreactivity of in-house prepared Hp 1-1, 2-1, and 2-2 was evaluated by chicken antibodies provided from the kit. Immunoreactivity of the single calibrator provided by the kit (dotted line) was almost superimposable to that of Hp 2-1. (B–E) Correlation of plasma Hp levels determined between our in-house and GenWay ELISA, when using type-matched Hp 1-1, 2-1, or 2-2 calibrator.

diabetes mellitus [1,33]. Hp 2-2 patients with coronary artery disease (CAD) are more likely to develop restenosis after percutaneous transluminal coronary angioplasty [34]. Hp 1-1 individuals are found to possess a significantly high rate of CAD mortality [35]. It is conceivable that the plasma Hp concentrations can even play a provocative role in addition to its phenotype difference. However, the reports of Hp concentrations associated with the clinical diseases are rare for the past decades. This is probably due to the heterogeneous structure of Hp 2-1 and 2-2 (Fig. 1) making the isolation procedure virtually difficult. Recently, we have reported simple procedures for the

isolation of Hp 1-1, 2-1, and 2-2 using a mAb affinity column, followed by an execution of HPLC Superose-12 chromatography [11,12]. The method provides a utility for the study of structural and functional relationship among the Hp phenotypes. In contrast to that of Hp 2-1 and 2-2, purification of human Hp 1-1 can be simply achieved by a single HPLC procedure similar to the purification of porcine Hp 1-1 previously described [36]. Ideally, it is convenient to utilize Hp 1-1 for setting up a standard curve. However, in the present study, we demonstrated that the simplest form of Hp 1-1 alone as a calibrator was not suitable for immunodiffusion, immunoturbidimetric, or ELISA

Table 1
Comparison of Hp levels (mg/dL) determined between our in-house and GenWay ELISA

Method	Hp 1-1 (n=16)	Hp 2-1 (n=28)	Hp 2-2 (n=24)	P value		
				2-2 vs. 1-1	2-1 vs. 1-1	2-2 vs. 2-1
In-house ELISA using type-matched calibrator	184±42	153±55	93±54	<0.001	0.045	0.016
GenWay ELISA kit using single calibrator provided ^a	134±38	147±48	137±87	0.903	0.317	0.749
GenWay ELISA kit adjusted by type-matched calibrator	186±53	152±50	91±55	<0.001	0.031	0.009

^a There was only one Hp calibrator provided by the GenWay ELISA kit. All data were expressed as mean±S.D.

Table 2
Analytical recovery of the addition of purified Hp in aheptoglobinemic plasma

	Hp added to plasma (mg/dL)	Measured	Expected	Recovery (%)
Hp 1-1	120	118.1±2.6	120	98.4
	240	249.6±12.2	240	104.0
Hp 2-1	120	114.4±3.4	120	95.3
	240	230.4±1.8	240	96.0
Hp 2-2	120	115.7±4.2	120	96.4
	240	233.6±4.6	240	97.3

Plasma was diluted to 1:160,000 prior to the assay.

methods. For example, in mostly used ELISA, the Hp 1-1 ($\alpha\beta$ homodimer) captured by immobilized polyclonal antibody possesses only 2 copies of a given epitope resulting in a limited binding for the secondary mAb (Fig. 5). Whereas, Hp 2-1 or 2-2 is polymeric containing up to 6 or more repeated copies of an epitope for the mAb (Figs. 1 and 5), so that it increases the availability for the final secondary antibody and HRP-conjugated antibody binding. The radial immunodiffusion method is easy and less tedious for evaluating a small size of the population, but the precipitation ring produced by the Hp 1-1 is significantly larger than 2-1 and 2-2 (Fig. 3A). The latter method would overestimate the plasma Hp concentrations in Hp 1-1 subjects. A similar situation was seen in the immunoturbidimetric assay (Fig. 3B). These results led us to hypothesize the possible use of separate and type-matched Hp calibrators for the assays.

Using a type-matched Hp standard corresponding to each phenotype subject, we show that the mean value of Hp 1-1 was notably higher than 2-1 and 2-2 and reached a statistical

significance ($p < 0.05$) (Fig. 7A). The trend shown, however, was not consistent when employing only one standard curve for calibration (Figs. 7B–D). We compared the recently published values of Hp measured by immunodiffusion, immunonephelometry, and ELISA, in which none of them used type-matched calibrators. As shown in Table 3, the data are varied depending on the method of choice. As expected, the Hp 2-2 level measured by ELISA is significantly higher than the Hp 1-1 levels [18]. This could be the reason that is explained in Fig. 5, in which Hp 2-2 possesses redundant-identical epitopes. Notably, all the immunoturbidimetric and immunonephelometric assays show a differentially higher Hp 1-1 value than 2-1 or 2-2 [13–15,19]. The latter data were expected owing to the high immunoreactivity of Hp 1-1 in solution (Fig. 3). One immunodiffusion assay shows that Hp 2-1 value is higher than 2-2 or 1-1 [16]. Regardless, the inconsistency of reported plasma values indicates that the immunoreactivity of each Hp type is diverse and heterogeneous; a type-matched Hp calibrator should be therefore used for each type of the specific subject.

With respect to the commercially available GenWay ELISA kit, we show the inaccuracy of the determination of Hp concentration in each phenotype using the sole Hp calibrator provided (Table 1). Although the type of provided Hp is not informed, we speculate that it belongs to Hp 2-1 or a mixture of three types as its immunoreactivity is almost superimposable to our Hp 2-1 calibrator (Fig. 9A). Remarkably interesting, the value could be normalized when using our in-house type-matched calibrators. After normalization, the kit method (using chicken anti-Hp) correlated well with our ELISA (Figs. 9B–D) suggesting that different sources of antibody would not affect Hp determination while using the same calibrators. The kit method is sensitive, similar to our ELISA, with a detection limit at the $\mu\text{g/dL}$ level and is relatively convenient with HRP-conjugated anti-Hp as a secondary antibody. In terms of cost and efficiency, the average cost of each sample determination (in duplicates) using the kit-method is about \$0.50 as compared to \$0.25 using our in-house ELISA. Should GenWay kit provide calibrators with each type-specific Hp, it could be widely used in clinical laboratories.

Furthermore, using human HepG2 cell line with Hp 2-1 genotype, we show the inducible biosynthesis of Hp mRNA (Fig. 10). It is of remarkable interest that synthesis rate of mRNA corresponding to $\alpha_1\beta$ (for Hp 1-1) alleles is greater than that of $\alpha_2\beta$ (for Hp 2-2) alleles upon the stimulation, although

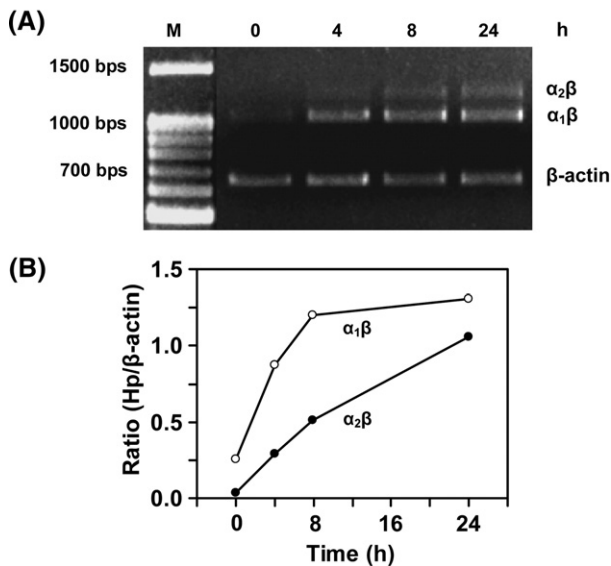


Fig. 10. Expression of Hp mRNA in HepG2 cells. HepG2 cell line used was identified to be of a Hp 2-1 genotype. (A) By RT-PCR, HepG2 expresses two linear forms of Hp mRNA, namely $\alpha_1\beta$ (Hp 1) and $\alpha_2\beta$ (Hp 2) with 988 and 1164 base pairs, respectively. Hp mRNA was induced by LDL (100 $\mu\text{g/mL}$) in cultured HepG2 cells at 37 °C over time. Expression of β -actin (657 bp) was used as a house-keeping gene control. (B) Relative synthesis rate of $\alpha_1\beta$ or $\alpha_2\beta$ mRNA was expressed as a ratio of imaged Hp area over that of β -actin.

Table 3
Historical methods for plasma Hp determinations (mg/dL)

Methods	Hp 1-1	Hp 2-1	Hp 2-2	Reference
The present study ELISA (type-matched calibrator)	182	153	93	
ELISA	126		164	[18]
Radial immunodiffusion	140	210	165	[16]
Immunoturbidimetry	210	178	130	[15]
Immunonephelometry	126	108	84	[13]
Immunonephelometry	258	171	149	[14]
Immunonephelometry	88	90	66	[19]
Not mentioned	184	198	198	[17]

the mechanism and induction pathways involved remain elusive. Each full-length of Hp mRNA ($\alpha_1\beta$ or $\alpha_2\beta$) in human liver cells is post-transcriptionally cleaved between residues arginine and isoleucine (Arg-Ile) to yield α and β chains by a specific protease [37,38]. The assembling of the α and β chains is relatively simple in Hp 1-1 or 2-2 homozygotes, which in turn produces either a dimeric form or cyclic polymers, respectively (Fig. 1). It remains puzzling for the assembling of Hp 2-1 molecules, since Hp 2-1 contains the mixture of both 1-1 dimers and linear polymers, without the 2-2 cyclic trimers (Fig. 1). This has been evidenced using electron microscopy by the structural and assembling analysis [39]. The overexpression of $\alpha_1\beta$ in our cell line suggests that the formation of 1-1 dimers in Hp 2-1 subjects was probably due to the excessive production of $\alpha_1\beta$. As a consequence, the other linear polymers are assembled without containing 2-2 cyclic polymers. Our data may help to explain why the structure of Hp 2-1 protein contains the mixture of Hp 1-1 molecules and other linear polymers but with no cyclic trimers, as shown in Fig. 1.

In conclusion, our data explore the complexity by accurately determining the concentrations of plasma Hp due to the diverse immunochemical structure among the Hp phenotypes. A type-matched Hp calibrator corresponding to each phenotype subject should be used for the construction of a standard curve. Using ELISA, the plasma levels of Hp 1-1 are significantly and differentially greater than Hp 2-1 and 2-2. Higher molecular expression of $\alpha_1\beta$ mRNA compared to $\alpha_2\beta$ in human HepG2 cells offers the explanation for the association of high levels of Hp with Hp 1-1 subjects. Phenotyping should be performed prior to the determination process of plasma Hp levels to ensure accuracy in use for clinical diagnosis.

Acknowledgments

We gratefully acknowledge the support of this work from the grants NHRI-EX94-9229SI (S. J. T. M) of National Health Research Institute, Taiwan, ROC, NSC 95-2313-B-009-003-MY2 (S. J. T. M) of National Science Council, Taiwan, ROC, and VGH90-2-60A (J. P. P) of Taipei Veterans General Hospital, Taiwan, ROC. We acknowledge James Lee for his critiques and comments which have made this paper more fluent.

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