[PG9303-1187] NHRI-EX93-9229SI (23.P)

計畫編號：NHRI-EX93-9229SI

國家衛生研究院整合性醫藥衛生科技研究計畫

Haptoglobin 在動脈硬化中所扮演之功能角色

九十三年度成果報告

執 行 機 構：交通大學 生物科技系/生化工程研究所

計 畫 主 持 人：毛仁淡

本年度執行期間： 93 年 1 月 1 日 至 93 年 12 月 31 日

＊＊本研究報告僅供參考用，不代表本院意見＊＊

計畫名稱：Haptoglobin 在動脈硬化中所扮演之功能角色

計畫編號：NHRI-EX93-9229SI

執行機構：交通大學 生物科技系/生化工程研究所

計畫主持人：毛仁淡

研究人員：毛仁淡

關鍵字：Haptoglobin、動脈硬化、抗氧化、血紅素

壹、 九十三年度計畫研究成果摘要

※撰寫注意事項※

一、 此部分將**彙整成冊並上網公告**，如果內容及研究成果有涉及專利或其他智慧財產權，而暫不宜彙編入冊者，請事先以書面通知本院

二、 請以**中、英文**分別簡述計畫成果摘要，中、英文摘要以**各不超過六頁**為原則，且不得包含圖表且不得僅描述研究目的

三、 若為群體計畫(PPG)，此章節內**必須彙整所有子計畫內容**，不得分別撰寫各子計畫，並以具體說明總計畫研究成果並強調子計畫間相輔相成之功能

四、 若擬詳述計畫研究內容(包含摘要、研究目的、研究方法、研究結果、討論與結論等)，或群體計畫擬寫出各子計畫內容者，可使用***中文***或***英文***補充，再附於附錄

五、 此部份若撰寫型式或頁數不符規定，將寄還計畫主持人更正，若因此造成超過成果報告繳交期限，將依合約書第十二條第四款處理

中文摘要

這是三年計畫中的第二年計畫報告，首先我們非常感謝國家衛生研究院的支持及給予我們機會執行此計畫，目前本計畫已出版 3 篇 SCI 報告與 16 篇研討會論文（其中 5 篇為國際研討會論文）。除此之外，另有 3 篇報告已經完成，並預定於 2004 年底投出。

在第二年的報告中，我們共發表了三篇 SCI 報告，其中二篇是著眼於發展並建立簡單純化大量 Hp 的方法，另一篇則是探討 Hp 之抗氧化特性研究。在計畫發展初期，由於 Hp 具有多種型態，且同一型態中又具有多種組合之聚合體，因此相當不容易大量純化並進行研究，所以我們首先利用血紅素與 Hp 之高親和力特性，發展了血紅素親和性管柱，但是此方法最終所得到的產物摻有少量的血紅素。後來，再利用抗體親和性管柱純化，再過一個 gel filtration 管柱，結果發現這是一個方便而有效的方法去除了之前純化方法傳統的缺點，如純度不夠、純化條件太嚴苛、太花時間等等。

我們也發展出一個快速辨別 Hp 類型的方法，不需要經過傳統的 starch gel，血漿（或血清）只需要先與血紅素混合，再跑個 native PAGE，再以 DAB 呈色，即可由呈色的樣式判斷 Hp 的類型，因此 3 種形式的 Hp 就可輕易的判別出來。

再者，6 株特異性辨識 Hp 的單株抗體也成功的製造出來並已對其加以分析。每一株單株抗體與 3 種形式的 Hp 之間的親和力也以藉由競爭型與非競爭型 ELISA 計算出來。其中最強的單株抗體與 Hp 1-1、2-1 及 2-2 的親和力分別為 5.58×10^9、2.81×10^9 與 2.19×10^9。由這樣的結果推測

Hp 1-1 的『功能性』表面可能較 2-1 及 2-2 為多，這樣的結構性差異可能可以解釋部分 Hp 類型與感染、動脈硬化以及一些自體免疫疾病之間的關係。

每一種 Hp 表現型 CD 的光譜圖可用於評估 Hp 的二級結構。依據這好的結果我們發現在 α 螺旋結構中 Hp 1-1、Hp 2-1 及 Hp 2-2 分別有 29%、22%及 21%。Hp 1-1 比聚合體 Hp 2-1 及 Hp 2-2 的結構排序上更為整齊。

在 Cu2+及 AAPH 所誘發的氧化試驗中，Hp 比現有的抗氧化物" propucol" 的抗氧化效力還強。Hp 的抗氧化效力是依據濃度的改變而改變，依據 IC 50 抗氧化效力的大小排序為 Hp 1-1 > Hp 2-1 > Hp 2-2 > probucol > vitamin E。利用化學修飾法改變 Hp 的結構來研究 Hp 的結構是否為 Hp 擁有抗氧化能力的主要因素。有趣的是，carboxymethylation 破壞分子間與分子內的雙硫鍵的鍵結，不僅改變 α 螺旋結構的結合，並且在 Hb 的結合分析中發現破壞 Hp-Hb 的結合能力。更特別的是還原後的 Hp 比未還原的 Hp 的抗氧化能力還強。因此 Hp 的抗氧化能力並不是與血紅素結合所產生的複合物所導致的。

探討 Hp 在 in vivo 的抗氧化活性上，我們設計了 Hp1-1 的 cDNA 並利用 CMV 啟動子將其插入 pcDNA3.0 載體中，再將此載體轉殖到 CHO-K1 細胞中，並利用已存在選擇性標的物 (geneticin) 的培養液中培養， 接著使用競爭型 ELISA、西方點漬法及免疫化學染色法來確認已轉殖 Hp1-1 cDNA 的 CHO-K1 細胞是否有表現 Hp1-1。此細胞可以穩定的表現超過十個繼代後，轉殖的 CHO-K1 細胞再以不同濃度的過氧化氫培

養 24 小時，使用 MTT 分析法分析細胞的存活率，發現轉殖的 CHO-K1 細胞抗氧化壓力的耐受度會比一般的 CHO-K1 細胞抗氧化壓力的耐受度高三倍，因此表示 Hp 可以增加細胞抗氧化壓力的耐受度。

Hp 也是與血紅素具有高親和力的結合蛋白，在研究當中，我們共獲得了 24 株特異性辨識 Hp 的單株抗體，其中 8B1 可以與 Hp β chain 結合，並可以完全抑制 Hp-Hb 複合物的形成。而 Hp β chain 經過 carboxymethylation 後就無法被 8B1 辨識，或與 Hb 結合。結合 trypsin cleavage、carboxymethylation 以及 peptide array 的結果，β chain 上之 105-116 為 8B1 之結合位置。因為 chymotrypsin 與 trypsin 和 Hp β chain 的相似性，利用電腦模擬其結構，發現 Cys-Leu-Pro-Ser-Lys-Asp-Tyr-Ala-Glu-Val-Gly-Arg 位於表面結構的邊緣，並以 random coiled 的結構存在。統整而言，我們製作了一株能夠阻隔 Hb 與 Hp 結合之抗體，並且確切瞭解其結合位置為 β chain 上之 105-116，其中 Cys105 更是在與 Hb 的結合上扮演關鍵的角色。

在 Hp phentype 與 CAD 病患間的關聯性研究上，1132 個檢體中其中包含 559 個正常檢體以及 573 個 CAD 病患檢體。正常人 Hp phenotype 的分佈分別為 Hp 1-1　7.5%，2-1　42.2%，2-2　50.3%，而 CAD 病患之分佈則為 8.6%，40.1%，51.3%。統計上而言，Hp phenotype 與 CAD 患病與否並無明顯之關聯，然而 apoAI 的濃度在 Hp 1-1 的人身上平均為 138.2±35.0 mg/ml，相較於 Hp 2-1(121.4±29.6 mg/ml)，Hp 2-2 (124.5±29.7 mg/ml)為高($p = 0.024$)。而 CAD 病患的 apoAI 的濃度值(107.4±28.0 mg/ml)也較正常人(125.6±32.6 mg/ml)為低，然而在其他脂蛋白上並沒有如此明顯之差異性，因此，我們推測 apoAI 是 CAD 病患的主要危險因子。然而

過去的文獻報告曾經提及年齡與性別會影響 CAD 病患的發生率，所以我們嘗試將其他可能干擾的因素排除，只考慮 Hp phenotype 與 CAD 病患間的關聯性，將 Hp 1-1 與其他二種 phenotype 相比，分別得到 odds ratio 為 2.22 (Hp1-1/Hp 2-1)和 2.19 (Hp 1-1/Hp 2-2)。這個數據強烈地證明 Hp phenotype 與 CAD 病患間確實有強烈的關聯性。同時我們也發現台灣、大陸和東南亞之 Hp 1-1 的比例與世界上其他國家有極大之差異性，非洲國家（利比亞）為 20-49%，歐洲和北美則為 10-20%，而亞洲國家則低於 10%，而新幾內亞與智利則高達 56%。

Abstract

This is a second-year (2004) progress report for the 3-year contract grant funded by National Healthy Research Institute (NHRI). First, we deeply appreciate for the funding support from NHRI to provide us the opportunity to conduct this study. With this support, in the first year, 3 SCI papers and 16 conference reports (including 5 international conference reports) have been published. In addition, another 3 papers have been written and ready to submit by the end of 2004.

With respect to the antioxidant characteristic of haptoglobin (Hp), it is extremely essential to establish a method to purify large quantity of human Hp, because of the complicated and heterogeneous structure of Hp 1-1, 2-1, and 2-2. Two methods have been developed. First, hemoglobin (Hb)-affinity column was used to purify Hp, however, trace amount of Hb was co-eluted with Hp. Antibody-affinity column followed by a gel filtration chromatography was then established and appears to be superior. Since the purification was very difficult in the past, it has hampered for years in understanding the biochemical role of Hp phenotype in the clinical outcome.

An instant Hp-phenotyping method from human plasma without using a conventional starch gel has also been successfully implemented. Human plasma (or serum) incubated with supplymented hemoglobin was used to conduct a native PAGE, and the patterns of haptoglobin-hemoglobin complex was subsequently lit up using a chromogenic substrate. Thus, 3 phenotypes of Hp 1-1, 2-1, and 2-2 could be easily determined.

Moreover, 6 lines of monoclonal antibodies (mAb) prepared against Hp have been produced and characterized. The affinities among each mAb and each phenotype Hp were also determined using competitive and non-competitive ELISA. The highest-affinity monoclonal antibody exhibited an affinity (Ka) 5.58 x 10^9, 2.81 x 10^9, and 2.19 x 10^9 M^{-1} against Hp 1-1, 2-1, and 2-2, respectively. The result suggests that the availability of "functional" surfaces of Hp 1-1 may be greater than that of Hp 2-1 and 2-2. Such structural differences may explain, in part, the clinical outcome by which Hp phenotype is associated with differential susceptibility to infections, atherosclerosis, and autoimmune disorders.

The CD spectrum of each Hp phenotype was conducted to estimate the secondary structure of Hp. According to the best of our knowledge, we show for the first time that the α-helical content of Hp 1-1, 2-1, and 2-2 was 29%, 22%, and 21%, respectively. Hp 1-1 possesses a more ordered structure than that of polymeric forms of Hp 2-1 and 2-2.

In Cu^{2+}- and AAPH-induced lipid peroxidation, Hp exerts more potent antioxidant activity than the known, most potent antioxidant compound, probucol. The antioxidant activity of Hp was found to be dose-dependent with an IC50 Hp 1-1> Hp 2-1> Hp 2-2> probucol> vitamin E in ranking. To determine whether or not conformation of Hp plays a role in its antioxidant property, chemical modifications on Hp were conducted to alter its conformation. Interestingly, carboxymethylation which breaks the inter- and intra- disulfide linkages, not only altered α-helical content but also abolished its formation of a Hp-Hb complex in the Hb-binding assay. Notably, the reduced form of Hp exerts even much greater antioxidant activity than native Hp. Thus, the antioxidant activity of Hp might not be resulted from forming

a complex with hemoglobin.

To further study the *in vivo* physiologic role of Hp, the cDNA of Hp 1-1 was cloned and inserted into the pcDNA3.0 vector with CMV promoter. CHO-K1 cells were then transfected with the inserted vector and cultured in the presence of the selection marker, geneticin. Expression of Hp 1-1 in transfected CHO-K1 cell was confirmed by competitive ELISA, Western blot, and immunocytochemical stain. The expression was stable for more than ten passages. The transfected CHO-K1 cell was then challenged with variable amount of hydrogen peroxide (H_2O_2) for 24 hours. MTT assay was used to estimate the relative cell survival ratio. The transfected cell exhibits a twice-higher relative survival ratio than that untransfected CHO-K1 cell. Thus, the expressed Hp elevates the cell tolerance against the additive oxidative stress.

Haptoglobin (Hp) is a hemoglobin binding protein with an extremely high binding affinity. In this study, we screened twenty-four monoclonal antibodies (mAb) prepared against Hp. Only one mAb (8B1), which specific to Hp β-chain, is able to completely block the formation of Hp-Hb complex. Carboxymethylation on Cys-105 of Hp β chain diminishs the recognition for both 8B1 mAb and hemoglobin. Combining of limited trypsin cleavage, chemical modification, and direct solid-phase peptide synthesis on nitrocellulose paper (protein array), residues of 105-116 of Hp βchain is defined as an epitope for the inhibitory mAb 8B1. Hypothetically this sequence, Cys-Leu-Pro-Ser-Lys-Asp-Tyr-Ala-Glu-Val-Gly-Arg, is located at the edged surface with a random coiled structure as probed from the crystal structure of serine proteases (chymotrypsin and trypsin), which has strong homology with Hp β chain in primary structure. In conclusion, we have

produced a hemoglobin-blocking antibody recognizing the residues 105-116 of Hp β-chain, where we suggest that Cys-105 is critically involved in the hemoglobin binding. This study provides a potential insight for the future delineation of Hp binding site for hemoglobin which is currently unknown.

To investigate the relationship between Hp phenotype and CAD patients, a total of 1132 subjects containing 559 hospital normal control and 573 CAD patients were investigated for their Hp phenotypes. All the hospital normal controls and CAD patients were identified from the hospitalized coronary angiography. The distribution in hospital normal controls is 7.5 %, 42.2 %, and 50.3 % for Hp 1-1, 2-1, and 2-2. The phenotype distribution in CAD is 8.6 %, 40.1 %, and 51.3 % for Hp 1-1, 2-1, and 2-2, respectively. The distribution of Hp 2-1 and 2-2 is almost equal between CAD and hospital controls. Statistically, the phenotype difference between patients with and without CAD was not significant (P=0.69). There was no association found between the Hp phenotypes and lipid profiles (plasma total cholesterol, HDL cholesterol, LDL-cholesterol, and triglyceride). However apoAI, a major apolipoprotein of HDL, in Hp 1-1 (138.2±35.0 mg/mL) was significantly higher than that in Hp 2-1 (121.4±29.6 mg/mL) and Hp 2-1 (124.5±29.7 mg/mL) ($p = 0.024$). In patients with CAD, the mean apoA-I levels are found to be remarkably lower in all the phenotypes (107.4±28.0 mg/mL) than in controls (125.6±32.6 mg/mL). However, none of the other lipoid (except HDL) levels are significantly different from that of control subjects. Thus, we demonstrate that low apoA-I level in plasma is a superior risk factor in patients with CAD. Except apoAI, age in CAD group (67.3±9.5) is older than normal control (61.4±12.3). It matches the reported that the incidence of cardiovascular disease in adults is increase with age of life. The proportion of male in CAD (91%) is higher than normal control (70%). Men

show higher risk scores of coronary artery disease risk than women.

We have attempted to only address whether Hp phenotypes are associated with coronary artery disease and tested the hypothesis whether or not Hp phenotypes may become a risk factor when and other established risk factors (age, sex, hypertension, and apoAI levels) are adjusted. Stepwise conditional logistic regression analysis, confirms that more than doubling in susceptibility of coronary artery disease rates in the Hp 1-1 group compared with the rest, with an adjusted odds ratio of 2.22 in Hp 1-1/ Hp 2-1and 2.19 in Hp 1-1/ Hp 2-2. Thus, the data strongly suggest that Hp 1-1 was associated with CAD.

We also show that the Hp 1-1 phenotype distribution in Taiwan, China, and Southeast Asia are dramatically different from the other countries. Hp 1-1 distribution in Africa is more than 20 % and up to 49% in Liberia, between 10~20% in Europe and North America, and less than 10 % in Asia. The dominant Hp 1-1 distribution is among New Guinea and Chile (Indians) with values greater than 56%.

貳、九十三年度計畫著作一覽表

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若為群體計畫，請勾選本表屬於：■子計畫； 或 □總計畫(請自行整合)

1.列出貴計畫於本年度中之所有計畫產出於下表，包含已發表或已被接受發表之文獻、已取得或被接受之專利、擬投稿之手稿（manuscript）以及專著等

2.「計畫產出名稱」欄位：請依「臺灣醫誌」參考文獻方式撰寫

3.「產出型式」欄位：填寫該產出為國內期刊、國外期刊、專利、手稿或專著等

4.「SCI/SSCI」欄位：Social/Science Citation Index，若發表之期刊為 SCI/SSCI 所包含者，請在欄位上填寫該期刊當年度之 impact factor

5.「致謝與否」欄位：請註明該成果產出之致謝單位。若該成果產出有註明衛生署資助字樣者，請以 DOH 註明；若該成果產出有註明國家衛生研究院委託資助字樣者，請以 NHRI 註明；若該成果產出有註明衛生署及國家衛生研究院資助字樣者，請合併以 DOH&NHRI 註明；若該成果產出有註明非上述機構資助字樣者，請以機構全銜註明，舉例如下：

序號	計畫產出名稱	產出型式	SCI/SSCI	致謝與否
1.	Liau CY, Chang TM, Pan JP, Chen WL, Mao SJ. Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography. J. Chromatogr. B Analyt. Technol. Biomed Life Sci. 2003, 790(1-2):209-16	國外期刊	2.085	NHRI
2.	Tseng CF, Huang HY, Yang YT, Mao SJ. Purification of human haptoglobin 1-1, 2-1, and 2-2 using monoclonal antibody affinity chromatography. Protein Expr Purif. 2004 Feb;33(2):265-73	國外期刊	1.470	NHRI
3.	Tseng CF, Lin CC, Huang HY, Liu HC, Mao SJ. Antioxidant role of human haptoglobin. Proteomics. 2004 Aug;4(8):2221-8	國外期刊	5.766	NHRI
4.	Wang SS, Yueh CH, Yang MC, Mao SJ. Epitope mapping of a haptoglobin monoclonal antibody that blocks the haptoglobin-hemoglobin interaction: Implication of the involvement of residues 105-116 of haptoglobin in hemoglobin binding. **To be submitted**	國外期刊		NHRI
5.	Association of Haptoglobin 1-1 phenotype with angiographically documented coronary artery disease patients. **To be submitted**	國外期刊		NHRI
6.	Yang YT, Lai SM, Tseng CF, Wu CF, Mao SJ. Molecular properties of human plasma haptoglobin as probed by monoclonal antibodies. **To be submitted**	國外期刊		NHRI

*本表如不敷使用，請自行影印。

參、九十三年度計畫重要研究成果產出統計表

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於：■子計畫； 或 □總計畫(請自行整合)

(係指執行九十三年度計畫之所有研究產出成果)

<table>
<tr><td colspan="3">科 技 論 文 篇 數</td><td colspan="3">技 術 移 轉</td></tr>
<tr><td></td><td>國 內</td><td>國 外</td><td>類 型</td><td>經 費</td><td>項 數</td></tr>
<tr><td>期 刊
論 文</td><td>篇</td><td>6 篇</td><td>技 術
輸 入</td><td>千元</td><td>項</td></tr>
<tr><td>研討會
論 文</td><td>11 篇</td><td>5 篇</td><td>技 術
輸 出</td><td>千元</td><td>項</td></tr>
<tr><td>專 著</td><td>篇</td><td>篇</td><td>技 術
擴 散</td><td>千元</td><td>項</td></tr>
<tr><td colspan="2">技術報告</td><td colspan="2">技術創新</td><td colspan="2">著作權</td><td>專利權</td></tr>
<tr><td colspan="2" rowspan="2">篇</td><td colspan="2" rowspan="2">項</td><td colspan="2">(核准) 項</td><td>(核准) 項</td></tr>
<tr><td colspan="2">(申請中) 項</td><td>(申請中) 項</td></tr>
</table>

期刊論文：指在學術性期刊上刊登之文章，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部分，未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內

研討會論文：指參加學術性會議所發表之論文，且尚未在學術性期刊上發表者

專　　著：為對某項學術進行專門性探討之純學術性作品

技術報告：指從事某項技術之創新、設計及製程等研究發展活動所獲致的技術性報告且未公開發表者

技術移轉：指技術由某個單位被另一個單位所擁有的過程。我國目前之技術轉移包括下列三項：一、技術輸入。二、技術輸出。三、技術擴散

技術輸入：藉僑外投資、與外國技術合作、投資國外高科技事業等方式取得先進之技術引進國內者

技術輸出：指直接供應國外買主具生產能力之應用技術、設計、顧問服務及專利等。我國技術輸出方包括整廠輸出、對外投資、對外技術合作及顧問服務等四種

技術擴散：指政府引導式的技術移轉方式，即由財團法人、國營事業或政府研究機構將其開發之技術擴散至民間企業之一種單向移轉（政府移轉民間）

肆、九十三年度計畫重要研究成果

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於：■子計畫； 或 □總計畫(請自行整合)

※請依下列項目簡述計畫重要之研究成果※

一、 計畫之新發現、新發明或對學術界、產業界具衝擊性（impact）之研究成果。計畫之研究成果，請勾選下列項目並敘述其執行情形。

■ 1.研發或改良國人重要疾病及癌症的早期診斷方式及治療技術
□ 2.發展新的臨床治療方式
□ 3.發展新生物製劑、篩檢試劑及新藥品
■ 4.瞭解常見疾病及癌症之分子遺傳機轉
□ 5.瞭解抗癌藥劑對癌細胞之作用機制
□ 6.提供有效的疾病預防策略
□ 7.利用生物統計與生物資訊研究，推動台灣生技醫藥研究，促進生物技術與基因體醫學之發展
□ 8.醫療保健政策相關研究
□ 9.瞭解環境毒理機制及重金屬對人體健康的影響
□ 10.研發適合臨床使用的人造器官及生醫材料
□ 11.縮短復健流程並增加復健效果的醫療輔助方式或器材之研究應用
□ 12.改進現有醫療器材的功能或增加檢驗影像的解析能力
□ 13.其他重要疾病或醫藥衛生問題研究_____

In the last decades, all Hp related studies have been hampered with slow progress because of the difficulty of the purification of this protein. One major rationale is the heterogeneity and the nature of the molecule itself. Up-to-date, we have developed two isolation methods for purifying Hp from human plasma and reported in peer-reviewed journals. It definitely facilitates the future functional studies in this area. Few previous reports have pointed out that phenotypes of Hp (1-1. 2-1, 2-2) are related with coronary artery disease, especially with atherosclerosis. In our published results, Hp was found to possess an extremely potent antioxidant activity, which was 5-10 times greater than that of probucol, a well-known potent antioxidant in treating patients with atherosclerosis. The finding provides a

critical milestone in currently related area, which we have recently published in *PROTEOMICS* of 2004. In a collaboration with the Cardiology Division of Veterans General Hospital at Taipei, approximately of 1,200 subjects with and without coronary artery disease have been investigated for studying whether Hp phenotype is an independent risk-factor associated with atherosclerosis. These data including the analyses of age, gender, lipid, and lipoprotein profiles have recently been completed and gone through statistical evaluation. The manuscript will be submitted by the end of year 2004 in a well-reputed Journal. With respect to the binding of Hp to hemoglobin, we have produced a monoclonal antibody capable of blocking the interaction between Hp and hemoglobin. Epitope mapping indicates residues 105-116 of Hp β-chain is recognized by the antibody, suggesting this domain is responsible for hemoglobin binding and Cys-105 was critically involved. This study provides a potential insight for the future delineation of Hp binding site for hemoglobin which is currently unknown. Moreover, we are excited by finding the existence of a point deletion in Hp allele 1 mRNA in all phenotypes of human samples. The deletion mutation results in forming a stop codon. As such, a truncated short peptide of Hp is putatively expressed without physiologic function as compared to the wild type. The existence of this truncated mRNA has not been reported to the best of knowledge. We plan to submit this critical report by the first quarter of year 2005.

二、 計畫對民眾具教育宣導之研究成果（此部份將為規劃對一般民眾教育或宣導研究成果之依據，請以淺顯易懂之文字簡述研究成果，內容以不超過 300 字為原則）

Haptoglobin，簡稱 Hp，是血漿裡面的蛋白質。Hp 之結構複雜並有三個亞型，因此相當難以大量純化以進行相關研究。在此研究計畫中，我們利用血紅素及抗體親和性管柱成功純化出人類 Hp，這是相當重要的突破，並皆已發表於國際知名期刊。過去有些國外文獻提到 Hp 與心血管疾

病有一定的關聯性，本研究報告更是發現 Hp 之抗氧化特性極強，比目前已知最強的抗氧化物 probucol 還要更強，如果能對其抗氧化特性再深入研究，對於預防檢測心血管疾病甚至發展治療藥物都是非常有機會的，而針對這方面，我們更是積極努力進行當中。除此之外，我們更針對台灣地區進行 Hp 與心血管疾病之關聯性統計分析，並與全球其他地區做比較，希望能將這方面之相關研究成果與全球進行交流探討。

三、 簡述全程計畫成果之討論與結論，如有技術移轉、技術推廣或業界合作，請概述情形及成效

This is a second-year report (2003-2004) for the 3-year contract grant funded by National Healthy Research Institute (NHRI). First, we deeply appreciate for the funding support from NHRI to provide us the opportunity to conduct this study. With this support, in the last two years, 3 SCI papers and 16 conference reports (including 5 international conference reports) have been published and 3 manuscripts will be submitted by the end of year 2004.

The initial goal of this proposal is to test the hypothesis whether or not the phenotypes of Hp (Hp 1-1, 2-2, and 2-1) are related to the patients with coronary artery disease (CAD) and to explore the functional role of Hp in atherogenesis. The specific aims in the next 3 years are to:

1. Develop a new and fast approach for human Hp phenotping (Hp 1-1, 2-2, and 2-1) using monoclonal antibodies, and to test the hypothesis whether or not Hp phenotypes are correlated to the patients with coronary artery disease in Taiwan (2003-2005).

2. Study the Hp mRNA expression in macrophages and SMC in the presence of oxidized LDL and lipoproteins VLDL, LDL, and HDL

(2003-2004).

3. Test the hypothesis that Hp may exert an antioxidant role in macrophages and SMC transfected with sense and antisense Hp cDNA; to define the antioxidant activity of Hp phenotypes in vitro (2003-2004).

4. Investigate the functional role of Hp in the progression of atherosclerosis using cholesterol-fed rabbits and antiatherogenic effect (if any) using Hp transgenic mice (2004-2005).

Haptoglobin (Hp) is an acute-phase protein, its plasma levels increase consistently in response to infection and inflammation. The concentration of human plasma Hp is ranged between 1 and 1.5 mg/ ml. Similar to blood types, each individual of human Hp is classified as Hp 1-1, 2-1, or 2-2. The structural and functional analysis of the Hp, however, has not been studied in detail due to its difficult isolation procedure. In this study, we have established two purification methods using a high capacity hemoglobin-affinity column and a antibody-affinity column chromatography. Briefly, DEAE-purified human hemoglobin was first coupled to Sepharose 4B to prepare an affinity column in a 15-ml bed volume. Following a flow through of human plasma and an extensive wash, the bound material was eluted with a solution of 0.15 M NaCl, pH 11 (adjusted by ammonium), to remove low-affinity bound proteins. The high-affinity bound Hp was then eluted with 0.15 M NaCl containing 5 M urea, pH 11, and collected in tubes containing 100 ml of 1 M Tris buffer, pH 7.0. The biological activity of dialyzed Hp was retained as it formed a complex with hemoglobin on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Using this procedure, approximately 10 mg of Hp 1-1, with homogeneity

greater than 96%, was obtained from 15 ml of human plasma. The purified Hp 2-1 or 2-2, however, contained trace amounts of apoA-I with the similar approach. The Hp could be further purified by HPLC using a Superose 12 gel-permeation chromatography, if desired, to achieve 100% purity. All the phenotypes of purified Hp consisted of α and β chains on SDS–PAGE in the presence of a reducing reagent, further confirmed by a Western blot analysis. We conclude that human hemoglobin-affinity column was most suitable for the isolation of Hp 1-1 in large quantities. Whereas, one additional step using a gel-permeation was necessary for that of Hp 2-1 and 2-2.

The antibody affinity column provides a simple protocol that can be used to purify each Hp phenotype. Plasma was first passed through an affinity column coupled with a high affinity Hp monoclonal antibody. The bound material was washed with a buffer containing 0.2 M NaCl and 0.02 M phosphate, pH 7.4, eluted at pH 11, and collected in tubes containing 1 M Tris-HCl, pH 6.8. The crude Hp fraction was then chromatographed on a HPLC Superose 12 column in 0.05 M ammonium bicarbonate at a flow rate of 0.5 ml/min. The homogeneity of purified Hp 1-1, 2-1, or 2-2 was greater than 95% as judged by SDS polyacrylamide gel electrophoresis. Essentially, each Hp isolated was not contaminated with hemoglobin and apolipoprotein A-I as that reported from the other methods, and was able to bind hemoglobin. Neuraminidase treatment demonstrated that the purified Hp possessed a carbohydrate moiety, while Western blot analysis confirmed and chains corresponding to each Hp 1-1, 2-1, and 2-2 phenotype. The procedures described here represent a significant improvement in current purification methods for the isolation of Hp phenotypes. Circular dichroic spectra showed that the α-helical content of Hp 1-1 (29%) was higher than that of Hp 2-1 (22%) and 2-2 (21%)

We also explored that Hp was an extremely potent antioxidant, which directly prevented LDL from Cu2+-induced oxidation. Its potency was markedly superior to probucol: one of the most potent antioxidants. The IC50 of antioxidant activity in ranking was: Hp 1-1> Hp 2-1> Hp 2-2> probucol> vitamin E. Blockage of disulfide linkages between Hp subunits, not only abolished the α-helical content but also diminished its ability to form a complex with hemoglobin (Hb). It exerted almost 4 x greater antioxidant activity than that of native Hp. To investigate antioxidant role of Hp on the cellular level, the cDNA of Hp 1-1 was cloned, constructed (containing the pcDNA3.0 vector with CMV promoter) and transfected to CHO-K1 cells. Following the transfection, these CHO cells were able to express Hp 1-1 protein and significantly ($P<0.001$) elevated the tolerance against the oxidative stress. The elevation was about twice-higher than that normal CHO cells when challenged with hydrogen peroxide for 24 h. Thus, Hp plays a provocative antioxidant role as demonstrated in our in vitro and *ex vivo* studies.

Previously, we have prepared several lines of mAb against human plasma Hp and employed them for isolation of Hp. In the present study, we test the hypothesis whether these mAb could be used to explore the Hp-hemoglobin interaction. Each antibody was purified as an IgG form to at least 90% homogeneity prior to carry out the entire studies.

From more than 20 mAb screened using a previous established method, only 8B1 could inhibit the binding of hemoglobin to Hp. We than randomly chose the other 5 mAb as negative controls. A typical example showing the formation of Hp-hemoglobin complex on gel-exclusion HPLC and its

blockage by mAb 8B1 is depicted. The properties of the mAb with respect to their subunit specificity, binding affinity, and sensitivity to limited trypsin digestion for Hp were then determined. The data exhibit that the inhibitory mAb 8B1 recognized Hp β-chain, but failed to bind Hp upon the trypsin treatment. Whereas, G2D and an α-chain specific mAb W1 possessing no inhibitory activity were not affected on trypsinized Hp. The inhibitory activity for the hemoglobin binding appears not due to the extraordinary binding affinity, since the other non-inhibitory mAb were with similar binding affinity to Hp. Furthermore, combination of the other 5 mAb did not reveal the inhibition for Hp-hemoglobin binding

.

Next, we attempted to map out the antigenic epitope recognized by this 8B1 mAb. Since the limited trypsin cleavage on Hp resulted in a total loss of its immunoreactivity, suggesting that either Lys or Arg or both was essential to maintain the antigenic structure or probably lied in the epitope. In the next experiment, we addressed whether the Cys residues of Hp may participate the antigenic role for 8B1 mAb. The Cys residues were then chemically modified by carboxymethylation. Previously, we have demonstrated that carboxymethylated reduction disassembled the cross-linking between α (Cys-72) and β (Cys-105) chains of Hp on SDS-PAGE confirming Cys-105 of β-chain was carboxymethylated. Each chain was identical to Hp subunit in the presence of reducing reagent β-mercaptoethanol. The present study shows that the carboxymethylation substantially attenuated the immunoreactivity for 8B1 mAb, but not for G2D mAb. The finding implicated that Cys-105 might be involved in the binding for 8B1. It was extremely interesting that carboxymethylation on Cys-105 of Hp β-chain also abolished its binding to hemoglobin. Therefore, we speculated that Cys-105 participated for both hemoglobin and inhibitory mAb binding. However,

carboxymethylation also altered the overall structure of Hp; we could not rule out that the loss of immunoreactivity might be correlated to the overall conformational changes of Hp. In other words, 8B1 could be a "conformational" mAb.

Hp β-chain consists of 246 amino acid residues and has been suggested responsible for the binding to hemoglobin. A recent study using recombinant β-chain of Hp indicates that residues 80-161 are capable of binding hemoglobin with the ability almost equivalent to the full length 1-246. Although the result has not been reconfirmed by the others, it was legitimate to assume that our inhibitory 8B1 mAb also recognized the epitope within residues 80-161. From our carboxymethylation and trypsin-treatment experiments, Cys-105, Lys, or Arg residue could be involved in maintaining the antigenic structure. In considering Pro residues are usually located at or near to the immunoreactive domain by forming a loop at the surface of a given protein, we hypothesized that there was one domain, namely residues 105-119, highly possible for the binding of 8B1.

Accordingly, a solid-phase peptide array containing this predicted region (residues 105-119) and other eight either overlapped or non-overlapped synthetic peptides (each with 15 residues) within the residues 80-161 was prepared. These peptides were directly synthesized on a nitrocellulose membrane. After binding of our mAb followed by a HRP-conjugated secondary antibody, the array was developed using a chemiluminescent agent. We found that the epitope was exactly located in the predicted region: residues 105-119 (peptide 7) containing Cys-105 with other Arg, Lys, and Pro residues. Fig. 3 suggests that residues Ala-112 and Glu-113 were crucial for 8B1 recognition, since peptides 2 and 3 cleaved between these two residues

did not yield any immunoreactivity.

To further delineate the antigenic residues in the COOH-terminus of peptide 105-119, peptides 10, 11, and 12 were then prepared. Peptide 10 without COOH-terminal Gly-115 and Arg-116 did not exhibit immunoreactivity, while equal immunoreactivity was found between peptides 11 and 12. Interpretation of the activity among peptides 7, 10, 11, and 12, and the carboxymethylated data; the putative antigenic determinant appeared to be between residues 105-116 or Cys-Leu-Pro-Ser-Lys-Asp-Tyr-Ala-Glu-Val-Gly-Arg. Notably this region contains two positively and two negatively charged residues, formation a stable ionic pair over the overall structure of Hp is likely. Although the crystal graphic structure of Hp has not as yet been elucidated, in theory this region with the insertion of Pro-117 (usually lies on the loop) should be located at or near to the surface of Hp for rendering the antibody interaction. Nevertheless, our antigenic mapping suggests that residues 105-116 of Hp β-chain was located at the surface and could be involved in the binding for hemoglobin, which has not been shown previously. It also confirms an early report that the hemoglobin binding domain is located between residues 80-161.

Several lines of evidence indicate that the antigenic epitope we defined was reasonably correct. First, the size of an epitope is relatively small usually containing 8-12 amino-acid residues as demonstrated by us and the others. Second, the 105-116 epitope contains both acidic and basic residues and Pro which are commonly present in an antigenic site. Third, the epitope recognized by inhibitory 8b1 mAb consisting of Cys-105 was consistent to the effect of carboxymethylation data, in which Hp binding either for

antibody or for hemoglobin was diminished.

Furthermore, to test the accuracy of defined epitope region, we first compared the sequence homology between human and mouse. The key rationale is that part of the antigenic determinant of human Hp (residues 105-116) has to differ from that of mouse, so that the monoclonal antibody can be made from the mouse. It confirms our prediction with the difference lies in the COOH-terminus of mouse Hp. Other species such as rabbit, pig, and rat were also examined. Mutation of Ala-112 and Glu-113 significantly reduced (in pig) or abolished (in mouse and rat) the immunoreactivity. The finding was again consistent to the peptide array assay, in which cleavage between Ala-112 and Glu-113 resulted in a total loss of the immunoreactivity. A slight decrease in activity was found in that of rabbit; presumably substitution of Ala-112 with Thr-112 did not significantly alter the solution property of Hp. Thus, the sequence homology depicted may aid us to interpret the difference in immunoreactivity among the species.

Following the comparison of sequence homology of epitope among the different species, we were puzzling the relevant binding site of Hp involved for hemoglobin when considering the fact that this site should be well conserved. However, analysis of the NH2-terminal half (residues 105-111 or Cys-Leu-Pro-Ser-Lys-Asp-Tyr) of the epitope, it reveals that sequence 105-111 is completely identical among the species we observed. It is plausible to interpret this conserved region was responsible for both inhibitory antibody and hemoglobin recognition, whereas two or three residues lie in the COOH-terminus determined the antigenic specificity among the species. Another possibility is that this 8B1 mAb binds to a region in proximity to the hemoglobin binding site and hence possesses the inhibitory activity via a

steric hindrance.

Nevertheless, the epitope (residus 105-116) we mapped should be theoretically on the surface of Hp molecule for facilitating the antibody binding. We examined the crystal structures of chymotrpsin and trypsin, since these two serine proteases possess strong homology to Hp β chain in primary structure (greater than 50% as calculated from the protein data bank). The sequence alignment among these three molecules revealing a good homology between the epitope region 105-116 of Hp and the proteases. It is of striking interest that this homology motif is exactly located at the edged surface of chymotrpsin and trypsin with a random coil structure. It is of worth mentioning that the authentic structure of this region in Hp should await the establishment of the Hp 3D-structure. In conclusion, we have produced a hemoglobin-blocking antibody recognizing the residues 105-116 of Hp β-chain. It provides a potential tool for the future delineation of Hp binding site for hemoglobin which is currently unknown.

Regarding to the relationship between Hp phenotypes and CAD patients, less than 8% of the normal population in Taiwan corresponded to Hp 1-1, while Hp 2-1 and 2-2 were about 42% and 50%. Such distribution was similar to other Far East Asian areas: China, Korea, and Japan. Hp 1-1 or 2-2 represents the homozygous individuals from human allele-1 or allele-2, respectively, located at chromosome 16q22.1. It is puzzling, however, that the heterozygous Hp 2-1 individuals generally account for 50% among all the populations, even though the frequency of Hp 1-1 is as low as 8% in Southeast Asia. Genetically, since α 2 structure of Hp 2-2 contains one complete α 1 copy and a partial repeat of α 1 (as a result of α 1 insertion) and the α 2 structure has not been found in non-human subjects, as such human

phenotype 2-2 may be evolved later than Hp 1-1. Hp 2-2 homozygous is then reproduced from heterozygous 2-1. Haptoglobin genotype is an independent risk factor for cardiovascular disease and that this relationship is specific for diabetes.

Hp 2-2 molecule was significantly less than Hp 2-1 and 1-1 in protection from Cu2+-induced LDL oxidation. We respected to the mechanism involved in the pathogenesis of Hp phenotypes in CAD, The present study demonstrates that Hp phenotype was associated with CAD. The data was reported by Melamed-Frank et al, 2002, in which they demonstrate that the antioxidant effect of Hp 1-1 is differentially superior to Hp 2-1 and 2-2 using hemoglobin-induced LDL oxidation, a protection mechanism thought to be mediated through Hp-Hb complex formation. In patients with diabetes mellitus developing nephropathy, Nakhoul et al (2001, Diabetologia) have postulated that the differences in the molecular shape and size between the Hp 1-1 and 2-2 are involved. Recently, we have shown that a significant overall difference in structure between Hp 1-1 and Hp 2-1 or 2-2 using a circular dichroic spectrum analysis. We suggest that the availability of "functional surfaces" of Hp 1-1 and 2-1 may be greater than that of Hp 2-2. This is because that only the Hp 2-2 can form heterogeneous cyclic polymers with some of the functional domains "buried" inside the bulky arrangement. Such structural diversity may account, in part, for the clinical outcome by which Hp 2-2 phenotype is associated with susceptibility to CAD and other related diseases. Nevertheless, identification and understanding of the biochemical basis for the differences among Hp phenotypes may lead to a rational design in intervening new pharmacological agents, such as mini-Hp, which have been recently proposed.

In control group, Hp 1-1 has higher concentrate of apoAI. It was readily known that apo AI concentration was a marker of atherosclerosis. Plasma level of apoAI is an important determinant and is more useful than HDL cholesterol for identifying patients with coronary artery disease. So to estimate other risk factors for CAD, it must adjust the apoAI level before statistic. The higher concentration of apo AI, the lower of atherosclerosis frequency. Apo AI is thought to be better predictor of coronary artery disease than HDL cholesterol. Apo AI is the major component of HDL, and it displays anti-atherogenic properties. Michelle C. et al reported that apoAI concentration was an independent predictor of CAD. On the other hand, the increase risk of coronary artery disease with the increase in age occurred due to a combination of factors. According to Kannel and Wilson, the incidence of cardiovascular disease in adults doubles approximately at each decade of life. Men had higher risk scores and were more concentrated in the highest positions of the ranges of coronary risk than women did. The biological explanation for this fact could be the female protection provided by estrogen, which has a direct effect on the circulatory system, causing vasodilatation and inhibiting the progression of the atherosclerotic processes, avoiding, therefore, ischemic events. Hypertension and coronary artery disease (CAD) are often associated, reflecting common origins in lifestyle and the role of hypertension as a risk factor for CAD.

To estimate the really effect of Hp phenotypes on CAD, these existed conventional risk factors should be considered to modulate and predict the susceptibility in the general population to atherosclerotic coronary artery disease. Therefore, we used the conditional stepwise logical regression to export the masked effects. Compare the phenotypes distribution of Hp show dramatic geographical differences. Haptoglobin polymorphism is associated

the prevalence and clinical evolution of many inflammatory diseases, including infections, atherosclerosis, and autoimmune disorders. The strong genetic pressure favoring the Hp 2-2 phenotype suggests an important role of Hp in human pathology. The Phenotype distribution difference of various populations is probability programmed in geographic differences in diseases (diabetes, cardiovascular disease, certain cancers, and infection diseases). For example, Hp 1-1 is associated with susceptibility to falciparum malaria and the development of severe complications; alternatively, the other phenotypes may confer resistance. It had been reported that women with the Hp 1-1 reproduce at an earlier age and have higher natural fertility potential than women with other Hp genotype. The highest distribution of Hp 1-1 in the world, it could partially explain the earlier reproduce in Africa than other area. It has been proposed that the Hp-2 allele was originated at about 2 million years ago and had since spread over the world under a strong genetic pressure, which favors the Hp 2-2 phenotype displacing the monopoly of Hp 1 allele gradually. The greatest value of Hp 1-1 distribution is being in the West Africa (49%) and Indians of Chile (56-63%), with the Hp 2-2 distribution less than 10%. Its distribution decreases from West Africa (49%) in the direction of East Africa (25%), Northwest Europe (16%), Southeast Europe (15%) to Asia (2-8%) (5-12,26-46). Thus, the data suggest that Hp phenotype distribution variance might be regarded as an index factor in relation to ancient human population migration. In the patient with diabetes increased oxidative stress, differences in genetically determined endogenous antioxidant protection may have exaggerated importance.

Finally, since Hp phenotype is an independent risk factor in developing CAD and its provocative antioxidant role in protecting atherosclerosis, determination of Hp concentrations in human plasma would be essential.

四、 成效評估（技術面、經濟面、社會面、整合綜效）

One concept that has received much recent attention for the pathogenesis of atherosclerosis is the LDL-modification hypothesis, which postulates that atherosclerotic plaque resulted from the uptake of oxidized LDL by macrophages in the arterial wall followed by smooth muscle (SMC) migration and proliferation. Inflammatory phenomena at sites of atherosclerotic plaques are increasingly thought to be major determinants of the progression and clinical outcome of atherosclerosis disease. Therefore, attention is being paid to systemic markers that may reflect the inflammatory activity in the plaques. Recently we found that haptoglobin (Hp), an acute phase protein elevated sharply during the infection and inflammation, was substantially accumulated in atherosclerotic lesions. Subsequently, we demonstrated that Hp was endogenously synthesized in macrophages, but not in SMC. We also demonstrated in vitro that Hp is a highly potent antioxidant superior to probucol. Nevertheless, while the pathogenesis of atherosclerosis has been continuously explored, the Hp molecule that may protect against the progression of atherosclerosis has never been reported. The clinical outcome as from this study should provide the highlight of the mechanism involved in the atherosclerosis. The clinical test in determination of the concentration of Hp may bring some commercial values in the diagnosis of Hp related diseases. On the other hands, students trained in our laboratory should provide significant contribution in future development of biotechnology and biomedical research.

五、 下年度工作構想及重點之妥適性

In the next year, we plan to explore the detailed antioxidant mechanism of Hp and to define the exact antioxidant domain. Thus, it may develop a mini-Hp

as a therapeutic agent in treating free radical associated diseases. We also plan to prepare si-RNA as a probe to knock-out the expression of Hp to study the physiologic role in cells containing Hp. In addition, we have identified minipigs and 3 patients deficient with Hp, we attempt to address the molecular mechanism involved. Some of the task force are described below in Section 6.

六、 檢討與展望

While reporting the first 2 year progress, we have published 3 SCI papers and 16 conference abstracts providing the new insights in Hp purification and physiologic significance. There are about 10 students in PI's laboratory who are currently engaging the studies of Hp. The solution property of Hp shows that Hp 1-1 possesses high surface area as demonstrated by its immunoreactivity. Antioxidant activity study reveals that Hp is an extremely potent molecule in protecting cell from oxidative stress as judged by the CHO cells transfected with Hp cDNA. Clinical study using 1200 subjects shows that Hp 1-1 phenotype is associated with the development of atherosclerosis, when apolipoprotein A-I concentration is adjusted. Currently, we have developed a new approach for the determination of Hp level in those patients. In an another preliminary study, we have identified 3 subjects whose Hp appealing to be deficient by a hemoglobin binding study. A follow-up study is underway to further explore its molecular mechanism. Another exciting finding is that we have also identified few minipigs, which contain no Hp in their plasma using hemoglobin binding, gel electrophoretic, and Westernblot analyses. This year we have purchased a peptide synthesizer, by which two major peptide corresponding to the β-chain of Hp have been chemically synthesized to define their functional domain. Hopefully, a mini-Hp can be identified as a therapeutic agent for atherosclerosis.

In addition, we are now designing the si-RNA to knockout the expression of cells containing Hp to delineate the function of this protein. Furthermore, cloning of Hp promoter is underway to study the molecular regulation of cellular Hp, and attempts to perform microinjection in preparing the Hp transgenic mice. We are confident that we are capable of taking the leadership in the area Hp research in the next 5 years, since all the biochemical and molecular tools are available. It is worth mentioning that we have identified a mutation or a deletion of a single nucleotide that causing a trauncated β-chain in human subjects with Hp 2-2 phenotype. In conclusion, many new dimensions of the structure-function studies of Hp have been emerged from our laboratory, we hope to bring the excitement of these findings into future clinical outcomes. Finally, we would like to thank all the research supports of National Health Research Institute (NHRI) for funding our ongoing studies and to keep sponsoring such a meaningful program.

伍、九十三年度計畫所培訓之研究人員

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於: □子計畫； 或 □總計畫(請自行整合)

種類				人數	備註
專任人員	1.	博士後研究人員	訓練中		
			已結訓		
	2.	碩士級研究人員	訓練中		
			已結訓		
	3.	學士級研究人員	訓練中		
			已結訓		
	4.	其他	訓練中		
			已結訓		
兼任人員	1.	博士班研究生	訓練中	4	鄭財木、曾繼鋒、陳文亮、賴以祥
			已結訓		
	2.	碩士班研究生	訓練中	6	宋俊瑩、殷韶嬋、林宏輝、楊明誌、王文昭、高立品
			已結訓	2	蔡忠義、劉惠君
醫師			訓練中		
			已結訓		
特殊訓練課程					

註： 1.特殊訓練課程請於備註欄說明所訓練課程名稱

2.本表如不敷使用，請自行影印

陸、參與九十三年度計畫所有人力之職級分析

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於: ■子計畫； 或 □總計畫(請自行整合)

職級	所含職級類別	參與人次
第一級	研究員、教授、主治醫師	3 人
第二級	副研究員、副教授、總醫師、助教授	人
第三級	助理研究員、講師、住院醫師	4 人
第四級	研究助理、助教、實習醫師	8 人
第五級	技術人員	人
第六級	支援人員	人
合計		15 人

第一級：研究員、教授、主治醫師、簡任技正，若非以上職稱則相當於博士滿三年、碩士滿六年、或學士滿九年之研究經驗者

第二級：副研究員、副教授、助研究員、助教授、總醫師、薦任技正，若非以上職稱則相當於博士、碩士滿三年、學士滿六年以上之研究經驗者

第三級：助理研究員、講師、住院醫師、技士，若非以上職稱則相當於碩士、或學士滿三年以上之研究經驗者

第四級：研究助理、助教、實習醫師，若非以上職稱則相當於學士、或專科滿三年以上之研究經驗者

第五級：指目前在研究人員之監督下從事與研究發展有關之技術性工作，且具備下列資格之一者屬之：具初（國）中、高中（職）、大專以上畢業者，或專科畢業目前從事研究發展，經驗未滿三年者

第六級：指在研究發展執行部門參與研究發展有關之事務性及雜項工作者，如人事，會計、秘書、事務人員及維修、機電人員等

柒、參與九十三年度計畫所有人力之學歷分析

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於：■子計畫；　或　□總計畫(請自行整合)

類別	學歷別	參與人次
1	博士	1　人
2	碩士	人
3	學士	2　人
4	專科	人
5	博士班研究生	4　人
6	碩士班研究生	8　人
7	其他	人
合計		15　人

捌、參與九十三年度計畫之所有協同合作之研究室

群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於：□子計畫　　□總計畫(請自行整合)

機構	研究室名稱	研究室負責人

玖、九十三年度之著作抽印本或手稿

依「貳、九十三年度計畫著作一覽表」所列順序附上文獻抽印本或手稿

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 790 (2003) 209–216
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography

Chun Yi Liau[a], Tsai Mu Chang[a], Ju Pin Pan[b], Wen Liang Chen[a], Simon J.T. Mao[a,*]

[a]*Research Institute of Biochemical Engineering, Department of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsinchu, Taiwan*
[b]*Division of Cardiology, Veterans General Hospital and Yang-Ming Medical College, Taipei, Taiwan*

Abstract

Haptoglobin (Hp) is an acute-phase protein; its plasma levels increase consistently in response to infection and inflammation. The concentration of human plasma Hp is ranged between 1 and 1.5 mg/ml. Similar to blood type, individual human Hp is classified as Hp 1-1, 2-1, or 2-2. The structural and functional analysis of the Hp, however, has not been studied in detail due to its difficult isolation procedure. Previously, we reported a single step for the purification of porcine Hp. In this study, we established a purification method using a high capacity hemoglobin-affinity column. Briefly, DEAE-purified human hemoglobin was first coupled to Sepharose 4B to prepare an affinity column in a 15-ml bed volume. Following a flow through of human plasma and an extensive wash, the bound material was eluted with a solution of 0.15 *M* NaCl, pH 11 (adjusted by ammonium), to remove low-affinity bound proteins. The high-affinity bound Hp was then eluted with 0.15 *M* NaCl containing 5 *M* urea, pH 11, and collected in tubes containing 100 μl of 1 *M* Tris buffer, pH 7.0. The biological activity of dialyzed Hp was retained as it formed a complex with hemoglobin on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Using this procedure, approximately 10 mg of Hp 1-1, with homogeneity greater than 96%, was obtained from 15 ml of human plasma. Affinity purified Hp 2-1 or 2-2, however, contained trace amounts of apoA-I with the similar approach. The Hp could be further purified by HPLC using a Superose 12 gel-permeation chromatography, if desired, to achieve 100% purity. All the phenotypes of purified Hp consisted of α and β chains on SDS–PAGE in the presence of a reducing reagent, further confirmed by a Western blot analysis. We conclude that human hemoglobin-affinity column was most suitable for the isolation of Hp 1-1 in large quantities. Whereas, one additional step using a gel-permeation was necessary for that of Hp 2-1 and 2-2.
© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Affinity adsorbents; Haptoglobin; Proteins; Glycoproteins

1. Introduction

Haptoglobin (Hp), also known as an α-2 glycoprotein, is a hemoglobin-binding protein present in plasma of all vertebrates and is believed to participate in hemoglobin transport. The concentration of Hp in human plasma is relatively high ranging from 1.0 to 1.5 mg/ml [1,2], which may increase as an acute-phase protein in response to a variety of injuries and inflammatory disease states [3,4]. For this reason, Hp is useful as a diagnostic marker and as a clinical evaluation of many inflammatory diseases. Human Hp is a tetrameric structure linked by disulfide linkages among the two α and two β chains

*Corresponding author. Tel.: +886-3-571-2121x56939 or 56948; fax: +886-3-572-9288.
E-mail address: mao1010@ms7.hinet.net (S.J.T. Mao).

1570-0232/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S1570-0232(03)00128-4

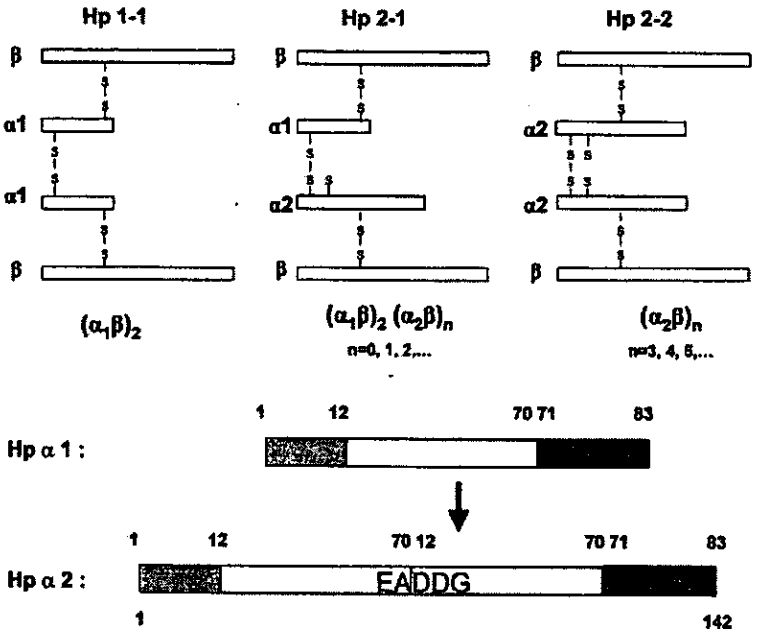

Fig. 1. Schematic drawing of the phenotype structure of human Hp 1-1, 2-1, and 2-2 (top). All three phenotypes share a common structure of β chains. The degree of polymerization within the inter-molecular arrangement is shown. The difference between α1 and α2 chains and their combinations determine the Hp phenotype (bottom). α2 is a duplicate of α1 with a repeat insert of residues 12–70. Making it simple, α2 (142 amino acids) contains two repeated domains showing a unique sequence of EADDG (residues 69–73) at the splicing site.

[5–7]. Based on the length of α chains, there are three phenotypes of Hp in the population, Hp 1-1, 2-1, and 2-2 (Fig. 1), which are similar to that of blood types. All the phenotypes share the same two β chains [each with about molecular mass (M_r) 40 000 including 243 amino acids and approximate 30% (w/w) carbohydrate moiety] [5–7]. A typical structure of homozygous Hp 1-1 is composed of two identical α 1 chains (each with about M_r 9000 including 83 amino acids). Whereas, Hp 2-2 is composed of two identical α 2 chains (142 amino acids) as compared to that of heterozygous Hp 2-1 containing each α 2 and α 1 (Fig. 1). Likewise, the tetrameric arrangement is also found in other animal species such as rat, rabbit, and pig [3,8–10]. However, the two αβ units joined by a non-covalent interaction, rather than a disulfide bridge, are found in dog, cat, and bear [11,12].

Several functional differences between Hp phenotypes have been demonstrated, appearing to have important biological and clinical consequences [1,2,13,14]. For example, patients with phenotype Hp 1-1 are less prone to the development of inflammatory-related cardiovascular diseases and diabetics than that with Hp 2-1 and 2-2 [13–15]. Although the protein has been well characterized genetically, the exact physiological role and the biochemical mechanism by which Hp 1-1 is more resistance to those inflammatory-related diseases are not well understood. The later has been hampered by the availability of Hp, which is mainly due to the considerably difficult procedures for Hp purification. Commercially prepared Hp is not only expensive lacking the biological activity, but also heterogeneous containing the mixture of three phenotypes isolated from the plasma pools.

Currently, the most common procedures involved for the purification of human Hp are associated with electrophoresis, affinity chromatography using a monoclonal antibody, and multiple high-performance liquid chromatography (HPLC) steps [16–20]. These methods are useful but are troublesome and time-consuming, and the quantity of Hp obtained is relatively small. Affinity column purification using chicken hemoglobin has been reported [19,21]. The binding affinity of chicken hemoglobin to human Hp,

however, is less than that of human hemoglobin [1,2]. The method [21] was satisfactory for Hp purification in species other than humans, but required a few column-steps for human Hp [19]. Previously, we established a simple purification method with high yield for porcine plasma Hp. The method, however, was not practical in the isolation of human samples due to the heterogeneity of human Hp [22]. In the present report, we describe a purification procedure for human Hp 1-1, 2-1, and 2-2 using an affinity column that was immobilized with highly purified human hemoglobin. Approximately 8–10 mg of human Hp can be obtained from 15 ml of plasma. The procedure can be easily scaled up for Hp 1-1 purification. A simple hemoglobin isolation procedure using an isocratic DEAE HPLC system is also described.

2. Experimental

2.1. Materials

Goat anti-human haptoglobin was purchased from Calbiochem-Novabiochem (San Diego, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany) without any further purification.

2.2. Preparation and purification of human hemoglobin

Fresh human blood collected in 0.1% EDTA was immediately centrifuged at 3000 *g* for 25 min, after which time plasma was removed by aspiration. The remaining red blood cells (RBCs) were washed five times with three volumes of phosphate-buffered saline (PBS) containing 0.12 *M* NaCl and 12 m*M* phosphate, pH 7.2, and then lysed with two volumes of deionized water at 4 °C. Cell debris was removed by centrifugation at 3500 *g* for 30 min. The supernatant containing mostly hemoglobin was fractionated by 50% saturated ammonium sulfate at 4 °C for 30 min followed by a centrifugation at 4500 *g* for 40 min at 4 °C. The supernatant was dialyzed against 0.02 *M* sodium phosphate, pH 8.0, at 4 °C overnight followed by a filtration through a 0.45 μm nylon fiber prior to HPLC.

The HPLC system (Waters) consisted of two pumps, an automatic sample injector, a photodiode array detector, and an interface module [23,24]. A Bio-Scale DEAE column (10×1.5 cm) packed with an anion-exchange Macro-Prep and equilibrated with 20 m*M* phosphate buffer, pH 8.0, at a flow-rate of 1 ml/min, was used for hemoglobin purification. Partially purified ammonium sulfate fraction of hemoglobin (total 50 mg in 2 ml) was applied to the column followed by an elution with the initial buffer at a flow-rate of 1 ml/min. The chromatographic profile was monitored by a photodiode array detector and read at 280 nm.

2.3. Preparation of human hemoglobin-affinity column

DEAE-purified human hemoglobin was first coupled to CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's procedures. Briefly, 5 g of freeze–dried Sepharose was swollen and suspended in 1 m*M* HCl and immediately washed 3× within 15 min with the same solution on a glass filter [23,25]. The gel was then washed with a coupling buffer containing 0.1 *M* $NaHCO_3$, and 0.5 *M* NaCl, pH 8.0, and subsequently degassed. A 2-ml volume of hemoglobin (25 mg/ml), pre-dialyzed in the coupling buffer, was slowly added to the gel (in 25 ml), while gently stirring for 1 h at room temperature. After coupling, the gel was washed 3× with the coupling buffer (200 ml) to remove uncoupled hemoglobin via a glass filter. Finally, the gel was treated with 0.1 *M* Tris–HCl, pH 8.0, for 2 h at room temperature to saturate the remaining reactive sites of Sepharose. The coupling efficiency of hemoglobin to gel was approximately of 98%. The degassed gel was then packed onto a 20×1.5 cm column and extensively washed with two cycles of PBS, pH 7.2, and 0.15 *M* NaCl, pH 11, which was adjusted by ammonium as previously described [25].

2.4. Isolation of Hp by human hemoglobin-affinity column

Initially, 15 ml of human plasma was loaded onto the hemoglobin-affinity column (15 ml in bed volume) at room temperature without incubation, fol-

lowed by an extensive wash with 200 ml of PBS. The bound materials were first eluted with three volumes of 0.15 *M* NaCl, pH 11 (adjusted by ammonium), as fraction 1 [25] and then eluted with three volumes of 5 *M* urea in 0.15 *M* NaCl, pH 11 (freshly prepared and filtered), as fraction 2. A 5-ml volume of each fraction was collected in a tube containing 0.1 ml of 1 *M* Tris–HCl, pH 7.0, to immediately neutralize the pH value. Pooled fractions containing Hp were then dialyzed at 4 °C overnight with three changes of PBS.

2.5. Gel electrophoresis and densitometry

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and native PAGE were performed according to the Laemmli's method [26] with some modification as previously described [22]. Samples (typically 10 μg) for SDS–PAGE were preheated at 100 °C for 10–15 min in an SDS loading buffer [50 m*M* Tris–HCl, 2% (w/v) SDS, 100 m*M* 2-mercaptoethanol, pH 6.8]. For molecular mass calibration, a subset of the following standards was included in each gel: β-galactosidase (116 000), phosphorylase B (97 000), bovine serum albumin (BSA, 66 000), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), lysozyme (14 400), and aprotinin (6500). The samples were run for 0.5 to 1 h at 120 V and stained by a Coomassie brilliant blue G-250. Densitomertic analysis of SDS–PAGE gel was performed using a Molecular Dynamics densitometer for data acquisition and Image Quant software for integration and analysis.

2.6. Immunoblot analysis

Following the separation of proteins by SDS–PAGE, the gel was soaked in a transfer buffer containing 50 m*M* Tris–HCl, 50 m*M* boric acid, and 1 m*M* EDTA, pH 8.2, for 30 min. The gel was then transferred to a nitrocellulose membrane (Pharmacia) at 100 mA for 1 h in a semi-dry transfer cell (Bio-Rad) containing a transfer buffer. The membrane was immersed in 1% BSA, Tween-containing Tris-buffered saline (TTBS) [20 m*M* Tris–HCl, 50 m*M* NaCl, 0.05% (w/v) Tween 20, pH 7.4] for 1 h with gentle shaking at room temperature. Following a wash with TTBS for 3 min, the membrane was incubated with a primary antibody [1:2500 dilution in TTBS containing 1% (w/v) BSA] for 1 h at room temperature and washed three times with TTBS. The membrane was then incubated with 1:5000 diluted antiserum against goat immunoglobulin G (IgG) conjugated with horseradish peroxidase for 1 h in TTBS containing 1% (w/v) BSA. Finally, the membrane was washed three times with TTBS and developed into a color immunoblot with 3,3′-diaminobenzidine (DAB)-stabilized substrate for horseradish peroxidase [22].

3. Results

3.1. Purification of human hemoglobin

A typical HPLC profile for the purification of ammonium sulfate fractionated hemoglobin is shown in Fig. 2. Both SDS–PAGE and native-PAGE analyses show that the homogeneity of purified hemoglobin was greater than 96% (Fig. 3).

3.2. Preparation of hemoglobin-affinity column

In theory, the purity of human hemoglobin obtained from ammonium sulfate fractionation was

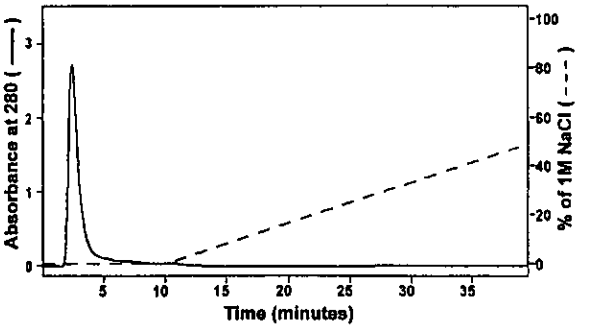

Fig. 2. Typical purification profile of human hemoglobin on DEAE HPLC. About 5 mg of 50% saturated ammonium sulfate top fraction was applied to a DEAE column (10×1.5 cm) pre-equilibrated with 20 m*M* phosphate, pH 8.0. A mobile phase containing the same buffer was run through for 10 min at a flow-rate of 1 ml/min. A linear gradient was produced from 0 to 0.3 *M* of NaCl to regenerate the DEAE resin. The same procedure was used for the purification of hemoglobin in large scale (total of 50 mg in 2 ml) as described in the Experimental section.

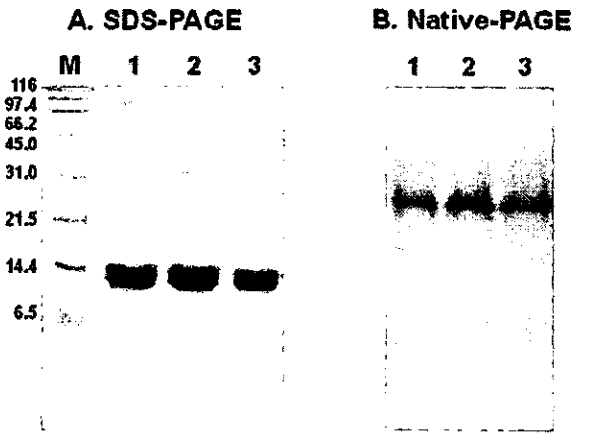

Fig. 3. Analyses of purified human hemoglobin using SDS–PAGE (A) and native-PAGE (B). (A) Lane M represents the molecular mass markers (molecular masses $\times 10^{-1}$). Lanes 1–3 represent hemoglobin obtained from the RBC lysate, top fraction of 50% saturated ammonium sulfate precipitation, and DEAE HPLC, respectively. Approximately 15 μg of each protein was loaded on an 18% SDS–PAGE in the presence of a reducing reagent. (B) Lanes 1–3 represent hemoglobin obtained from RBC lysate, top fraction of 50% saturated ammonium sulfate precipitation, and DEAE HPLC, respectively. Each protein was loaded on a 10% native-PAGE.

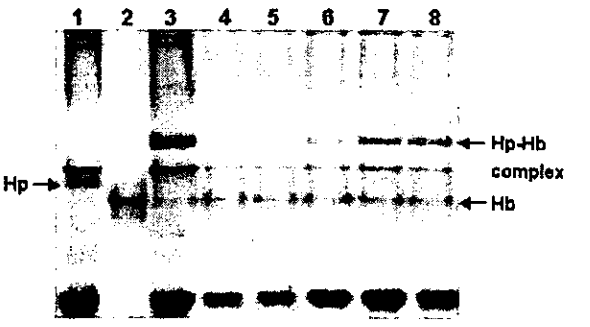

Fig. 4. Evaluation of binding capacity of hemoglobin-conjugated Sepharose to Hp in plasma. Briefly, plasma containing Hp was passed through 1 ml of conjugated Sepharose column. The pass-through fraction was collected and mixed with free hemoglobin. The unbound or remaining Hp, if any, was able to form a Hp–hemoglobin complex displaying an extra band in a 7% native-PAGE. Lanes: 1=human plasma prior to the affinity column; 2=purified hemoglobin; 3=plasma spiked with purified hemoglobin showing a Hp–hemoglobin complex; 4–8=samples of 0.25, 0.5, 0.75, 1.0, and 2.0 ml plasma passed through a hemoglobin-Sepharose containing 1 mg of hemoglobin, respectively. No Hp was detected in lanes 4 and 5 when 0.25–0.5 ml of plasma was applied onto the conjugated Sepharose. According to our calculation, 1 mg of hemoglobin coupled on Sepharose could bind about 0.75 to 1.13 mg Hp (lanes 5 and 6).

adequate in preparing affinity column for Hp purification as that described using chicken hemoglobin [19]. However in a preliminary application, we found that column immobilized with ammonium sulfate fraction of hemoglobin could produce significant plasma clots and subsequently demolished the chromatography (data not shown). This clotting effect, however, was not observed when DEAE-purified hemoglobin was employed for affinity column. Using native-PAGE to evaluate the binding capacity of Sepharose 4B immobilized with human hemoglobin, the capacity we estimated was approximately between 0.75 and 1.13 mg of Hp 1-1 per mg of hemoglobin (Fig. 4). This binding capacity was 20-times greater than that reported using chicken hemoglobin [19].

3.3. Isolation of human Hp by hemoglobin-affinity column chromatography

Fig. 5 shows a typical chromatography using an affinity column conjugated with highly purified human hemoglobin. Initially, 15 ml of plasma of Hp 1-1 was applied to the column followed by an extensive wash (Fig. 5); the bound protein was first eluted with 0.15 *M* NaCl, pH 11 (fraction 1) to remove the low-affinity binding proteins such and apoA-I. The column was then eluted with 0.15 *M* NaCl containing 5 *M* urea, pH 11 (fraction 2) for high-affinity binding Hp. Each eluent was immediately neutralized in the tube containing 100 μl of 1 *M* Tris–HCl, pH 7.0 (Fig. 5). SDS–PAGE analysis on fraction 1 revealed that it contained mostly high-molecular-mass proteins and apoA-I (Figs. 6 and 7), but not in fraction 2. The purity of Hp 1-1 in fraction 2 was approximately 96%. The recovery of Hp in fraction 2 was approximately 45.5% from the plasma with a final of 77-fold purification (Table 1). Under the same condition, however, some apoA-I was found to be co-eluted in the fraction 2 of Hp 2-1 and 2-2 (Figs. 6 and 7). The contaminated apoA-I could be further removed (data not shown) using a single step on HPLC Superose 12 as previously described by us [22]. A typical Western blot analysis showing three isolated phenotypes of Hp is depicted in Fig. 7. The presence of apoA-I in Hp 2-1 and 2-2 was

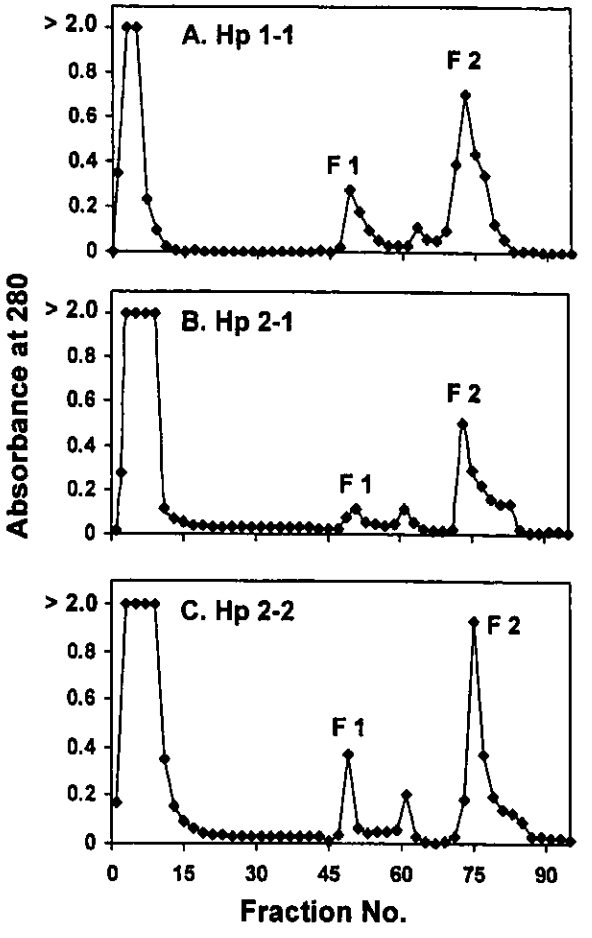

Fig. 5. Typical elution profile of hemoglobin-affinity column chromatography of plasma containing Hp 1-1 (A), Hp 2-1 (B), and Hp 2-2 (C). Initially, 15 ml of human plasma was applied to the hemoglobin-affinity column followed by an extensive wash with 200 ml of PBS. The bound materials were first eluted with three volumes of 0.15 *M* NaCl, pH 11 (adjusted by ammonium), as fraction 1 and then eluted with three volumes of freshly prepared and filtered 5 *M* urea in 0.15 *M* NaCl, pH 11, as fraction 2. A 5-ml volume of each fraction was collected in a tube containing 0.1 ml of 1 *M* Tris–HCl, pH 7.0, to immediately neutralize the pH value.

unavoidable using hemoglobin-based affinity column and was confirmed by a monoclonal antibody prepared against apoA-I (Fig. 7). Nevertheless, the major isolation procedure was simple and can be achieved within a few hours. This procedure should be widely used for the purification of Hp and particularly for 1-1 phenotype.

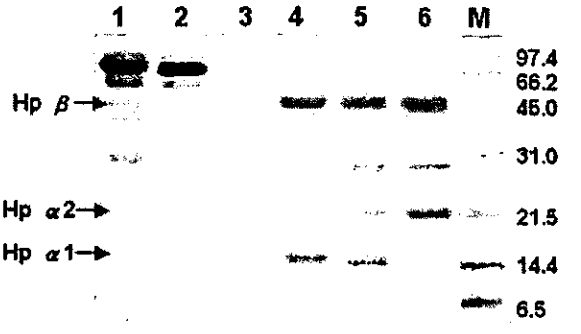

Fig. 6. Analyses of isolated Hp from affinity column on 15% SDS–PAGE. Lanes: M=molecular mass markers, 1=plasma of Hp 1-1, 2=a typical pass-through fraction (Hp 1-1 plasma) from hemoglobin-affinity chromatography, 3=a typical sample from fraction 1 (Hp 1-1) contaminated mostly apoA-I and proteins with large molecular mass. Lanes 4 and 6 represent fraction 2 of isolated Hp 1-1, 2-1, and 2-2, respectively. Notably, apoA-I is co-eluted in Hp 2-1 and 2-2.

4. Discussion

The acute phase serum protein, Hp, response to infection, inflammation, and trauma has been identified in a number of species. Methods designed for large isolation of human Hp have been complicated and time-consuming. We have recently described a single-step purification procedure for porcine Hp

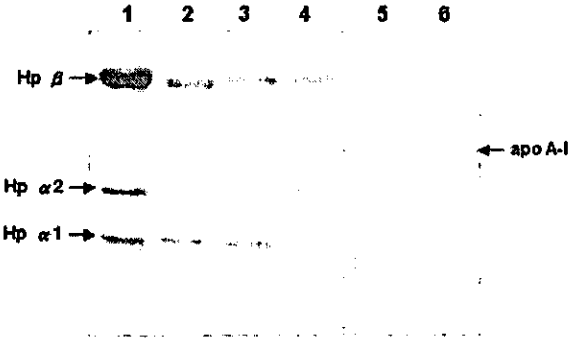

Fig. 7. Western blot analyses on affinity-purified human Hp 1-1, 2-1, and 2-2 by a goat antibody prepared against human haptoglobin. Lanes: 1=Hp standard purified from a human plasma pool, 2–4=affinity-purified Hp 1-1, 2-1, and 2-2, respectively, 5 and 6=the apoA-I co-eluted in affinity-purified Hp 2-1 and 2-2 fractions by a mouse monoclonal antibody prepared against human apoA-I. Purified Hp 1-1 did not reveal immunoreactive apoA-I (data not shown).

Table 1
Analytical recovery of haptoglobin 1-1 purified from 15 ml human plasma

Total protein from plasma (mg)	Theoretical amount of Hp (mg)	Total Hp yield (mg)	Final yield (%)	Purity (%)	Fold purification
1755	22	10	45.5	>96	77

using HPLC gel-permeation chromatography in the presence of 5 *M* urea [22]. The procedure, however, could not be reproduced in human Hp isolation. Presumably, the human Hp structure is more complicated in its polymerization nature (Fig. 1) than that of pig. Thus, the purification for human Hp has been hampered by its structural diversity as each Hp 1-1, 2-1, and 2-2 has average molecular masses of 100 000, 220 000, and 400 000, respectively (Fig. 1). Although the procedure using a salting-out of plasma proteins followed by anion-exchange chromatography has been recommended, the reproducibility (including the yield) is rather poor due to the heterogeneity of its polymerization form of Hp 2-1 and 2-2 [20,27,28]. It is almost not feasible to isolate Hp 2-2 as a pool and to study its biochemical properties.

An immunoaffinity chromatography method to purify human Hp had been developed using a two-monoclonal antibody system [20], in which the phenotypes and the final purity of Hp were not specified. The yield, on the other hand, is limited and utilized only for the preparation of antigen and polyclonal antibodies [20].

With respect to hemoglobin-affinity column, Rademacher and Steele [19] have reported use of Sepharose immobilized with chicken hemoglobin. However, an attempt using human hemoglobin for the purification of human Hp was unsuccessful [19]. The method we employed was different from that of chicken hemoglobin-Sepharose chromatography. First, our human hemoglobin-Sepharose had a binding capacity 0.75–1.00 mg Hp/mg hemoglobin that was about 20 times greater than that of using chicken hemoglobin (Fig. 4). Second, our results demonstrated that highly purified hemoglobin via DEAE chromatography should be used for the affinity column rather than a crude extract of hemoglobin from ammonium sulfate fraction described previously [19]. Under this condition, the formation of plasma clots in the column could be eliminated. Third, the pH of each eluted fraction was immediately neutralized by a 1 *M* Tris buffer, pH 7.0, to restore the biological activity (complex formation between Hp and hemoglobin). It is worth mentioning that ammonium, rather than a high-capacity buffer solution, was used for adjusting the final pH of the saline solution (pH 11) in eluting the Hp; this was because the eluent could be easily neutralized by a Tris buffer. A similar procedure was employed previously in our laboratory [25]. Fourth, the most contaminants of proteins that bound weakly or non-specifically to the affinity column were differentially removed using pH 11 saline solution (Fig. 5, fraction 1). Fifth, unlike phenotypes Hp 2-1 and 2-2, Hp 1-1 could be isolated without apoA-I contaminant, as confirmed by a Western blot analysis (Fig. 7).

In the present study, about 8–10 mg of Hp 1-1 could be isolated from 15 ml of human plasma in one isolation. A similar yield of Hp 2-1 and 2-2 was obtained, but it required a further gel-filtration to remove apoA-I. The mechanism by which the affinity column favored the Hp 1-1 purification is not readily clear. Since Hp 2-1 and 2-2 molecules are largely polymerized by disulfide linkages with molecular weights ranging from 153 000 to 1 200 000 [6], these polymers may more accessibly "trap" the apoA-I than that of monomeric Hp 1-1. To address this assumption, we applied purified-apoA-I [25] directly to the affinity column. There was no apoA-I binding to the column suggesting that apoA-I did not interact with hemoglobin in the absence of Hp (data not shown). On the other hand, apoA-I may weakly bind to Hp and therefore was co-eluted with Hp during the purification. Regardless, the apoA-I deficient plasma, which can be easily obtained by a simple ultra-centrifugation for the removal of high-density lipoproteins [25], may be ultimately considered for the purification of all Hp phenotypes. This experimental procedure is currently in progress in our laboratory.

In conclusion, human hemoglobin could be con-

veniently isolated in large quantities by ammonium sulfate fractionation followed by a HPLC DEAE column. Immobilized human hemoglobin had a binding capacity about 20-times greater than that of chicken hemoglobin and could be more suitable for the purification of phenotype Hp 1-1. Accordingly, the procedure described in this report can be simply scaled up using a 100-ml bed affinity column for even larger Hp purification. This Hp purification procedure is currently used in our laboratory; the resulting Hp has been utilized in studying the structural and functional relationship and preparing polyclonal and monoclonal antibodies.

Acknowledgements

This work was supported by grants NHRI-EX92-9229SI (S.J.T.M.) from the National Health Research Institute, and NSC 89-2313-B-009-001-A20 (S.J.T.M.) and NSC 90-2314-B-075-099 (J.P.P.) from the National Science Council, Taiwan. The authors thank Ms. Yu-Chi Pong for her dedicated administrative assistance.

References

[1] W. Dobryszycka, Eur. J. Clin. Chem. Clin. Biochem. 35 (1997) 647.
[2] M.R. Langlois, J.R. Delanghe, Clin. Chem. 42 (1996) 1589.
[3] J.M. Hanley, T.H. Haugen, E.C. Heath, J. Biol. Chem. 258 (1983) 7858.
[4] M. Morimatsu, B. Syuto, N. Shimada, T. Fujinaga, S. Yamamoto, M. Saito, M. Naiki, J. Biol. Chem. 266 (1991) 11833.
[5] N. Maeda, O. Smithies, Annu. Rev. Genet. 20 (1986) 81.
[6] D. Patzelt, G. Geserick, H. Schroder, Electrophoresis 9 (1988) 393.
[7] J. Javid, Curr. Top. Hematol. 1 (1978) 151.
[8] I.H. Fraser, D.B. Smith, Can. J. Biochem. 49 (1971) 141.
[9] H. Baumann, G.P. Jahreis, J. Cell Biol. 97 (1983) 728.
[10] V. Chow, A. Kurosky, R.K. Murrary, J. Biol. Chem. 258 (1983) 7858.
[11] A. Kurosky, R.E. Hay, B.H. Bowman, Comp. Biochem. Physiol. 62B (1978) 339.
[12] K. Mominoki, N.N. Tosa, M. Morimatsu, B. Syuto, M. Saito, Comp. Biochem. Physiol. 110B (1995) 785.
[13] A. Roguin, F. Ribichini, V. Ferrero, G. Matullo, P. Herer, W. Wijns, A.P. Levy, Am. J. Cardiol. 89 (2002) 806.
[14] I. Hochberg, A. Roguin, E. Nikolsky, P.V. Chanderashekhar, S. Cohen, A.P. Levy, Atherosclerosis 161 (2002) 441.
[15] D.D. Bacquer, G.D. Backer, M. Langlois, J. Delanghe, H. Kesteloot, M. Kornitzer, Atherosclerosis 157 (2001) 161.
[16] N. Tosa, M. Morimatsu, M. Nakagawa, F. Miyoshi, E. Uchida, M. Niiyama, B. Syuto, M. Saito, J. Vet. Med. Sci. 55 (1993) 27.
[17] B.H. Bowman, D.R. Barnett, J.B. Lum, F. Yang, Methods Enzymol. 163 (1988) 452.
[18] M.K. O'Bryan, J. Grima, D. Mruk, C.Y. Cheng, J. Androl. 18 (1997) 637.
[19] B. Rademacher, W.J. Steele, Anal. Biochem. 160 (1987) 119.
[20] I. Katnik, J. Jadach, Arch. Immunol. Ther. Exp. 41 (1993) 303.
[21] F. Delers, C. Lombart, M. Domingo, S. Musquera, Anal. Biochem. 118 (1981) 353.
[22] S.J. Yang, S.J.T. Mao, J. Chromatogr. B 731 (1999) 395.
[23] S.J.T. Mao, M.T. Yates, T.J. Owen, J.L. Krstenansky, Biochemistry 27 (1988) 8170.
[24] L.F. Chu, W.C. Lee, P.C. Yang, R. Chu, T.Y. Huang, S.J.T. Mao, Protein Expr. Purif. 10 (1997) 180.
[25] S.J.T. Mao, J.P. Miller, A.M. Gotto Jr., J.T. Sparrow, J. Biol. Chem. 255 (1980) 3448.
[26] U.K. Laemmli, Nature 227 (1970) 680.
[27] W. Dobryszycka, E. Lisowska, Biochim. Biophys. Acta 121 (1966) 42.
[28] M. Morimatus, B. Syuto, N. Shimada, T. Fujinaga, S. Yamamoto, M. Saito, M. Naiki, J. Biol. Chem. 266 (1991) 11833.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Protein Expression and Purification 33 (2004) 265–273
Protein Expression & Purification
www.elsevier.com/locate/yprep

Purification of human haptoglobin 1-1, 2-1, and 2-2 using monoclonal antibody affinity chromatography

Chi Feng Tseng, Hsing Yi Huang, Yuan Ting Yang, and Simon J. T. Mao*

Department of Biological Science and Technology, Research Institute of Biochemical Engineering, National Chiao Tung University, Hsinchu, Taiwan, ROC

Received 11 August 2003, and in revised form 23 September 2003

Abstract

Similar to blood type, human plasma haptoglobin (Hp) is classified as 3 phenotypes: Hp 1-1, 2-1, or 2-2. The structural and functional relationship between the phenotypes, however, has not been studied in detail due to the complicated and difficult isolation procedures. This report provides a simple protocol that can be used to purify each Hp phenotype. Plasma was first passed through an affinity column coupled with a high affinity Hp monoclonal antibody. The bound material was washed with a buffer containing 0.2 M NaCl and 0.02 M phosphate, pH 7.4, eluted at pH 11, and collected in tubes containing 1 M Tris–HCl, pH 6.8. The crude Hp fraction was then chromatographed on a HPLC Superose 12 column in 0.05 M ammonium bicarbonate at a flow rate of 0.5 ml/min. The homogeneity of purified Hp 1-1, 2-1, or 2-2 was greater than 95% as judged by SDS–polyacrylamide gel electrophoresis. Essentially, each Hp isolated was not contaminated with hemoglobin and apolipoprotein A-I as that reported from the other methods, and was able to bind hemoglobin. Neuraminidase treatment demonstrated that the purified Hp possessed a carbohydrate moiety, while Western blot analysis confirmed α and β chains corresponding to each Hp 1-1, 2-1, and 2-2 phenotype. The procedures described here represent a significant improvement in current purification methods for the isolation of Hp phenotypes. Circular dichroic spectra showed that the α-helical content of Hp 1-1 (29%) was higher than that of Hp 2-1 (22%), and 2-2 (21%). The structural difference with respect to its clinical relevance is discussed.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Human haptoglobin 1-1, 2-1, 2-2; Affinity purification; α-helix; Monoclonal antibodies

Hp is known as an acute phase protein and its plasma level elevates in response to inflection or inflammation. For this reason, Hp is a useful indicator for some infectious diseases [1–3]. It is also a hemoglobin-binding protein present in the plasma of all vertebrates and believed to participate in hemoglobin transport [4].

Human Hp is a tetrameric structure joined by disulfide linkages among the 2α and 2β chains [4–6]. Based on the length of α chains, there are three phenotypes of Hp in the population, Hp 1-1, 2-1, and 2-2 (Fig. 1). All the phenotypes share the same 2β chains (each with about M_r 40,000 kDa containing 243 amino acids and approximately 30% carbohydrate). A typical structure of homozygous Hp 1-1 is composed of two identical α1 chains (each with about M_r 9000 kDa containing 83 amino acids). Homozygous Hp 2-2 is composed of two identical α2 chains (each with about M_r 16,500 kDa containing 142 amino acids) as compared to that of heterozygous Hp 2-1 containing one each of α2 and α1 (Fig. 1). Likewise, the tetrameric arrangement is also found in other animal species such as rat, rabbit, and pig [7–12]. However the two identical αβ units (Hp 1-1), joined by a non-covalent interaction rather than a disulfide bridge, are found in dog, cat, and bear [13,14].

Clinically, polymeric form of Hp 2-1 or 2-2 is associated with the complications of myocardial infarction [15], kidney failure [16], and diabetics [17].

* Corresponding author. Fax: +88635729288.
E-mail address: mao1010@ms7.hinet.net (S.J.T. Mao).

1046-5928/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2003.09.006

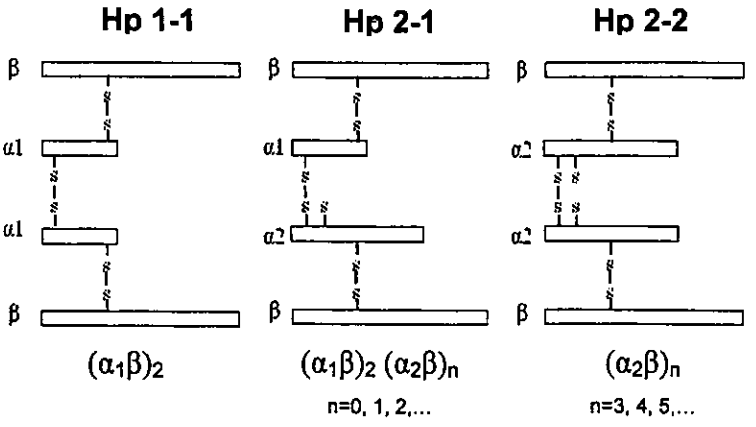

Fig. 1. Schematic drawing of proposed structure of human Hp 1-1, 2-1, and 2-2. All three phenotypes share a common structure of β chains (please also see [4]). The degree of polymerization within the inter-molecular arrangement is shown.

Presumably, this was due to the complicated structure of Hp 2-1 and 2-2 as it forms heterogeneous polymers, in which some of the biologically functional groups are not fully expressed on the surface (Fig. 1). The assumption, however, has not been tested because the structural and functional studies are hampered by lack of a straightforward isolation procedure in preparing sufficient Hp phenotypes. The methods currently used for the purification of Hp frequently suffer some drawbacks. For example, Rademacher et al. [18] utilize the chicken hemoglobin–Sepharose affinity column to isolate human Hp; the harsh-elution condition (8 M urea) causes the dissociation of a hemoglobin subunit from the Sepharose. Meanwhile, human apolipoprotein A-I appears to be another major contaminant. Wassdal et al. [19] use rabbit hemoglobin–Sepharose; the hemoglobin is still co-eluted from the column. Travis et al. [20] employ Sephadex G-200 gel filtration, but the purified Hp is accompanied with large amounts of IgM and α-2 macroglobulin. Morimatsu et al. [21] provide a modified method using HPLC with anion-exchange, Sephacryl S-300, TSK Phenyl-5PW, and TSK DEAE-5PW columns together; the procedures however are time-consuming and the yield is relatively low (2.5 mg per 130 ml acute phase serum). Although Katnik et al. [22] have shown a single-step isolation for Hp using an antibody-affinity column, the phenotypes, final purity, and the biological properties of Hp are not fully reported. Presumably, the purpose of their report was to use isolated Hp for raising monoclonal antibodies [22]. The similar antibody affinity-column procedure [22] was employed in our laboratory, but the isolated Hp was not pure. In the present report, we established simple two-step procedures for each Hp 1-1, 2-1, and 2-2 purification using a monoclonal antibody affinity-column followed by a HPLC Superose 12 gel filtration. Finally, some of the biochemical and physical properties with respect to each Hp phenotype were characterized and discussed.

Materials and methods

Materials

Goat polyclonal antibody against human Hp was purchased from Sigma (St. Louis, MO, USA). Rabbit anti-Goat IgG was purchased from Chemicon. CNBr-activated Sepharose 4B was purchased from Pharmacia. All other chemicals were purchased from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany) without any further purification. The buffers used in this report were all filtered through a 0.45 μm filter before using.

Preparation of monoclonal antibody against Hp

Six monoclonal antibodies: 8B1-3A, W1-11G, 2-3H, G2D-7G, 12B-1, and 4A2-4H, against human Hp were produced and characterized according to the standard procedures established in our laboratory [23]. Monoclonal antibody 8B1-3A, which possessed the highest binding affinity to Hp, was selected for preparation of the affinity column. Briefly, 120 ml of cultured medium from the 8B1-3A hybridoma was first precipitated in 50% saturated ammonium sulfate. The precipitate was dissolved in 12 ml of phosphate-buffered saline containing 0.02 M phosphate and 0.15 M NaCl, pH 7.4 (PBS). The solution was then dialyzed exhaustively in PBS to remove the remaining ammonium sulfate, followed by a dialysis in coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3.

Preparation of antibody affinity column

Dialyzed monoclonal antibody was first coupled to CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to manufacturer's procedures. Briefly, 2.86 g of freeze-dried Sepharose (1 g of freeze-dried powder gave about 3.5 ml final volume of gel) was swollen and suspended in 1 mM HCl and immediately washed with 20× volume of the same solution within 15 min on a sintered glass filter [24–26]. The gel was then washed with coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3, and degassed. About 10 ml (18.7 mg/ml) of ammonium-sulfate fraction of monoclonal antibody in coupling buffer was slowly added into the gel (in 15 ml), while gently stirring by a magnetic bar for 1 h at room temperature. After coupling, the gel was washed with 10× volume of PBS to remove unbound materials via a sintered glass filter. The gel was then treated with a blocking solution containing 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0, for 2 h at room temperature to saturate the remaining reactive-sites. The degassed gel was then washed with 3 cycles of blocking buffer and a 0.15 M NaCl solution of pH 11.0 (adjusted by ammonium) according to the procedures previously described by us [26]. Finally, the gel was equilibrated in PBS and packed onto a 1.5 × 20 cm column.

Purification of human Hp using antibody affinity-column chromatography

Initially, 1 ml of filtered human plasma of each Hp-phenotype batch was loaded onto the antibody affinity-column (10 ml in bed volume) at room temperature. The column was then washed with 50 ml PBS. The bound materials were washed further 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 with pH 11 adjusted by ammonium [26]. Five milliliters of each fraction was collected in a tube containing 0.25 ml of 1 M Tris–HCl buffer, pH 6.8, to immediately neutralize the pH value. Pooled fractions containing Hp were then concentrated to a final volume of 1 ml using an Amicon centrifugal filter (Millipore).

Further purification of Hp by gel filtration column

Concentrated solution with Hp was filtered with a 0.45 μm nylon fiber prior to HPLC. The HPLC system (Waters) consisted of two pumps, an automatic sample injector and a photodiode array detector. Superose 12 column (1 × 30 cm) (Pharmacia) was used for further Hp purification. The column was pre-equilibrated with 50 mM ammonium bicarbonate. Partially purified Hp (0.8 ml) was applied to the column at a flow rate of 0.5 ml/min. Fractions containing Hp were pooled and concentrated to a final volume of 1 ml using an Amicon centrifugal filter and then lyophilized. The lyophilized Hp was stored at −80 °C until analyzing.

Gel electrophoresis and densitometry

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli's method [27] with some modifications in using 5% polyacrylamide (w/v) on the stacking gel as previously described [7]. Samples (typically 5 μg) for SDS–PAGE were preheated at 100 °C for 10 min in a loading buffer (12 mM Tris–HCl, pH 6.8, 0.4% SDS (w/v), 5% glycerol (v/v), 2.88 mM of 2-mercaptoethanol, and 0.02% bromphenol blue (w/v)). For molecular weight calibration, a subset of the following standards was included in each gel: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). The samples were run for about 1.5 h at 100 V and stained using Coomassie brilliant blue R-250. Densitometric analysis of SDS–PAGE was performed using a Molecular Dynamics densitometer for data acquisition and Image Quant software for integration and analysis.

Immunoblot analysis

Following the separation of proteins by SDS–PAGE, the gel and nitrocellulose- and 3MM filter-papers were soaked in a transfer buffer containing 48 mM Tris–HCl, 39 mM glycine, 0.037% SDS (w/v), and 20% methanol (v/v) at pH 8.3 for 30 min. The gel was then electrotransferred to a nitrocellulose membrane (Pharmacia) at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad) containing a transfer buffer. The transferred membrane was then immersed in 5% skimmed milk (w/v) in PBS for 1 h at room temperature while shaking gently. After three times washing with PBS for 5 min, the membrane was incubated with a primary goat polyclonal antibody against human Hp (1:5000 dilution in PBS washing buffer containing 1% (w/v) skimmed milk and 0.05% Tween 20 (v/v) for 1 h) at room temperature and washed three times for 5 min. The membrane was then incubated with 1:10,000 diluted rabbit anti-goat IgG conjugated with horseradish peroxidase in washing buffer for 1 h. In addition, the membrane was washed two times with washing buffer and further washed once with PBS. Finally, the membrane was developed using 3,3′-diaminobenzidine (DAB) as a substrate for horseradish peroxidase [7,25].

Circular dichroic spectra

The lyophilized Hp was dissolved in 10 mM phosphate buffer at pH 7.4 with a final concentration of 0.2 mg/ml. About 300 μl of Hp solution was used to analyze within a cuvette of 1-mm path length. Circular dichroic spectrum was conducted between 190 and 300 nm in a Jasco J-715 spectropolarimeter. The obtained spectrum of each type of Hp was accumulated for 20 times at a scanning rate of 50 nm/min and the percentage α-helical content was estimated from the mean residue molar elliplicity (θ_{222}). % α-helix $= [(\theta_{222} + 3000)/(36,000 + 3000)] \times 100$ [28].

Results

Preparation of monoclonal antibody against Hp

Six monoclonal antibodies prepared against Hp were characterized, in which 8B1-3A possessed the highest binding affinity ($K_a = 5.6 \times 10^9\ M^{-1}$) and was chosen to prepare an affinity column. The binding capacity estimated was greater than 100 μg of Hp per ml of coupled Sepharose (data not shown).

Purification of human Hp using antibody affinity column chromatography

Fig. 2 shows a typical chromatographic profile for Hp 1-1, 2-1, and 2-2 purification on the affinity column. Human plasma was applied to the column followed by an extensive wash with a phosphate buffer containing 0.2 M NaCl. It is worth mentioning that this pre-wash step differed from the conventional method in which 0.12 M NaCl was used. Using 0.2 M NaCl, most of the low-affinity binding proteins were eliminated (Fig. 3). Hp was then eluted at pH 11 and collected in tubes containing 0.25 ml of 1 M Tris-HCl, pH 6.8, to immediately neutralize the pH. The purity of each Hp phenotype was approximately 60–80% in homogeneity as analyzed on SDS–PAGE. Apolipoprotein A-I appeared to be a major contaminant. All the phenotypes of Hp converted to α (α1 or α2 or both) and β subunits in the presence of the reducing reagent (Fig. 3). A typical Western blot analysis showing 3 isolated phenotypes is depicted in Fig. 4. The recovery of Hp at this step accounted for 75–94% of the Hp from the plasma with a final step of 51- to 54-fold purification (Table 1).

Further purification of Hp on HPLC gel-filtration column

The obtained Hp 1-1, 2-1, or 2-2 fraction was concentrated and applied onto a gel-filtration Superose 12 column pre-equilibrated with 0.05 M of ammonium bicarbonate, pH 8.0. Chromatographic profiles (Fig. 5)

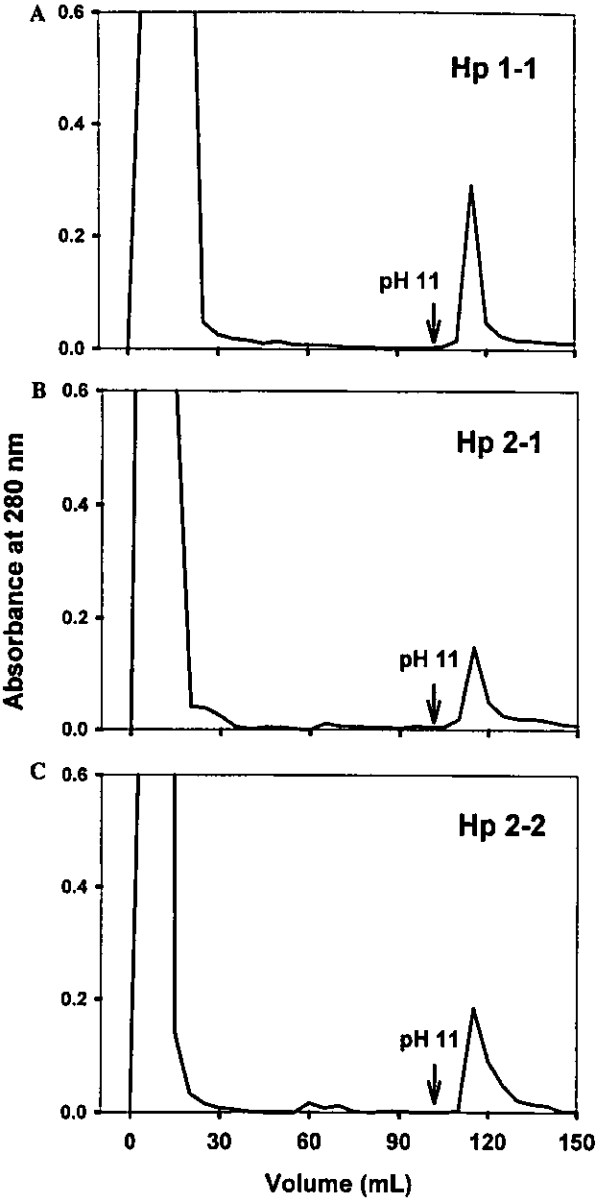

Fig. 2. Typical purification profile of human Hp on antibody affinity-column. One milliliter of human plasma was applied to an antibody affinity-column (pre-equilibrated with PBS, pH 7.4) followed by a wash in 10 mM phosphate buffer containing 0.2 M NaCl, pH 7.4. The bound Hp was then eluted in a solution at pH 11 and collected in tubes containing 0.25 ml of 1 M Tris, pH 6.8.

revealed that the solution property of each Hp phenotype was consistent with its molecular form, in which Hp 1-1 was more homogeneous in size with longer elution time than those of Hp 2-1 and 2-2. Purity of each phenotype was then analyzed on SDS–PAGE containing the reducing reagent 2-mercaptoethanol. Homogeneity of each phenotype was greater than 95% (Fig. 6). Thus, HPLC Superose column was markedly effective in removing the apoA-I contaminant.

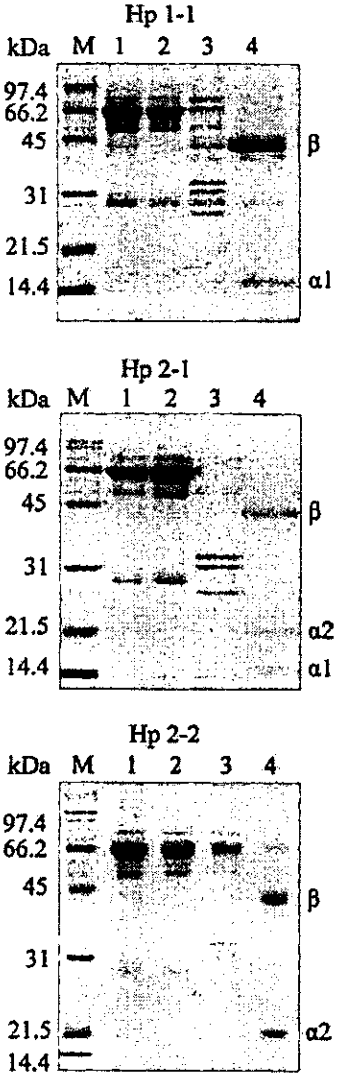

Fig. 3. Analysis of isolated Hp from affinity column using 12% SDS–PAGE in the presence of reducing reagent. Lane M: molecular markers (expressed as kDa). Lanes 1–2: plasma before and after flowing through the affinity column, respectively. Lane 3: low-affinity binding proteins washed with 10 mM phosphate buffer containing 0.2 M NaCl, pH 7.4. Lane 4: the eluted Hp as described in Fig. 2.

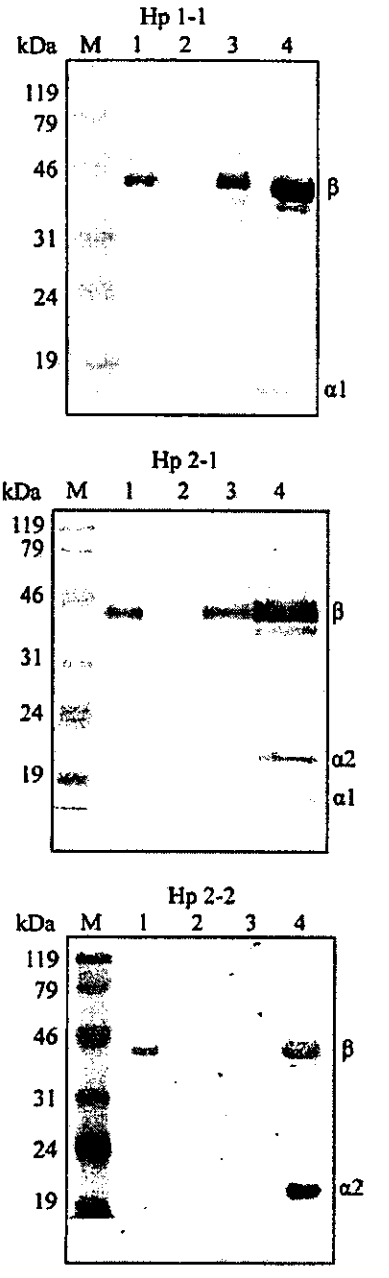

Fig. 4. Western blot analysis of Hp isolated from antibody affinity-column. Following a 12% SDS–PAGE, the separated proteins were transferred, blocked, and developed by a goat anti-human Hp. Lane M: molecular markers. Lanes 1-2: plasma before and after flowing through the affinity column, respectively. Lane 3: low-affinity binding proteins washed with 10 mM phosphate buffer containing 0.2 M NaCl, pH 7.4. Lane 4: the eluted Hp.

Western blot analysis in the absence of a reducing reagent demonstrated that Hp 2-1 and 2-2 were all polymeric (Fig. 7), in which Hp 2-2 was devoid of monomer and dimer consistent with the proposed structure of Hp (Fig. 1). Thus, our purification procedures did not apparently alter the structural characteristics of Hp phenotypes.

The polymeric structure of isolated Hp and its binding to hemoglobin

We studied further the ionic property of isolated Hp 1-1, 2-1, and 2-2 on a native-PAGE; the distinct polymorphism of each phenotype was also observed (Fig. 8). Hp 2-2 was the most basic among the Hp phenotypes. Since hemoglobin (Hb) is able to bind Hp and to form a Hb–Hp complex [10], Fig. 8 demonstrates that the Hb could form Hb-Hp complex with each Hp phenotype we isolated.

Table 1
Analytical recovery and yield of haptoglobin purified from 1 ml of human plasma

	Plasma	Affinity column	Gel filtration
(A) Hp 1-1			
Total protein (mg)	102	1.41	0.88
Moiety of Hp (mg)	1.46[a]	1.09[b]	0.81[b]
Purity (%)[b]	1.43	77	92
Fold purification	1	54	64
Recovery (%)	100	75	55
Yield (mg)	—	—	0.81
(B) Hp 2-1			
Total protein (mg)	101	1.80	0.72
Moiety of Hp (mg)	1.17[a]	1.10[b]	0.71[b]
Purity (%)[b]	1.16	61	98
Fold purification	1	53	84
Recovery (%)	100	94	61
Yield (mg)	—	—	0.71
(C) Hp 2-2			
Total protein (mg)	100	1.53	0.81
Moiety of Hp (mg)	1.26[a]	0.98[b]	0.76[b]
Purity (%)[b]	1.26	64	94
Fold purification	1	51	75
Recovery (%)	100	78	60
Yield (mg)	—	—	0.76

[a] The concentration of each human Hp phenotype was determined using an ELISA currently used in our laboratory.
[b] Determined by densitometer using digital Image Quant software.

Circular dichroic spectra

To characterize the secondary structure of each Hp phenotype, we determined the conformation of Hp by CD (Fig. 9). The estimated α-helical content was about 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively (Table 2). Statistically, the α-helical content in Hp 1-1 was significantly higher than those in Hp 2-1 and 2-2 ($P < 0.001$).

Discussion

Purification of human Hp has been hampered for years due to its structural diversity as described above [7,18–21]. In the present study, plasma without any additional manipulations (e.g., ammonium-sulfate precipitation) was utilized for Hp isolation. With the use of a 0.2 M NaCl wash in our procedures, most of the nonspecific low-affinity binding proteins were eluted from the column. Nevertheless, the affinity purified Hp 1-1, 2-1 or 2-2 analyzed on SDS–PAGE was only 60–80% pure (Fig. 3). HPLC Superose 12 column appeared to be superior to the other methods in the second-step purification since apolipoprotein A-I, a major contaminant, and other unknown high molecular-weight proteins were almost eliminated. The solution property of each Hp phenotype on this Superose column was consistent with its molecular forms, of which Hp 1-1 was more homogeneous than the other two species in size. Notably, the elution time of each phenotype was also consistent with the size of Hp, in which the polymeric forms of Hp 2-2 and 2-1 were eluted earlier, respectively (Fig. 5). Western blot analysis on the polymeric structure of isolated Hp using 6% SDS–PAGE without reducing reagent (Fig. 7) revealed its structural identity to that originally present in plasma (data not shown). It also confirmed that the isolated Hp 2-1 was heterogeneous in nature containing Hp monomer and dimer, while the polymeric Hp 2-2 contained neither.

Subsequently, we show that each phenotype of isolated Hp possessed the ability to bind hemoglobin (Fig. 8), although we cannot presently address whether or not the binding potency might be attenuated during the isolation. Meanwhile, we demonstrated the presence of carbohydrate moiety in isolated Hp as neuraminidase treatment could remove the terminal sialic acid residues from the Hp in a time-dependent manner similar to our previous study [7] (data not shown).

Taking together, the Hp isolated from the antibody affinity-column combined with HPLC Superose 12 exhibited advantages over the conventional methods. First, the heterogeneous particles of Hp 2-1 or 2-2 could be obtained in one pool as polymeric forms. Second, the co-eluted hemoglobin from hemoglobin affinity-column [19] was eliminated in the present study. Using an ELISA for hemoglobin assay, we could not detect hemoglobin in the Hp we isolated (data not shown). Third, the isolated Hp not only retained its ability to bind

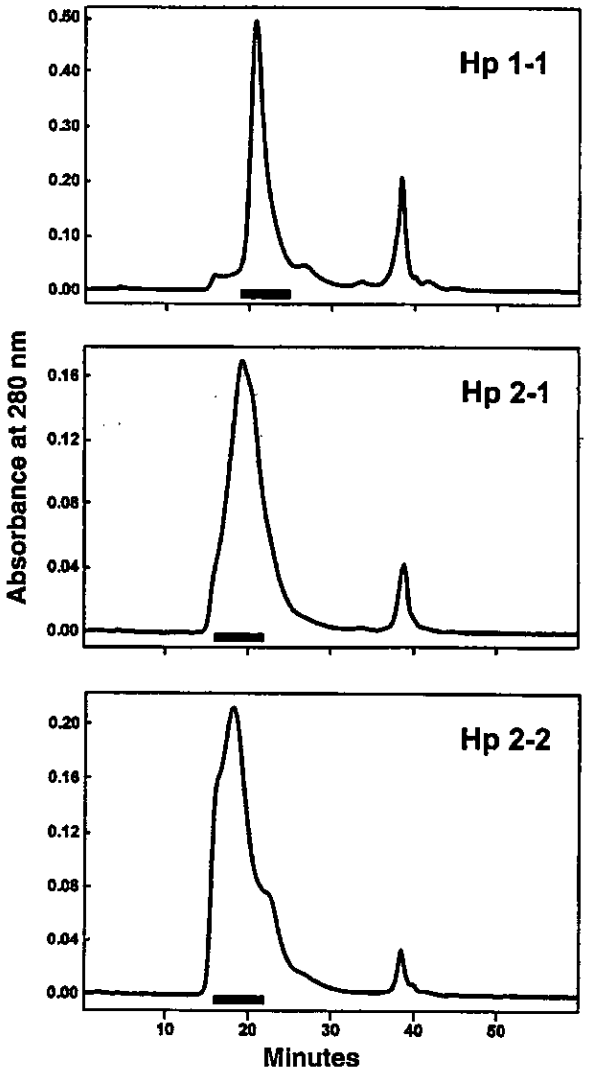

Fig. 5. Typical chromatographic profile of affinity isolated Hp on HPLC Superose 12 column. Isolated Hp 1-1, 2-1, or 2-2 from the affinity column was first concentrated and applied to a HPLC Superose 12 pre-equilibrated in 50 mM of ammonium bicarbonate, pH 8.0. The same solution was used in the mobile phase at a flow rate of 0.5 ml/ml. The filled bar represents the pooled fractions corresponding to isolated Hp.

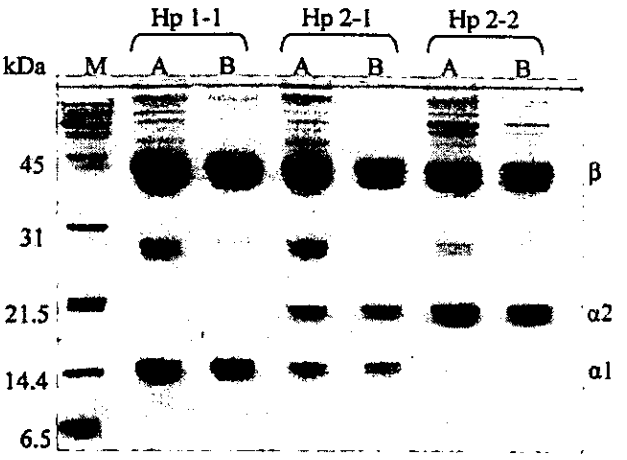

Fig. 6. Analysis of purified Hp from antibody affinity and HPLC gel filtration column on 15% SDS–PAGE in the presence of reducing reagent. M: molecular markers. (A) Isolated Hp from affinity column alone. (B) Purified Hp from an additional separation on HPLC Superose 12 column.

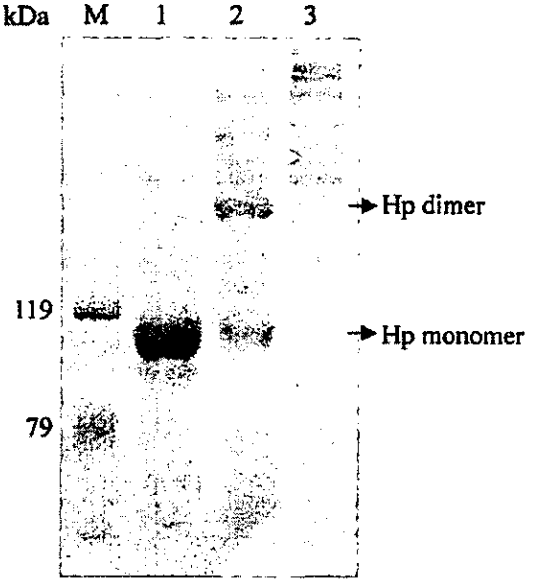

Fig. 7. Western blot analysis of polymeric structure of purified Hp on 6% SDS–PAGE in the absence of reducing reagent. Lane M: molecular markers. Lanes 1-3: purified Hp 1-1, 2-1, and 2-2, respectively. Notably, the Hp 2-1 contains monomeric and dimeric forms of Hp 1-1: whereas the Hp 2-2 is devoid of both.

hemoglobin (Fig. 8), but also blocked the peroxidase activity of hemoglobin (data not shown).

The CD spectrum of each Hp phenotype was determined to estimate the secondary structure of Hp. For the first time, according to the best of our knowledge, we showed that the α-helical content of Hp 1-1, 2-1, and 2-2 was 29, 22, and 21%, respectively. Hp 1-1 possessed a more ordered structure than those of polymeric forms of Hp 2-1 and 2-2. Moreover, we identified that the immunoreactivity of Hp 1-1 was also greater than those of Hp 2-1 and 2-2. For example, the monoclonal antibody used in our affinity column (8B1-3A) exhibited an affinity (K_a) of 5.58×10^9, 2.81×10^9, and 2.19×10^9 M^{-1} against Hp 1-1, 2-1, and 2-2, respectively. The latter result suggests that the availability of "functional" surfaces of Hp 1-1 may be greater as compared to those of Hp 2-1 and 2-2. Such structural differences may explain, in part, the clinical outcome by which Hp phenotype is associated with differential susceptibility to infections, atherosclerosis, and autoimmune disorders [3,29]. These effects are correlated with a phenotype-dependent modulation of oxidative stress and prostaglandin

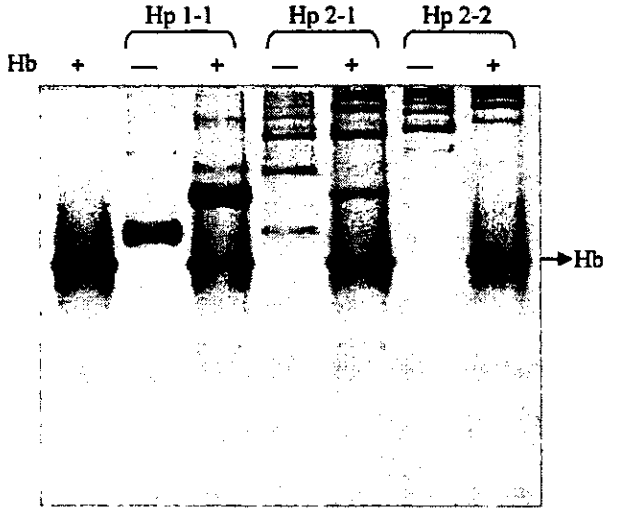

Fig. 8. Analysis of hemoglobin-binding property of purified Hp 1-1, 2-1, and 2-2 on a 7% native-PAGE. Briefly, each Hp phenotype (5 μg) was incubated with and without hemoglobin (Hb) (5 μg) at room temperature for 30 min before conducting the native-PAGE. The gel was then stained with Coomassie Blue R-250. Hp of each phenotype was shifted to basic upon the binding of Hb.

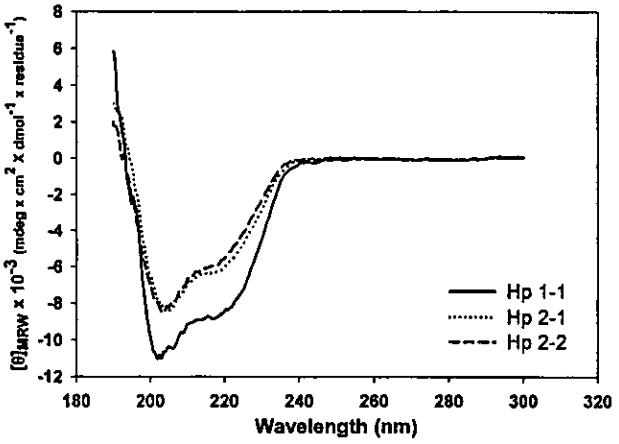

Fig. 9. Circular dichroic spectra of Hp 1-1, 2-1, and 2-2. Each Hp in 10 mM phosphate buffer, pH 7.4, at a final concentration of 0.2 mg/ml was monitored by a circular dichroism. Each spectrum represents a mean of 20× determinations.

Table 2
α-Helical content of each Hp phenotype as determined by CD

	Hp 1-1	Hp 2-1	Hp 2-2
α-Helix	29%*	22%	21%

*Significant difference as compared to Hp 2-1 or Hp 2-2 ($P < 0.001$).

synthesis. In general, patients with Hp 2-2 are more susceptible to develop severity of the diseases mentioned above. Identification of the biochemical basis for the differences among Hp phenotypes may lead to a rational design in intervening new pharmacological agents, such as mini-Hp, which have been recently proposed [30].

In conclusion, due to the structural heterogeneity, it is difficult to purify Hp from human plasma, particularly with the Hp 2-1 and 2-2 polymers. The present report provides a simple method for the purification of Hp phenotypes with relatively high yield. Hp 1-1, 2-1, and 2-2 can therefore be prepared and used for the study of structural and functional relationship with the pathogenesis in the diseases of interest.

Acknowledgment

This work was supported by the Grant NHRI-EX92-9229SI (S.J.T.M.) from the National Health Research Institute, Taiwan, ROC.

References

[1] M.T.M. Raijmakers, E.M. Roes, R.H.M. te Morsche, E.A.P. Steegers, W.H.M. Peters, Haptoglobin and its association with the HELLP syndrome, J. Med. Genet. 40 (2003) 214–216.
[2] G. Engstrom, L. Stavenow, B. Hedblad, P. Lind, K.F. Eriksson, L. Janzon, F. Lindgarde, Inflammation-sensitive plasma proteins, diabetes, and mortality and incidence of myocardial infarction and stroke: a population-based study, Diabetes 52 (2003) 442–447.
[3] A.P. Levy, I. Hochberg, K. Jablonski, H.E. Resnick, E.T. Lee, L. Best, B.V. Howard, Haptoglobin phenotype is an independent risk factor for cardiovascular disease in individuals with diabetes: the strong heart study, J. Am. Coll. Cardiol. 40 (2002) 1984–1990.
[4] M. Kristiansen, J.H. Graversen, C. Jacobsen, O. Sonne, H. Hoffman, S.K.A. Law, S.K. Moestrup, Identification of the haemoglobin scavenger receptor, Nature 409 (2001) 198–201.
[5] N. Maeda, O. Smithies, The evolution of multigene families: human haptoglobin genes, Annu. Rev. Genet. 20 (1986) 81–108.
[6] D. Patzelt, G. Geserick, H. Schroder, The genetic haptoglobin polymorphism: relevance of paternity assessment, Electrophoresis 9 (1988) 393–397.
[7] S.J. Yang, S.J.T. Mao, Simple high-performance liquid chromatographic purification procedure for porcine plasma haptoglobin, J. Chromatogr. B. 731 (1999) 395–402.
[8] J.M. Hanley, T.H. Haugen, E.C. Heath, Biosynthesis and processing of rat haptoglobin, J. Biol. Chem. 258 (1983) 7858–7869.
[9] I.H. Fraser, D.B. Smith, Studies on porcine haptoglobin and its complex with human hemoglobin, Can. J. Biochem. 49 (1971) 141–147.
[10] H. Baumann, G.P. Jahreis, Regulation of mouse haptoglobin synthesis, J. Cell Biol. 97 (1983) 728–736.
[11] V. Chow, A. Kurosky, R.K. Murrary, Studies on the biosynthesis of rabbit haptoglobin, J. Biol. Chem. 259 (1984) 6622–6629.
[12] W.H. Busby Jr., J.C. Travis, Structure and evolution of artiodactyla haptoglobins, Comp. Biochem. Physiol. B 60 (1978) 389–396.
[13] A. Kurosky, R.E. Hay, B.H. Bowman, Canine haptoglobin: a unique haptoglobin subunit arrangement, Comp. Biochem. Physiol. B 62 (1979) 339–344.
[14] K. Mominoki, N. Nakagawa-Tosa, M. Morimatsu, B. Syuto, M. Saito, Haptoglobin in Carnivora: a unique molecular structure in bear, cat and dog haptoglobins, Comp. Biochem. Physiol. B 110 (1995) 785–789.
[15] G. Engstrom, P. Lind, B. Hedblad, P. Wollmer, L. Stavenow, L. Janzon, F. Lindgarde, Lung function and cardiovascular risk:

relationship with inflammation-sensitive plasma proteins, Circulation 106 (2002) 2555–2560.
[16] H. Miyoshi, S. Ohshiba, A. Matsumoto, K. Takada, E. Umegaki, I. Hirata, Haptoglobin prevents renal dysfunction associated with intravariceal infusion of ethanolamine oleate, Am. J. Gastroenterol. 86 (1991) 1638–1641.
[17] I. Hochberg, A. Roguin, E. Nikolsky, P.V. Chanderashekhar, S. Cohen, A.P. Levy, Haptoglobin phenotype and coronary artery collaterals in diabetic patients, Atherosclerosis 161 (2002) 441–446.
[18] B.E. Rademacher, W.J. Steele, A general method for the isolation of haptoglobin 1-1, 2-1, and 2-2 from human plasma, Anal. Biochem. 160 (1987) 119–126.
[19] I. Wassdal, A.H. Myrset, L. Helgeland, A simple and rapid method for purification of rat haptoglobin for production of antiserum, Scand. J. Clin. Lab. Invest. 51 (1991) 565–569.
[20] J.C. Travis, B.G. Sanders, Haptoglobin evolution: polymeric forms of Hp in the Bovidae and Cervidae families, J. Exp. Zool. 180 (1972) 141–148.
[21] M. Morimatsu, B. Syuto, N. Shimada, T. Fujinaga, S. Yamamoyo, M. Saito, M. Naiki, Isolation and characterization of bovine haptoglobin from acute phase sera, J. Biol. Chem. 266 (1991) 11833–11837.
[22] I. Katnik, J. Jadach, Immunoaffinity purification of human haptoglobin using monoclonal antibodies, Arch. Immunol. Ther. Exp. (Warsz) 41 (1993) 303–308.
[23] S.J. Mao, A.E. Rechtin, J.L. Krstenansky, R.L. Jackson, Characterization of a monoclonal antibody specific to the amino terminus of the alpha-chain of human fibrin, Thromb. Haemost. 63 (1990) 445–448.
[24] L.F. Chu, W.C. Lee, P.C. Yang, R. Chu, T.Y. Huang, S.J.T. Mao, One-step HPLC purification procedure for porcine brain 90-kDa heat shock protein, Protein Expres. Purif. 10 (1997) 180–184.
[25] C.Y. Liau, T.M. Chang, J.P. Pan, W.L. Chen, S.J.T. Mao, Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography, J. Chromatogr. B. 790 (2003) 209–216.
[26] S.J. Mao, J.P. Miller, A.M. Gotto Jr., J.T. Sparrow, The antigenic structure of apolipoprotein A-I in human high density lipoproteins. Radioimmunoassay using surface-specific antibodies, J. Biol. Chem. 255 (1980) 3448–3453.
[27] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[28] S.J. Mao, J.T. Sparrow, A.M. Gotto Jr., R.L. Jackson, The phospholipid-binding and immunochemical properties of amidinated, guanidinated and acetylated apolipoprotein A-II, Biochim. Biophys. Acta 617 (1980) 245–253.
[29] D.R. Bernard, M.R. Langlois, J.R. Delanghe, M.L. De Buyzere, Evolution of haptoglobin concentration in serum during the early phase of acute myocardial infarction, Eur. J. Clin. Chem. Clin. Biochem. 35 (1997) 85–88.
[30] M. Melamed-Frank, O. Lache, B.I. Enav, T. Szafranek, N.S. Levy, R.M. Ricklis, A.P. Levy, Structure–function analysis of the antioxidant properties of haptoglobin, Blood 98 (2001) 3693–3698.

Proteomics 2004, *4*, 2221–2228 DOI 10.1002/pmic.200300787

Antioxidant role of human haptoglobin

Chi Feng Tseng, Chia Ching Lin, Hsing Yi Huang, Huei Chun Liu and Simon J. T. Mao

Institute of Biochemical Engineering, College of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan

Human plasma haptoglobin (Hp) is classified according to three phenotypes: Hp 1-1, 2-1, and 2-2 attributed by their two common alleles 1 and 2. Clinically, the 2–2 phenotype is associated with the risk of cardiovascular diseases and diabetes mellitus in patients. In this study, we demonstrate that Hp is an extremely potent antioxidant, which directly protects low density lipoprotein from Cu^{2+}-induced oxidation. Its potency was markedly superior to probucol (one of the most potent antioxidants). Ranking of the IC_{50} of antioxidant activity was as follows: Hp 1-1 > Hp 2-1 > Hp 2-2 > probucol > vitamin E. Blockage of disulfide linkages between Hp subunits, not only abolished the α-helical content but also diminished the ability of Hp to form a complex with hemoglobin. The modified Hp subunits exerted almost 4 times greater antioxidant activity than that of native Hp. To investigate the antioxidant role of Hp on the cellular level, the cDNA of Hp 1-1 was cloned, introduced into the pcDNA3.0 vector which contains the cytomega lovirus promoter and transfected into chinese hamster ovary (CHO)-K1 cells. Following transfection, CHO cells were able to express Hp 1–1 protein and significantly ($p < 0.001$) elevated cell tolerance against oxidative stress. Transfected cells showed 2-fold higher resistance to hydrogen peroxide exposure for 24 h compared to control cells. Thus, Hp plays a provocative antioxidant role as demonstrated by our *in vitro* and *ex vivo* studies.

Received	16/12/03
Revised	1/2/04
Accepted	11/2/04

Keywords: Antioxidant / Haptoglobin cDNA / Haptoglobin phenotypes / Hemoglobin / Physiologic role

1 Introduction

Hemoglobin (Hb) is the most abundant and functionally important protein in erythrocytes. However, once it is released from red blood cells, it becomes highly toxic because of the oxidative nature of iron-containing heme, which participates in the Fenton reaction to produce reactive oxygen species which cause cell injury [1–2]. Human plasma haptoglobin (Hp), a known acute phase protein [3–5], can capture Hb by forming a high affinity Hp-Hb complex [1, 3, 4]. The complex is then metabolized through a receptor-mediated process involving the CD 163 receptor of macrophages [6]. Therefore, Hb binding by Hp is essential for rapid clearance of Hb from plasma [7]. For this reason, Hp plays a crucial role against Hb-induced oxidative stress by a mechanism thought to involve its high-affinity binding with Hb and prevents iron release from Hb. However, it has yet to be shown that Hp itself is an antioxidant molecule.

The Hp phenotypes 1-1, 2-1, and 2-2 are attributed to two common alleles, 1 and 2, located at chromosome 16q22.1. Structurally, the minimal unit of Hp (β-α-α-β) is joined by disulfide linkages of the two α and two β chains [6, 8, 9]. Fig. 1 shows that the phenotypes all share the same two β chains (each with M_r 40 000 kDa containing 245 amino acids and about 30% carbohydrate). A typical structure of homozygous Hp 1-1 (β-α1-α1-β) is composed of two identical α1 chains (M_r 9000 kDa containing 83 amino acids). Homozygous Hp 2-2 is composed of two identical α2 chains (M_r 16 500 kDa containing 142 amino acids) whereas heterozygous Hp 2-1 contains one α2 and one α1 chain (Fig. 1). Due to an extra thio group in the α-2 chain, Hp 2-1 and 2-2 form large polymers of monomeric, trimeric, tetrameric, pentameric, hexameric, and even larger arrangement through the disulfide-linkages (Fig. 1). Clinically, diabetic patients with the Hp 1-1 phenotype are markedly resistant to the development of diabetic retinopathy, diabetic nephropathy, and cardiovascular disease (CAD) [10–12]. In a prospective study, participants

Correspondence: Simon J. T. Mao, Ph.D., Professor and Dean, Department of Biological Science and Technology, College of Biological Science and Technology, National Chiao Tung University, 75 PO-Ai Street, Hsinchu, Taiwan
E-mail: mao1010@ms7.hinet.net
Fax: +886-3-572-9288

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane)-dihydrochloride; **CHO**, Chinese hamster ovary; **CM**, carboxymethylated; **CMV**, cytomegalovirus; **Hb**, hemoglobin; **Hp**, haptoglobin; **LDL**, low density lipoprotein

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

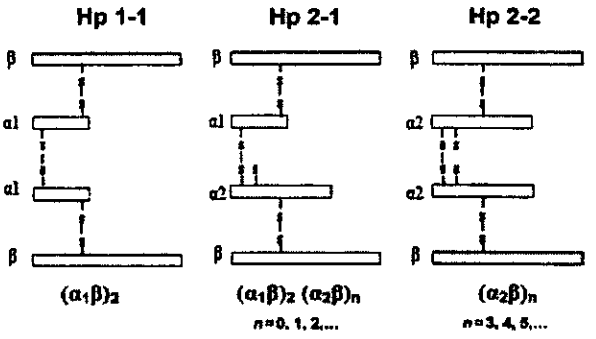

Figure 1. Schematic drawing of the proposed structure of human Hp 1-1, 2-1, and 2-2. All three phenotypes share a common structure of β chains, while Hp 2-1 and 2-2 are heterogeneous polymeric forms. Essentially, the α2 chain represents one entire copy of α1 (1–83) plus an insertion of a partial sequence of α1 (residues 12–70). The degree of polymerization within the intermolecular arrangement is shown. Due to an extra thio group in the β-chain Hp 2-1 and 2-2 can form large polymers (see Fig. 3).

homozygous for the Hp 2-2 allele had a 5-fold increased risk for the development of CAD as compared to participants homozygous for the Hp 1-1, allele [13]. The risk in heterozygous Hp 2-1 participates is intermediate [13].

In the present study, we show that Hp is an extremely potent antioxidant and the activity of Hp 1-1 was differentially and moderately greater than that of Hp 2-1 and 2-2. We also demonstrate that Hp 1-1 transfected Chinese hamster ovary (CHO) K1 cells (which normally do not express Hp) are significantly resistant to oxidative stress. The role of Hp as an antioxidant molecule is therefore identified. The clinical significance with respect to the structure and function of the Hp phenotype is discussed.

2 Materials and methods

2.1 Purification of human Hp

The Hp phenotype was first identified using plasma supplemented with Hb by PAGE, followed by a peroxidase substrate staining [14]. The plasma of each Hp phenotype was then chromatographed on an antibody affinity-column followed by gel filtration chromatography as previously described [14, 15]. The homogeneity of each Hp species employed was greater than 95%.

2.2 Gel electrophoresis

SDS-PAGE containing 15% w/v polyacrylamide gels with a top stacking gel of 5% polyacrylamide was performed for Hp characterization [14, 16]. Samples (typically 5 μg) for SDS-PAGE were preheated at 100°C for 10 min in loading buffer (12 mM Tris-HCl, pH 6.8, 0.4% w/v SDS, 5% v/v glycerol, 0.02% w/v bromphenol blue) with or without 2.88 mM 2-mercaptoethanol. The samples were run for about 1.5 h at 100 V and stained using CBB R-250.

2.3 Western blot

Following protein separation by SDS-PAGE, gels were soaked in a transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% w/v SDS, and 20% v/v methanol at pH 8.3. Electrotransfer to a nitrocellulose paper (Pharmacia Diagnostics, Uppsala, Sweden) was performed at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad, Hercules, CA, USA). Immunoblotting and developing were conducted according to standard procedures previously described [14–16].

2.4 Reduction and carboxymethylation of Hp

Tris-HCl buffer (pH 8.6, 3.3 mL of 0.01 M) containing 5.4 M urea, and 1% v/v β-mercaptoethanol was added to 1 mg HP. The reaction mixture was flushed with nitrogen and incubated at room temperature. After 2 h, 20 mg of iodoacetic acid was added and the reaction mixture was then maintained at pH 8.6 by the addition of 1 M NaOH for 30 min. Finally, carboxymethylated (CM)-Hp was desalted on a Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column eluted with 0.1 M ammonium bicarbonate and lyophilized [17].

2.5 Preparation of human low density lipoprotein

Human low density lipoprotein (LDL; 1.012–1.063 g/mL) was prepared from human plasma by sequential ultracentrifugation according to the method previously established [17]. Sodium azide (0.01%) was added to the plasma prior to ultracentrifugation and to LDL after isolation. Subsequently, freshly prepared LDL was dialyzed against PBS to remove EDTA and used for oxidation assays [1].

2.6 LDL oxidation

Thiobarbituric acid-reactive substances (TBARS) were used as an index to measure LDL oxidation [1, 18]. In a typical assay, 4 μM $CuSO_4$ and 40 μg of LDL were incubated with native Hp, CM-Hp, probucol, or vitamin E in a final volume of 100 μL. For oxidation induced by water-soluble initiator (5 mM 2,2'-azobis(2-amidinopropane)-dihydrochloride (AAPH)), only Hp 2-1 was employed. Incubation was carried out at 37°C for 2 h, after which

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

time 250 μL of 20% w/v TCA was added to precipitate proteins. Subsequently, 250 μL of 0.67% w/v 2-thiobarbituric acid was added and the reaction was incubated at 80°C for 30 min. The reaction mixtures were centrifuged at 3000 × *g* for 5 min. Supernatant (300 μL) in a 96-well plate was read at 540 nm [1].

2.7 Circular dichroic analysis

Lyophilized Hp was dissolved in 10 mM phosphate buffer (pH 7.4) with a final concentration of 0.2 mg/mL. About 300 μL aliquot was used for the analysis in a cuvette with a 1 mm path length. Circular dichroic spectrum recorded between 190 and 300 nm (in Jasco J-715 spectropolarimetry) was accumulated 20 times at a scanning rate of 50 nm/min. The α-helical content was estimated from the mean residue molar ellipticity (θ_{222}). % α-helix = (θ_{222} + 3000)/(36 000 × 3000) × 100 [17].

2.8 Analysis of Hp-Hb binding complex using HPLC

A HPLC experiment was performed to examine whether or not CM-Hp retains its ability to bind Hb. Hb was purified as previously described [16]. A gel-filtration column (Tosoh, Yamguchi, Japan; TSK-GEL G2000SWXL, 7.8 × 300 mm) pre-equilibrated in PBS was used to analyze the formation of Hp-Hb complex at a flow rate of 0.5 mL/min. A final reaction mixture (100 μL) containing Hp1-1 (24 μg) or CM-Hp 1-1 (24 μg) with and without Hb (16 μg) was subjected to HPLC. Typical chromatographic profiles were monitored at 280 nm, while Hb or Hb-Hp complex formation was monitored at 415 nm.

2.9 Preparation of Hp 1-1 cDNA

Total RNA was extracted from HepG2 cells using TRIzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using 1 μg of total RNA, 30 ng/μL oligo dT, 0.25 mM dNTP, and 3.5 μL diethyl pyrocarbonate water in a 40 μL volume. The mixture was incubated at 65°C for 5 min and chilled on ice. Subsequently, 8 μL 5 × reaction buffer, 4 μL 0.1 M DTT, 2 μL RNase inhibitor (Pharmacia Diagnostics, Uppsala, Sweden) and 1 μL M-MLV RTase (Invitrogen, Carlsbad, CA, USA) were added and incubated at 37°C for 50 min. The reaction was terminated by heating at 70°C for 15 min. PCR was performed in a 50 μL volume containing 0.1 μg of cDNA product, 5 μL 10 × Taq polymerase buffer, 4 μL 2.5 mM dNTP, 2.5 units Taq polymerase, and 50 ng of each specific Hp 1-1 primer (forward: 5'-TCGGTACCATGAGTGCCCTGGAAGCTGTCATTG-3'; reverse: 5'-TCGGTACCTTAGTTCTCAGCTATGGTCTTC TG-3'). The *Kpn I* restriction site is underlined. The thermal cycling program was 40 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The resulting PCR amplification products were visualized by ethidium bromide on a 1% agarose gel and then recovered using NucleoSpin Extraction Kit (Clontech, CA, USA). The extracted DNA fragment and pcDNA3.0 plasmid (Invitrogen, Carlsbad, CA, USA) were subsequently digested with *Kpn I* at 37°C for 2 h and recovered. Finally, the insert fragment and vector were ligated with a molar ratio of 3:1 at 16°C for 16 h.

2.10 Cell cultures

The CHO-K1 cell line CCL-61 and human hepatoblastoma (HepG2/C3A) CRL-10741 cells obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in DMEM/F12 (Gibco BRL, Grand Island, NY, USA) and α-minimal essential medium (Gibco), respectively, at 37°C. Media were supplemented with 10% bovine calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin Gibco.

2.11 Immunocytochemistry

Immunostaining was performed using the VECTOR M.O.M. Immunodetection kit (Burlingame, CA, USA) according to the manufacturer's instructions. Briefly, 10^5 CHO cells were cultured in a 6-well plate in which a 76 × 26 mm glass-slide was placed overnight. After washes in PBS, 4% paraformaldehyde was added for 30 min to fix cells. The slide was then immersed in PBS containing 0.1% Tween-20 for 15 min and washed four times with PBS. The slide was hydrogen peroxide and then washed twice immersed in 3% and blocked with M.O.M. mouse Ig blocking reagent for 1 h with two washes. Monoclonal anti-human Hp (1:5000 dilution) was added and incubated for 30 min. Following washes, the slide was incubated with biotinylated anti-mouse IgG for 10 min and washed twice. VECTASTAIN ABC reagent (Vector Laboratories, CA, USA) was added and incubated for 5 min. The slide was washed and developed using 3,3'-diaminobenzidine as a chromogenic substrate.

2.12 Transfection and hydrogen peroxide tolerance assay

The 1044-bp cDNA of Hp 1-1 obtained from human hepatoblastoma HepG2/C3A cells by RT-PCR was cloned into the pcDNA3.0 vector to generate a cytomegalovirus (CMV) promoter-driven Hp 1-1 construct. CHO-K1 cells were transfected with the inserted vector using the

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

SAINT-MIX Gene/Protein-Delivery System Kit (Groningen, The Netherlands), and selected in the presence of 400 μg/mL geneticin. Expression of Hp 1-1 was confirmed by Western blot, immunostaining, and competitive ELISA. Approximately 5×10^3 cells were cultured in a 96-well plate for 24 h and treated with variable amounts of hydrogen peroxide (0–5 mM) for another 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was then used to estimate the relative survival ratio [19].

3 Results

3.1 Antioxidant activity of Hp molecules

To determine Hp antioxidant activity, a method using Cu^{2+}-induced LDL oxidation was employed. Hp was an extremely potent antioxidant against LDL oxidation and acted in a dose-dependent manner (Fig. 2). It was 5 times more potent than probucol [1] and almost 20 times more potent than vitamin E. Notably, the antioxidant activity of monomeric Hp 1-1 was moderately higher than that of polymeric Hp 2-1 and 2-2 (Fig. 2).

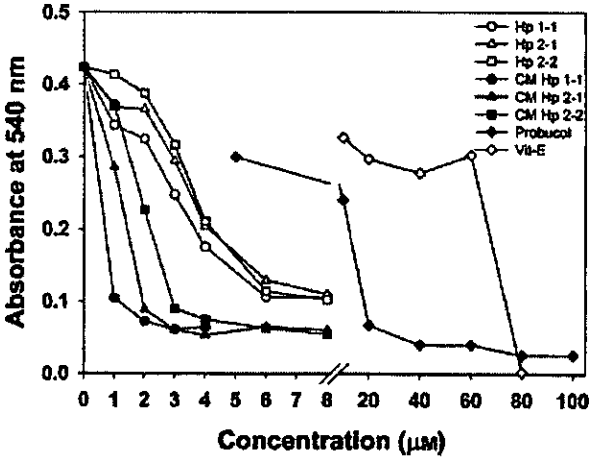

Figure 2. Antioxidant activity of Hp phenotypes. The assay was evaluated using the degree of inhibition of Cu^{2+}-induced formation of thiobarbituric acid-reactive substances from LDL. LDL (40 μg) was incubated with 4 μM Cu^{2+} in the presence of Hp, CM-Hp, probucol, or vitamin E at 37°C for 2 h in a final volume of 100 μL in PBS. BSA (10–100 μM) did not reveal any antioxidant activity (data not shown).

3.2 Effect of disulfide-linkages on the antioxidant property of Hp

Since the disulfide-linkages help maintain the tetrameric arrangement of each Hp monomer, we attempted to address whether or not the overall conformation of Hp plays a key role in its antioxidant function. We found that CM reduction disassembled the α and β chains of Hp on SDS-PAGE without requiring β-mercaptoethanol (Fig. 3). Each chain was identical to Hp subunits following β-mercaptoethanol reduction [14]. Second, using circular dichroic spectrum analysis, we observed a drastic conformational change in Hp following blockage of the disulfide linkage. A representative spectrum is shown in Fig. 4. The estimated α-helical content before CM was about 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively. Statistically, the α-helical content in Hp 1-1 was significantly higher than that in Hp 2-1 and Hp 2-2 ($p < 0.001$). CM-Hp 1-1 had a disordered structure. The conformational change, however, did not attenuate its antioxidant activity. Unexpectedly, it resulted in a marked increase in antioxidant activity (about 4 times; Fig. 2). The ranked antioxidant potency expressed as IC_{50} was: CM-Hp1-1 > CM-Hp 2-1 > CM-Hp 2-2 > Hp 1-1 > Hp 2-1 > Hp 2-2 > probucol > vitamin E (Table 1). BSA did not exhibit any antioxidant activity (data not shown).

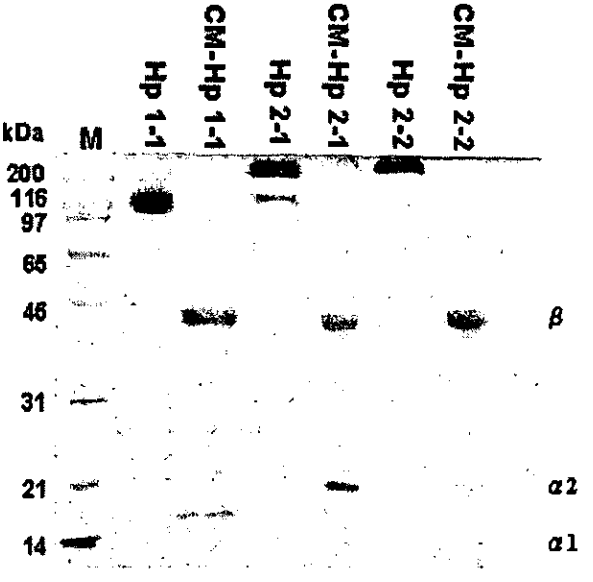

Figure 3. Analysis of CM-Hp on 15% SDS-PAGE without the reducing reagent β-mercaptoethanol. Lane M: protein marker. The α and β subunits are covalently linked in native Hp, but not in CM-Hp 1-1, 2-1, and 2-2 following chemical modification.

3.3 AAPH-induced LDL oxidation

We also tested the ability of Hp to scavenge free radicals generated from a water-soluble azo-compound, AAPH. Hp 2-1 showed dose-dependent antioxidant activity with an IC_{50} about 5 times greater than that of probucol (Table 1).

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

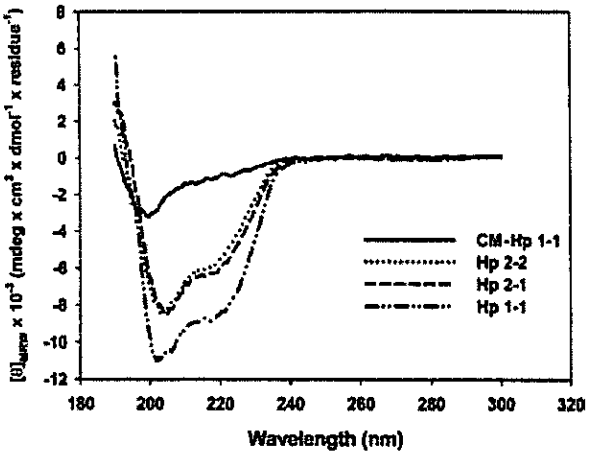

Figure 4. Circular dichroic spectra of native Hp 1-1, 2-1, 2-2, and CM-Hp 1-1. Hp in 10 mM phosphate buffer, pH 7.4, (final concentration 0.2 mg/mL) was monitored by circular dichroism. Each spectrum represents a mean of 20 determinations. The estimated α-helical content is about 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively. The structure of CM-Hp 1-1 is disordered. Statistically, the α-helical content in Hp 1-1 is significantly higher than that of Hp 2-1, 2-2, and CM-Hp 1-1 ($p < 0.001$).

Table 1. Antioxidant activity in Cu^{2+}- and AAPH- induced LDL lipid peroxidation

	Cu^{2+}-induced IC_{50} (μM)[a]	APH-Induced IC_{50} (μM)[a]
CM-Hp 1-1	0.8	nd
CM-Hp 2-1	1.5	nd
CM-Hp 2-2	2.2	nd
Hp 1-1	3.0	nd
Hp 2-1	3.5	6
Hp 2-2	3.7	nd
Probucol	15	30
Vitamin E	70	nd

a) IC_{50}: the concentration that inhibited 50% of LDL oxidation nd: not determined. See Fig. 2 legend for more details

3.4 Hb-binding ability of CM-Hp

Hp binds Hb forming a high affinity Hp-Hb complex [6]. Presumably, the domain of Hp which binds to Hb is dependent on the overall three-dimensional structure of Hp. We examined the effect of CM of Hp on the formation of the Hp-Hb complex using a HPLC technique. Binding was totally abolished when Hp was CM (Fig. 5). Since the antioxidant activity of CM-Hp was superior to native Hp (Fig. 2), the data indicate that the antioxidant nature of Hp was independent on its binding ability to Hb.

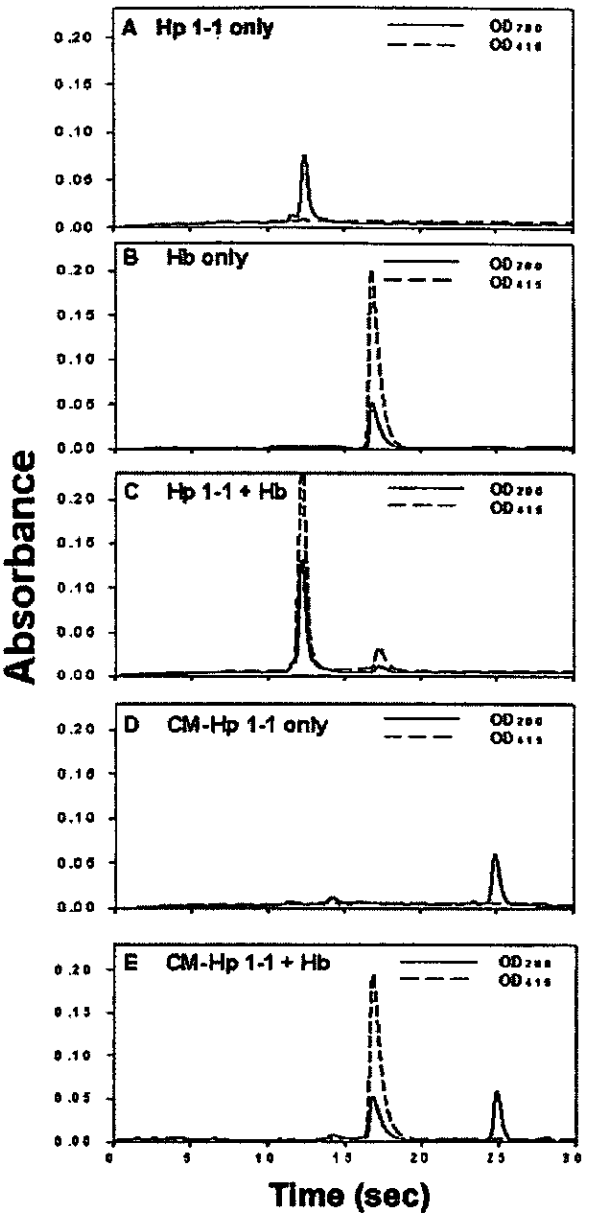

Figure 5. HPLC profile of Hb complexed to native and CM-Hp 1-1. A HPLC gel-filtration column (TSK-GEL G2000SWXL, 7.8 × 300 mm) was used to analyze the formation of the Hp-Hb complex. A final reaction mixture in 100 μL of PBS containing Hp1-1 (24 μg) or CM-Hp 1-1 (24 μg) with and without Hb (16 μg) was subjected to HPLC. Hb presence was monitored at 415 nm and to 280 nm.

3.5 Expression of Hp 1-1 in CHO-K1 cells

To create an antioxidant cell that may prevent oxidative stress, a CHO-K1 cell line was transfected with a pcDNA3.0 vector containing CMV promoter-driven Hp

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

1-1 cDNA. The plasmid construct also contained a selection marker, geneticin, for conditionally expressing Hp 1-1. Untransfected cells did not express endogenous Hp mRNA as shown by RT-PCR (Fig. 6). Expression of Hp 1-1 protein in the culture medium and CHO cells was confirmed by Western blot analysis (Fig. 7). Only cell lysate from transfected cells had Hp immunoreactivity in a competitive ELISA (data not shown). The specificity of Hp expression in transfected cells was also confirmed by immunocytochemical staining (Fig. 8). The nonimmuned serum did not stain transfected cells (data not shown).

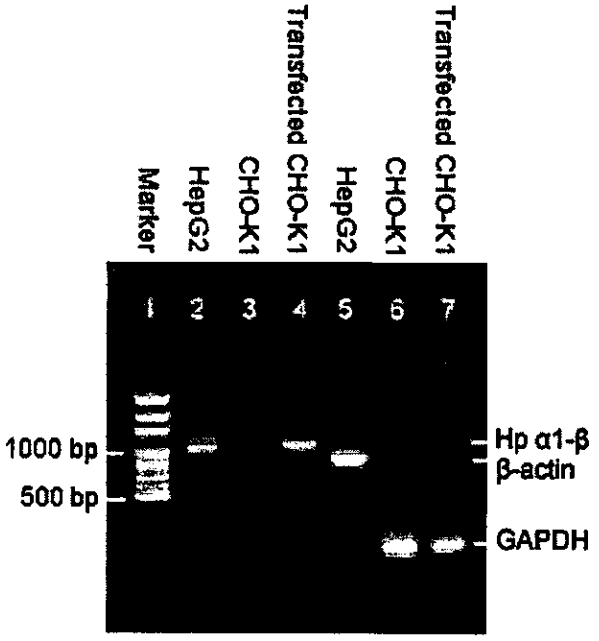

Figure 6. Expression of Hp α1-β mRNA in transfected CHO-K1 cells. PCR was performed to amplify the Hp α1-β cDNA. Lane 1: 100 bp ladder; Lane 2: HepG2 control (Hp α1-β cDNA, 1044 bp); Lane 3: CHO-K1 cells; Lane 4: transfected CHO-K1 cells (Hp α1-β cDNA, 1044 bp); Lane 5: HepG2 internal standard (β-actin, 838 bp); Lanes 6 and 7: CHO-K1 internal standard (GAPDH fragment, 120 bp). The PCR products were analyzed on a 1% agarose gel.

3.6 Activity of Hp transfected CHO cells against oxidative stress

To explore whether the antioxidant cells were resistant to oxidative stress, hydrogen peroxide was added to the CHO-K1 cell culture. Hp 1-1transfected and control cells were treated with variable dosages of hydrogen peroxide for 24 h. As shown in Fig. 9, the relative cell survival ratios of transfected CHO-K1 cells were statistically and significantly higher ($p < 0.001$) than that of untransfected CHO-K1 cells following hydrogen peroxide challenge, especially in the presence of 1 mM H_2O_2. Thus, the expression of Hp elevated the cell tolerance against hydrogen peroxide-induced oxidative stress.

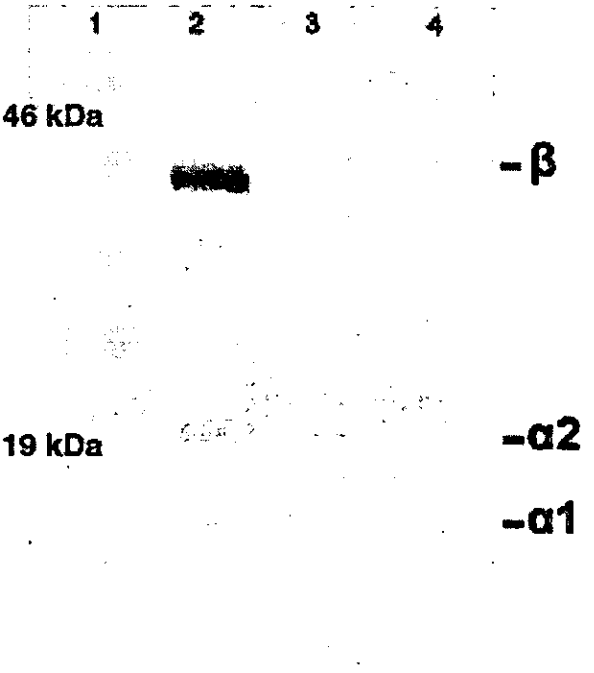

Figure 7. Western blot analysis of Hp expression in culture medium of transfected CHO-K1 cells. CHO-K1 cells were transfected with pcDNA3.0 vector containing CMV promoter-driven Hp α1-β cDNA and cultured in 400 μg/mL of the selection marker geneticin. Transfected CHO-K1 cells exhibited stable expression of Hp over at least 10 passages and more than one month in culture. The collected culture media were concentrated 40-fold, then subjected to 15% SDS-PAGE and Western blot analysis. Lane 1: protein marker; Lane 2: Hp 2-1 standard containing α1, α2, and β subunits; Lane 3: culture media of untransfected CHO-K1 cells; Lane 4: transfected CHO-K1 cells.

4 Discussion

It has recently been demonstrated that Hp can prevent Hb-induced oxidative tissue damage by virtue of its ability to form a high-affinity complex with Hb [7]. Furthermore, Hp 1-1 is superior to Hp 2-1 and 2-2 in binding to Hb [7]. As such, Hp is able to attenuate the release of heme from Hb. In addition to this function, we demonstrate that Hp is also an extremely potent antioxidant molecule in Cu^{2+}- and AAPH-induced LDL oxidation (Fig. 2 and Table 1). The rationale for choosing Cu^{2+}, rather than Fe^{3+}, as a free radical initiator for oxidation was that Cu^{2+} ist similar to Fe^{3+}, forming hydroxyl radicals in the Fenton reaction [1, 20].

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

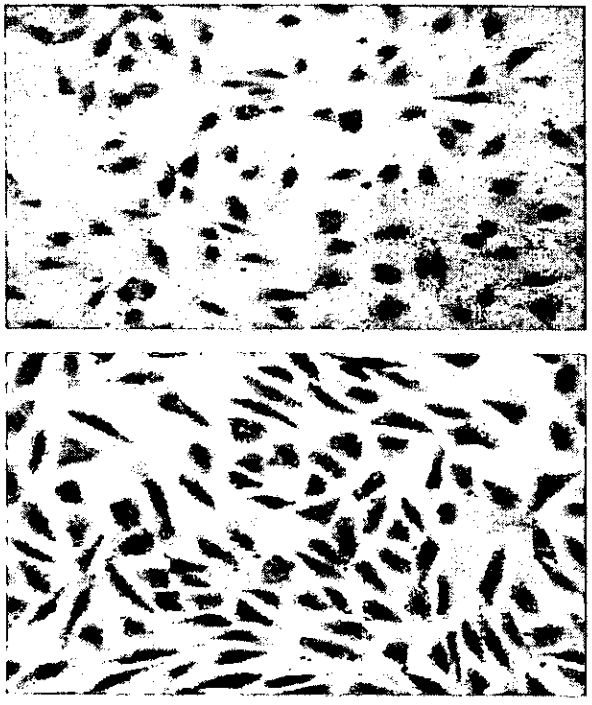

Figure 8. Immunochemical staining for Hp expression in untransfected (top) and transfected (bottom) CHO-K1 cells. 3,3'-Diaminobenzidine was used as the chromogenic substrate for Hp staining and hematoxylin was employed for the nuclear staining. Distribution of Hp (brown) and the nucleus (blue) were observed. The staining was negative when nonimmuned antiserum was used (data not shown).

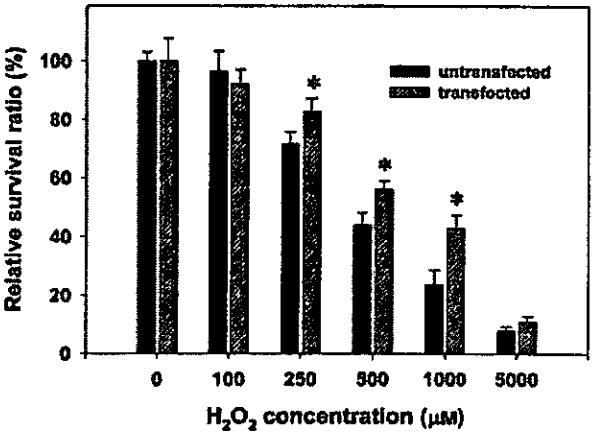

Figure 9. Hydrogen peroxide induced oxidative stress of CHO-K1 cells. Approximately 5×10^3 cells were cultured in a 96-well plate for 24 h, followed by an additional 24 h treatment with variable amounts of hydrogen peroxide (0–5 mM). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was then used to estimate the relative survival ratio. Each bar represents the mean ± SD in seven determinations. * $p < 0.001$.

Experimental conditions previously reported by us [1, 21–24] were mimicked to compare the antioxidant properties of Hp and probucol. A similar result was seen using Fe^{3+} [25]. Furthermore, a hydrophilic decomposed radical initiator, AAPH, also produced a similar antioxidant effect with Hp 2-1 (Table 1). The antioxidant potency of Hp significantly exceeded probucol [1]. Probucol has been used for the treatment of patients with xanthoma and atherosclerosis for decades [26–30]. Hypothetically, cellular Hp in the cellular level may also play a key role as a natural antioxidant in protection against atherosclerosis.

It is not clear, however, why Hp 1-1 possessed differentially and moderately greater antioxidant activity than Hp 2-1 and 2-2. Evidently, it is not solely due to the polymeric forms of Hp 2-1 and 2-1, since the dissociation of polymeric forms by chemical modification (CN; Fig. 3) did not equalize the antioxidant activity among the phenotypes (Fig. 2). On the contrary, the drastic conformational changes in CM-Hp (Fig. 4) resulted in its 4 times greater antioxidant potency (Fig. 2). We speculate that the region(s) which exert antioxidant activity were further exposed following structural unfolding of Hp. Since the differences in the Hp phenotypes are due to the amino acid sequence of the α-chains, it is essential to analyze the antioxidant domain in this region which may lead to the rational design of new pharmacological agents [1, 21–23]. We are currently in the process of exploring such possibilities.

The diversity in antioxidant activity of the Hp phenotypes may explain, in part, the clinical outcome by which Hp phenotype is associated with differential susceptibility to free-radical related atherosclerosis and autoimmune disorders [5, 31]. A correlation between phenotype-dependent modulation of oxidative stress and prostaglandin synthesis has been reported [31–34]. In general, patients with Hp 2-2 are more susceptible to developing nephropathy in diabetes mellitus. Nakhoul *et al.* [12] postulate that differences in the molecular shape and size of Hp 1-1 and Hp 2-2 may be responsible for the varying susceptibility. Abundant evidence has shown that Hp can be synthesized in the liver, lung, and some fibroblast cells [35]. We have recently reported that it can also be endogenously synthesized in macrophages [36]. At the cellular level Hp can stimulate angiogenin and remodel the arterial wall [37–39]. However, other functions of Hp have not been fully explored. We show that Hp cDNA transfected CHO-K1 cells attain the ability to resist oxidative damage (Fig. 9). This data further substantiates our hypothesis that Hp plays a provocative antioxidant role in the cell.

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

5 Concluding remarks

In conclusion, we demonstrate that Hp is an extremely potent antioxidant molecule and plays a role in cellular resistance to oxidative stress. Blockage of the disulfide linkages of Hp resulted in a loss of its ability to form a complex with Hb. Nevertheless, the modified Hp showed almost 4 times greater antioxidant activity than native Hp. Thus, in addition to its role of blocking the heme group of Hb by forming a complex, Hp may also have an antioxidant role. Whether or not Hp is directly beneficial for free-radical associated atherosclerosis and myocardial infarction [29, 31, 40] will be an interesting and callenging subject for the future.

This work was supported by grant NHRI-EX92-9229SI (S.J.T.M.) from the National Health Research Institute, Taiwan. We also thank the Hsinchu Blood Center of Chinese Blood Services Foundation for kindly providing human plasma.

6 References

[1] Mao, S. J., Yates, M. T., Jackson, R. L., *Methods Enzymol.* 1994, *234*, 505–513.

[2] Tolosano, E., Fagoonee, S., Hirsch, E., Berger, F. G. *et al.*, *Blood* 2002, *100*, 4201–4208.

[3] Raijmakers, M. T. M., Roes, E. M., Morsche, R. H. M., te Steegers, E. A. P., Peters, W. H. M., *J. Med. Genet.* 2003, *40*, 214–216.

[4] Engstrom, G., Stavenow, L., Hedblad, B., Lind, P. *et al.*, *Diabetes* 2003, *52*, 442–447.

[5] Levy, A. P., Hochberg, I., Jablonski, K., Resnick, H. E. *et al.*, *J. Am. Coll. Cardiol.* 2002, *40*, 1984–1990.

[6] Kristiansen, M., Graversen, J. H., Jacobsen, C., Sonne, O. *et al.*, *Nature* 2001, *409*, 198–201.

[7] Melamed-Frank, M., Lache, O., Enav, B. I., Szafranek, T. *et al.*, *Blood* 2001, *98*, 3693–3698.

[8] Maeda, N., Smithies, O., *Annu. Rev. Genet.* 1986, *20*, 81–108.

[9] Patzelt, D., Geserick, G., Schroder, H., *Electrophoresis* 1988, *9*, 393–397.

[10] Levy, A. P., Roguin, A., Hochberg, I., Herer, P. *et al.*, *N. Engl. J. Med.* 2000, *343*, 969–970.

[11] Nakhoul, F. M., Marsh, S., Hochberg, I., Leibu, R. *et al.*, *JAMA* 2000, *284*, 1244–1245.

[12] Nakhoul, F. M., Zoabi, R., Kanter, Y., Zoabi M. *et al.*, *Diabetologia* 2001, *44*, 602–604.

[13] Asleh, R., Marsh, S., Shilkrut, M., Binah, O. *et al.*, *Circ. Res.* 2003, *92*, 1193–1200.

[14] Tseng, C. F., Huang, H. Y., Yang, Y. T., Mao, S. J., *Protein Expr. Purif.* 2003, *33*, 265–273.

[15] Yang, S. J., Mao, S. J., *J. Chromatogr. B, Biomed. Sci. Appl.* 1999, *731*, 395–402.

[16] Liau, C. Y., Chang, T. M., Pan, J. P., Chen, W. L., Mao, S. J., *J. Chromatogr. B, Analyt. Technol. Biomed. Life Sci.* 2003, *790*, 209–216.

[17] Mao, S. J., Sparrow, J. T., Gotto, A. M. Jr., Jackson, R. L., *Biochim. Biophys. Acta* 1980, *617*, 245–253.

[18] Barnhart, R. L., Busch, S. J., Jackson, R. L., *J. Lipid Res.* 1989, *30*, 1703–1710.

[19] Lobner, D., *J. Neurosci. Methods* 2000, *96*, 147–152.

[20] Fenton, H. J. H., *Chem. Soc. J. Lond.* 1894, *65*, 899–910.

[21] Mao, S. J., Yates, M. T., Parker, R. A., Chi, E. M., Jackson, R. L., *Arterioscler. Thromb.* 1991, *11*, 1266–1275.

[22] Dage, R. C., Anderson, B. A., Mao, S. J., Koerner, J. E., *J. Cardiovasc. Pharmacol.* 1991, *17*, 158–165.

[23] Mao, S. J., Yates, M. T., Rechtin, A. E., Jackson, R. L. *et al.*, *J. Med. Chem.* 1991, *34*, 298–302.

[24] Chen, T. H., Tseng, H. P., Yang, J. Y., Mao, S. J., *Life Sci.* 1998, *62*, 277–282.

[25] Thomas, C. E., Jackson, R. L., *J. Pharmacol. Exp. Ther.* 1991, *256*, 1182–1188.

[26] Sia, Y. T., Lapointe, N., Parker, T. G., Tsoporis, J. N. *et al.*, *Circulation* 2002, *105*, 2549–2555.

[27] Roy, K., Saha, A., De, K., Sengupta, C., *Acta Pol. Pharm.* 2002, *59*, 231–234.

[28] Kim, M. H., Cha, K. S., Han, J. Y., Kim, H. J., Kim, J. S., *Catheter. Cardiovasc. Interv.* 2002, *57*, 424–428.

[29] Inazu, A., Koizumi, J., Kajinami, K., Kiyohar, T. *et al.*, *Atherosclerosis* 1999, *145*, 405–413.

[30] Stocker, R., *Curr. Opin. Lipidol.* 1999, *10*, 589–597.

[31] Bernard, D. R., Langlois, M. R., Delanghe, J. R., De Buyzere, M. L., *Eur. J. Clin. Chem. Clin. Biochem.* 1997, *35*, 85–88.

[32] Bernard, D., Christophe, A., Delanghe, J., Langlois, M. *et al.*, *Redox Rep.* 2003, *8*, 41–46.

[33] Langlois, M. R., Delanghe, J. R., *Clin. Chem.* 1996, *42*, 1589–1600.

[34] Frohlander, N., Ljunggren, O., Lerner, U. H., *Biochem. Biophys. Res. Commun.* 1991, *178*, 343–351.

[35] Kalmovarin, N., Friedrichs, W. E., O'Brien, H. V., Linehan, L. A. *et al.*, *Inflammation* 1991, *15*, 369–379.

[36] Mao, S. J., Yang, S. J., Wu, C. H., *FASEB J.* 2001, *15*, A247.

[37] Cid, M. C., Grant, D. S., Hoffman, G. S., Auerbach, R. *et al.*, *J. Clin. Invest.* 1993, *91*, 977–985.

[38] Wassell, J., *Clin. Lab.* 2000, *46*, 547–552.

[39] Cockerill, G. W., Gamble, J. R., Vadas, M. A., *Int. Rev. Cytol.* 1995, *159*, 113–160.

[40] Chapelle, J. P., Albert, A., Smeets, J. P., Heusghem, C., Kulbertus, H. E., *N. Engl. J. Med.* 1982, *307*, 457–463.

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Epitope mapping of a haptoglobin monoclonal antibody that blocks the haptoglobin-hemoglobin interaction: Implication of the involvement of residues 105-116 of haptoglobin in hemoglobin binding

S. S. Wang[a], Sunny C. H. Yueh, M. C. Yang, and Simon J. T. Mao*

Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan, R.O.C., and

[a]General Courses Center, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan, R.O.C

Correspondence: Simon J. T. Mao, Ph.D., Professor and Dean, Department of Biological Science and Technology, College of Biological Science and Technology, National Chiao Tung University, 75 PO-Ai Street, Hsinchu, Taiwan

E-mail: mao1010@ms7.hinet.net

Fax: +886-3-572-9288

Abstract

Haptoglobin (Hp) is a hemoglobin binding protein with an extremely high binding affinity. In this study, we screened twenty-four monoclonal antibodies (mAb) prepared against Hp. Only one mAb (8B1), which specific to Hp β-chain, was able to completely block the formation of Hp-Hb complex. Carboxymethylation on Cys-105 of Hp β chain diminished the recognition for both 8B1 mAb and hemoglobin. Combining of limited trypsin cleavage, chemical modification, and direct solid-phase peptide synthesis on nitrocellulose paper (protein array), residues of 105-116 of Hp β chain was defined as an epitope for the inhibitory mAb 8B1. Hypothetically this sequence, Cys-Leu-Pro-Ser-Lys-Asp-Tyr-Ala-Glu-Val-Gly-Arg, is located at the edged surface with a random coiled structure as probed from the crystal structure of serine proteases (chymotrypsin and trypsin), which has strong homology with Hp β chain in primary structure. In conclusion, we have produced a hemoglobin-blocking antibody recognizing the residues 105-116 of Hp β-chain, where we suggest that Cys-105 was critically involved in the hemoglobin binding. This study provides a potential insight for the future delineation of Hp binding site for hemoglobin which is currently unknown. The strategy for the antigenic epitope mapping and its relationship with the putative site participated in hemoglobin binding are discussed in detail.

Key words: antigenic epitope mapping, hemoglobin binding, haptoglobin structure

1. Introduction

Human plasma haptoglobin (Hp) is classified according to three phenotypes: Hp 1-1, 2-1, and 2-2 attributed by their two common alleles 1 and 2 [1-3]. Clinically, the phenotype 2-2 is associated with the risk in patients with diabetes mellitus [4-6]. Structurally, the minimal arrangement of Hp 1-1 (β-α-α-β) is joined by disulfide linkages among the 2 α and 2 β chains [1, 7-8], in which Cys-72 and Cys-105 is responsible for the cross linking between the α and β chains, respectively.

One of the key functions of Hp in plasma is to capture the hemoglobin released from red blood cells by forming a high affinity Hp-hemoglobin complex [9-10]. Heme in hemoglobin is highly toxic participating in Fenton reaction to produce reactive oxygen species which cause cell injury [11, 12]; Hp has therefore thought to play a protective role via its binding with Hb and prevent the iron "leaking" from the Hb. Recently, we have demonstrated that Hp is an extremely potent antioxidant, which directly protects low density lipoprotein from free-radical induced oxidation [1]. Its potency was markedly superior to probucol: one of the most potent antioxidants [11, 13]. Blockage of disulfide linkage between the Hp α and β chains diminishes its ability to form a complex with hemoglobin (Hb). However, the antioxidant activity is four times greater than that of native Hp suggesting that the antioxidant domain is independent from that hemoglobin binding site [1].

Physiologically, Hp-hemoglobin complex is metabolized through a receptor-mediated process including a recent report showing the CD 163 receptor of macrophages [2]. CD163 binds only Hp and hemoglobin in complex, which indicates the exposure of a new receptor-binding epitope. The detailed Hp binding domain to hemoglobin is currently unknown. Early studies have demonstrated that the binding

site lies in the β-chain of Hp [14-16]. A recent study using recombinant β-chain reveals that the binding domain is located in residues 80-161 [17]. Lacking of a crystal structure of Hp may explain not sufficient data to explore the binding site responsible for the Hp-hemoglobin interaction.

Using monoclonal antibodies (mAb) as a probe to study the structural and functional relationship of a given protein has been popular and reviewed [18]. It provides a power tool to define the functional location within the molecule [18]. For example a mAb specific to Hp-hemoglobin complex have been used for probing the specific domain responsible for the CD receptor [2, 19]. The purposes of this study were two folds: 1) To identify an inhibitory Hp mAb capable of blocking the Hp-hemoglobin binding. 2) To map out the epitope recognized by this inhibitory mAb and delineate its conserved region among the species that might be involved in the interaction with hemoglobin.

Our results show that the epitope recognized by the inhibitory mAb 8B1 was located in residues 104-116, where two or three residues in the COOH-terminus of this region were responsible for the antigenic specificity among the species. The relationship between the epitope with the hemoglobin binding is discussed.

2. Materials and Methods

2.1. Production and purification of monoclonal antibodies

A battery of mouse mAb prepared against human Hp 2-1 was produced according to the standard procedures established in our laboratory [20]. They were characterized for α or β chain specificity. The interested mAb were further purified as an IgG form via a Hp-affinity column chromatography [21].

2.2. Purification of plasma Hp

Hp phenotype was first identified using plasma supplemented with hemoglobin (Hb) on a native polyacrylamide gel electrophoresis (PAGE), followed by a peroxidase substrate staining [21]. In the present study, plasma of Hp 1-1 or Hp 2-1 phenotype was used for Hp purification using an antibody affinity-column followed by a gel-filtration high performance liquid chromatography (HPLC) as previously described by us [21-23]. The homogeneity of Hp employed was greater than 95% as judged by a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

2.3 Limited trypsin digestion and carboxymethylation of Hp

For trypsin treatment, 1 μl trypsin (0.1 mg/ml) was added into 100 μg of Hp 1-1 in 100 μl buffer containing 0.02 M phosphate, 0.12 M NaCl, pH 7.4 (PBS). The reaction was preceded at room temperature for 1 h and stopped by an addition of soy-bean trypsin inhibitor. Immunoreactivity of trypsinized Hp was determined on Western blot following a SDS-PAGE (18% polyacrylamide). Carboxymethylation of Hp was conducted and characterized according to the procedures previously descried by us [1]. The carboxymethylated Hp was desalted on a Bio-Gel P2 column eluted with 0.05 M ammonium bicarbonate and lyophilized [24]. Cys-105 of β-chain was completely modified as evidenced by the dissociation of α and β chains on SDS-PAGE without reducing reagent β-mercaptoethanol [1].

2.4 Preparation of Hp-Hemoglobin Complex

A HPLC experiment was performed to examine the formation of Hp-hemoglobin complex as described previously [1]. Hp 1-1 (10 ug) in 1.2-fold molar excess was mixed with purified hemoglobin in a final 100 ul of PBS and incubated at room temperature for 10 min, while allowing the formation of complex. To test the mAb capable of inhibiting the Hp binding to hemoglobin, each purified mAb (obtained from Hp-affinity column) containing about 50-60 ug was pre-incubated with Hp for 10 min before the addition of hemoglobin in 100 ul PBS. The reaction mixture was then applied onto a gel-filtration column (TSK-GEL G2000SWXL, 7.8 x 300 mm) pre-equilibrated in PBS. HPLC equipped with a photodiode array detector (Waters 996; Milford, MA, USA) was run at 0.5 mL/min using PBS as a mobile phase. Typical chromatographic profiles were monitored at 280 nm, while Hb or Hb-Hp complex was monitored at 415 nm.

2.5. Gel electrophoresis and Western blot analysis

SDS-PAGE was performed according to Laemmli's method with some modifications by using 15% polyacrylamide (w/v) on the stacking gel as previously described [1]. Samples (typically 5 ug) for SDS–PAGE were preheated at 100 °C for 10 min in a loading buffer [12 mM Tris–HCl, pH 6.8, 0.4% SDS (w/v), 5% glycerol (v/v), 2.88 mM of 2-mercaptoethanol, and 0.02% bromphenol blue (w/v)]. Samples were run for 1.5 h at 100 V and stained using Coomassie brilliant blue R-250.

Following the separation of proteins by SDS–PAGE, the gel was electro-transferred to a nitrocellulose membrane (Pharmacia) and incubated with Hp mAb followed by a horseradish peroxidase (HRP) labeled secondary antibody and developed according to the method previously described [21-23].

2.6. Peptide array and immunoassay

Twelve synthetic peptides, each containing 15 amino-acid residues, were designed corresponding to the sequence from residue 81 to 160 of Hp. The synthetic peptides were prepared under a contract with a local biotechnology company (Genesis Biotech Inc., Taipei, ROC). Briefly, the peptides were directly synthesized in situ on a nitrocellulose (NC) paper according to the method described [25]. The NC membrane in 0.01 M Tris buffered saline containing 0.05% (v/v) Tween-20 (TBST), was blocked with 5% (w/v) gelatin in TBST for 2 h at room temperature followed by 3x washes. After incubation with mAb for 2 h and 3 x washes, goat anti-mouse IgG conjugated with HRP in 5% gelatin/TBST was added and incubated. Finally, following the washes, chemiluminescent substrate (ECL™ Western Blotting System, Amersham) was added, washed, and immediately developed by exposing onto a film.

3. Results and Discussion

Previously, we have prepared several lines of mAb against human plasma Hp and employed them for isolation of Hp [21]. In the present study, we test the hypothesis whether these mAb could be used to explore the Hp-hemoglobin interaction. Each antibody was purified as an IgG form to at least 90% homogeneity prior to carry out the entire studies.

From more than 20 mAb screened using a previous established method [20], only 8B1 could inhibit the binding of hemoglobin to Hp. We than randomly chose the other 5 mAb as negative controls. A typical example showing the formation of Hp-hemoglobin complex on gel-exclusion HPLC and its blockage by mAb 8B1 is depicted in Fig. 1. The properties of the mAb with respect to their subunit specificity,

binding affinity, and sensitivity to limited trypsin digestion for Hp were then determined (Table 1). The data exhibit that the inhibitory mAb 8B1 recognized Hp β-chain, but failed to bind Hp upon the trypsin treatment. Whereas, G2D and an α-chain specific mAb W1 possessing no inhibitory activity were not affected on trypsinized Hp (Table 1). The inhibitory activity for the hemoglobin binding appears not due to the extraordinary binding affinity, since the other non-inhibitory mAb were with similar binding affinity to Hp (Table 1 and Fig. 1). Furthermore, combination of the other 5 mAb did not reveal the inhibition for Hp-hemoglobin binding (data not shown).

Next, we attempted to map out the antigenic epitope recognized by this 8B1 mAb. Since the limited trypsin cleavage on Hp resulted in a total loss of its immunoreactivity, suggesting that either Lys or Arg or both was essential to maintain the antigenic structure or probably lied in the epitope. In the next experiment, we addressed whether the Cys residues of Hp may participate the antigenic role for 8B1 mAb. The Cys residues were then chemically modified by carboxymethylation. Previously, we have demonstrated that carboxymethylated reduction disassembled the cross-linking between α (Cys-72) and β (Cys-105) chains of Hp on SDS-PAGE confirming Cys-105 of β-chain was carboxymethylated [1]. Each chain was identical to Hp subunit in the presence of reducing reagent β-mercaptoethanol. The present study shows that the carboxymethylation substantially attenuated the immunoreactivity for 8B1 mAb, but not for G2D mAb (Fig. 2). The finding implicated that Cys-105 might be involved in the binding for 8B1. It was extremely interesting that carboxymethylation on Cys-105 of Hp β-chain also abolished its binding to hemoglobin [1]. Therefore, we speculated that Cys-105 participated for both hemoglobin and inhibitory mAb binding. However, carboxymethylation also

altered the overall structure of Hp [1]; we could not rule out that the loss of immunoreactivity might be correlated to the overall conformational changes of Hp. In other words, 8B1 could be a "conformational" mAb.

Hp β-chain consists of 246 amino acid residues and has been suggested responsible for the binding to hemoglobin [14-16]. A recent study using recombinant β-chain of Hp indicates that residues 80-161 are capable of binding hemoglobin with the ability almost equivalent to the full length 1-246 [17]. Although the result has not been reconfirmed by the others, it was legitimate to assume that our inhibitory 8B1 mAb also recognized the epitope within residues 80-161. From our carboxymethylation and trypsin-treatment experiments, Cys-105, Lys, or Arg residue could be involved in maintaining the antigenic structure (Table 1 and Fig. 2). In considering Pro residues are usually located at or near to the immunoreactive domain by forming a loop at the surface of a given protein, we hypothesized that there was one domain, namely residues 105-119, highly possible for the binding of 8B1.

Accordingly, a solid-phase peptide array containing this predicted region (residues 105-119) and other eight either overlapped or non-overlapped synthetic peptides (each with 15 residues) within the residues 80-161 was prepared (Fig. 3). These peptides were directly synthesized on a nitrocellulose membrane (Fig. 3). After binding of our mAb followed by a HRP-conjugated secondary antibody, the array was developed using a chemiluminescent agent. We found that the epitope was exactly located in the predicted region: residues 105-119 (peptide 7) containing Cys-105 with other Arg, Lys, and Pro residues (Fig. 3). Fig. 3 suggests that residues Ala-112 and Glu-113 were crucial for 8B1 recognition, since peptides 2 and 3 cleaved between these two residues did not yield any immunoreactivity.

To further delineate the antigenic residues in the COOH-terminus of peptide 105-119, peptides 10, 11, and 12 were then prepared. Peptide 10 without COOH-terminal Gly-115 and Arg-116 did not exhibit immunoreactivity, while equal immunoreactivity was found between peptides 11 and 12 (Fig. 3). Interpretation of the activity among peptides 7, 10, 11, and 12, and the carboxymethylated data (Fig. 2); the putative antigenic determinant appeared to be between residues 105-116 or Cys-Leu-Pro-Ser-Lys-Asp-Tyr-Ala-Glu-Val-Gly-Arg. Notably this region contains two positively and two negatively charged residues, formation a stable ionic pair over the overall structure of Hp is likely. Although the crystal graphic structure of Hp has not as yet been elucidated, in theory this region with the insertion of Pro-117 (usually lies on the loop) should be located at or near to the surface of Hp for rendering the antibody interaction. Nevertheless, our antigenic mapping suggests that residues 105-116 of Hp β-chain was located at the surface and could be involved in the binding for hemoglobin, which has not been shown previously. It also confirms an early report that the hemoglobin binding domain is located between residues 80-161 [17].

Several lines of evidence indicate that the antigenic epitope we defined was reasonably correct. First, the size of an epitope is relatively small usually containing 8-12 amino-acid residues as demonstrated by us and the others [26-30]. Second, the 105-116 epitope contains both acidic and basic residues and Pro which are commonly present in an antigenic site [30]. Third, the epitope recognized by inhibitory 8b1 mAb consisting of Cys-105 was consistent to the effect of carboxymethylation data, in which Hp binding either for antibody or for hemoglobin was diminished.

Furthermore, to test the accuracy of defined epitope region, we first compared the sequence homology between human and mouse (Table 2). The key rationale is that part of the antigenic determinant of human Hp (residues 105-116) has to differ

from that of mouse, so that the monoclonal antibody can be made from the mouse. Table 2 confirms our prediction with the difference lies in the COOH-terminus of mouse Hp. Other species such as rabbit, pig, and rat were also examined. Their immunoreactivity of plasma Hp assayed on Western blot was given in Table 2. Mutation of Ala-112 and Glu-113 significantly reduced (in pig) or abolished (in mouse and rat) the immunoreactivity. The finding was again consistent to the peptide array assay (Fig. 3), in which cleavage between Ala-112 and Glu-113 resulted in a total loss of the immunoreactivity. A slight decrease in activity was found in that of rabbit; presumably substitution of Ala-112 with Thr-112 did not significantly alter the solution property of Hp. Thus, the sequence homology depicted in Table 2 may aid us to interpret the difference in immunoreactivity among the species.

Following the comparison of sequence homology of epitope among the different species, we were puzzling the relevant binding site of Hp involved for hemoglobin when considering the fact that this site should be well conserved. However, analysis of the NH_2-terminal half (residues 105-111 or Cys-Leu-Pro-Ser-Lys-Asp-Tyr) of the epitope, it reveals that sequence 105-111 is completely identical among the species we observed. It is plausible to interpret this conserved region was responsible for both inhibitory antibody and hemoglobin recognition, whereas two or three residues lie in the COOH-terminus determined the antigenic specificity among the species. Another possibility is that this 8B1 mAb binds to a region in proximity to the hemoglobin binding site and hence possesses the inhibitory activity via a steric hindrance.

Nevertheless, the epitope (residus 105-116) we mapped should be theoretically on the surface of Hp molecule for facilitating the antibody binding. We examined the crystal structures of chymotrpsin and trypsin, since these two serine proteases possess strong homology to Hp β chain in primary structure (greater than 50% as calculated from the protein data bank). Fig. 4 shows the sequence alignment among these three molecules revealing a good homology between the epitope region 105-116 of Hp and the proteases. It is of striking interest that this homology motif is exactly located at the edged surface of chymotrpsin and trypsin with a random coil structure (Fig. 4). It is of worth mentioning that the authentic structure of this region in Hp should await the establishment of the Hp 3D-structure.

In conclusion, we have produced a hemoglobin-blocking antibody recognizing the residues 105-116 of Hp β-chain. It provides a potential tool for the future delineation of Hp binding site for hemoglobin which is currently unknown.

Acknowledgement

References

[1] Tseng, C. F., Lin, C. C., Huang, H. Y., Liu, H. C., and Mao, S. J. (2004) Proteomics. 4(8), 2221-2228.

[2] Kristiansen, M., Graversen, J. H., Jacobsen, C., Sonne, O. et al. (2001) Nature. 409, 198 201.

[3] Patzelt, D., Geserick, G., and Schroder, H. (1988) Electrophoresis. 9, 393 397.

[4] Levy, A. P., Roguin, A., Hochberg, I., Herer, P. et al., (2000) N. Engl. J. Med. 343, 969 970.

[5] Nakhoul, F. M., Marsh, S., Hochberg, I., Leibu, R. et al. (2000) JAMA 284, 1244 1245.

[6] Nakhoul, F. M., Zoabi, R., Kanter, Y., Zoabi M. et al. (2001) Diabetologia, 44, 602 604.

[7] Maeda, N., and Smithies, O., (1986) Annu. Rev. Genet. 20, 81 108.

[8] Sawyer, J. T., Lukaczyk, T., and Yilla, M. (1994) J. Biol. Chem. 269(35), 22440-22445.

[9] Raijmakers, M. T. M., Roes, E. M., Morsche, R. H. M., teSteegers, E. A. P., and Peters, W. H. M. (2003) J. Med. Genet. 40, 214 216.

[10] Engstrom, G., Stavenow, L., Hedblad, B., Lind, P. et al. (2003) Diabetes 52, 442 447.

[11] Mao, S. J., Yates, M. T., and Jackson, R. L. (1994) Methods Enzymol. 234, 505 513.

[12] Tolosano, E., Fagoonee, S., Hirsch, E., Berger, F. G. et al. (2002) Blood 100, 4201 4208.

[13] Ishida, E., Managit, C., Kawakami, S., Nishikawa, M., Yamashita, F., and Hashida, M. (2004) Pharm Res. 21(6), 932-939.

[14] Lustbader, J. W., Acroleo, J. P., Birken, S., Greer, J. (1983) J. Biol. Chem. 258, 1227-1234.

[15] Geer, J., Liao, W. D. and Brown, W. E. (1981) J. Biol. Chem. 256, 8771-8774.

[16] Urushibara, N., Kumazaki, T., and Ishii, S. (1992) J. Biol. Chem. 267(19), 13413-13417.

[17] Melamed-Frank, M., Lache, O., Enav, B. I., Szafranek, T., Levy, N. S., Ricklis, R. M., and Levy, A. P. (2001) Blood. 98(13), 3693-3698.

[18] Miller, E. J., and Cohen, A. B. (1991) Am. J. Physiol. 260, L1-12.

[19] Moller, H. J., Peterslund, N. A., Graversen, J. H., and Moestrup, S. K. (2002) Blood. 99(1), 378-380.

[20] Chen, W. L., Huang, M. T., Liu, H. C., Li, C. W., and Mao, S. J. (2004) J. Dairy Sci. 87(8), 2720-2729.

[21] Tseng, C. F., Huang, H. Y., Yang, Y. T., and Mao, S. J. (2004) Protein Expr. Purif. 33(2), 265-273.

[22] Yang, S. J., and Mao, S. J. (1999) J. Chromatogr. B, Biomed. Sci. Appl. 731, 395-402.

[23] Liau, C. Y., Chang, T. M., Pan, J. P., Chen, W. L., and Mao, S. J. (2003) J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 790(1-2), 209-216.

[24] Mao, S. J., Sparrow, J. T., Gotto, A. M. Jr., and Jackson, R. L. (1980) Biochim. Biophys. Acta. 617, 245-253.

[25] Hasen, P. R., Holm, A., and Houen, G. (1993) Int. J. Pept. Protein Res. 41, 237-245.

[26] Mao, S. J., Yates, M. T., Owen, T. J., and Krstenansky, J. L. (1989) J. Immunol. Methods. 120(1), 45-50.

[27] Bhatnagar, P. K., Mao, S. J., Gotto, A. M. Jr., and Sparrow, J. T. (1983) Peptides. 4(3), 343-349.

[28] Mao, S. J., Miller, J. P., Gotto, A. M. Jr., and Sparrow, J. T. (1980) J. Biol. Chem. 255(8), 3448-3453.

[29] Mao, S. J., Sparrow, J. T., Gotto, A. M. Jr., and Jackson, R. L. (1979) Biochemistry. 18(18), 3984-3988.

[30] McCormick, D. J., Lennon, V. A., and Atassi, M. Z. (1985) Biochem. J. 226, 193-197.

Association of Haptoglobin 1-1 phenotype with angiographically documented coronary artery disease patients

Background

Haptoglobin (Hp) an acute phase protein, and its plasma level elevates in response to inflammation. It remained controversial whether Hp polymorphism is associated to the risk of coronary artery disease (CAD).

Methods and Results

In this study, we enrolled 1116 patients from Cardiology Division of Veterans General Hospital-Taipei for coronary arteriographic examination. These patients scheduled for percutaneous transluminal angioplasty or bypass surgery for documented CAD. Haptoglobin typing was performed by means of modified native polyacrylamide gel electrophoresis (native PAGE) of hemoglobin-supplemented serum or plasma. The developed method was consistent to that using Western blot analysis. We demonstrate again that low apoAI level in plasma is a superior risk factor in patients with CAD. Conditional logistic regression analysis, confirms that more than doubling in susceptibility of CAD rates in the Hp 1-1 group compared with the rest, with an adjusted odds ratio of 2.22 in Hp1-1 /Hp2-1 and 2.19 in Hp1-1/Hp2-2.

Conclusions

The procedure represents a definite improvement for Hp typing in clinical diagnoses. ApoAI level is found to be remarkable lower in CAD than in control consistent to our previous finding over the population in Minnesota

area. This study was the first group adjusted for apoAI in stepwise logistic regression and the presented data strongly suggest that Hp 1-1 was associated with CAD. Hp phenotypes may lead to a rational design in intervening new pharmacological agents.

Introduction

It remained controversial whether Hp polymorphism is associated to the risk of coronary artery disease. One of the key biological functions of human plasma haptoglobin (Hp) is to capture the free hemoglobin (Hb) in plasma allowing hepatic clearance of Hb and preventing the oxidative damage in kidney during hemolysis (1). On the other hand, Hp is known as an acute phase protein, and its plasma level elevates in response to infection or inflammation (1-3). Similar to blood types, there are three major Hp phenotypes 1-1, 2-1, and 2-2 in human plasma attributed by two common alleles (1 and 2). Structurally, Hp is tetramerically arranged (α-β-β-α) joined by disulfide linkages among α and β chains (4). Figure 1 shows that all the phenotypes share the same 2β (each with about Mr 40 kDa containing 243 amino acids and approximately 30 % carbohydrate). A typical structure of homozygous Hp 1-1 is composed of two identical α1 chains (each with about Mr 9 kDa containing 83 amino acids). Homozygous Hp 2-2 is composed of two identical α2 chains (each with about Mr 16.5 kDa containing 142 amino acids) as compared to that of heterozygous Hp 2-1 containing one each of α2 and α1 (fig. 1).

In patients with myocardial infarction, individuals with Hp 2-2 were found to have more frequent left ventricular failure than Hp 1-1 and Hp2-1 patient (5). Also, Hp 2-2 patients have demonstrated to be a predictor of the risk of

restenosis after PTCA (6) and diabetic nephropathy (7) in diabetes patients. Hp 2-2 carriers also showed higher rate of L-ascorbic acid oxidation which implicated that they might have less protection against Hb-driven perioxidation (8). Although Hp 2-2 has been suggested to be a risk phenotype with coronary artery disease (CAD), however, a recent survey (9) showed that Hp1-1 individual was at double risk for CAD mortality. Cid et al (10) found that Hp functioning as an angiogenic factor. Puified Hp stimulates endothelial cell differentiation and vascularization in a dose-dependent manner in vivo and in vitro model. They suggested that in systemic vasculitis, Hp may also compensate for ischemia by promoting development of collateral vessels. Delanghe et al (11) also found that in patients with peripheral arterial occlusive disease, Hp 2-2 individuals having a better walking ability than Hp 1-1 individuals. Hp is a member of the endogenous inhibitors of prostaglandin synthesis which has important biological consequences, including antiinflammtory action. The inhibitory effect in Hp 1-1 is stronger than the effects of Hp 2-2 and Hp 2-1 (12). Despite the atherogenic potential, Hp 2-2 might have a beneficial effect in more advanced atherosclerosis. Clinically, patients with polymeric form of Hp 2-1 or 2-2 are associated with the complications of myocardial infraction (13), kidney failure (14), and diabetics (15). There had been reported that Hp 2-2 is more likely to develop restenosis (16). Hp 2-2 patients have more severe myocardial infarctions than Hp 1-1 and Hp 2-1(17). Compared to controls, patients with Hp 2-2 phenotype showed a significantly increased risk for essential hypertension (18) and hypertension associated with ischemia heart disease. Belgium (BIRNH) individuals for the Hp 1-1 were found to be at significantly elevated risk for

CHD mortality as compared to individuals with the Hp 2 allele (Hp 2-1 or Hp 2-2) (19). However, in the Strong Heart Study, a population-based nested cased control study of North American Indians, individuals who were Hp 1-1 exhibited a dramatically reduced risk of CHD and stroke (21). All above did not direct evidence to estimate the influence of Hp phenotypes and coronary artery disease

The primary objective of the study was to determine whether Hp phenotype is associated to the risk of coronary artery disease.

Materials and methods

Subjects

The subjects with or without CAD were identified from cases that were admitted to Cardiology Division of Veterans General Hospital-Taipei for coronary arteriographic examination. The severity of CAD was determined by fixed stenotic lesion with luminal narrowing $\geqq$ 50% in at least one of the major or minor coronary arteries. These patients scheduled for percutaneous transluminal angioplasty or bypass surgery for documented CAD. A total 1116 subjects were examined, consisting of 559 hospital controls and 557 CAD patients. Patients with either acute or chronic infectious diseases and malignancy were excluded.

Clinical covariates were categorized at the index examination and included age, body mass index (BMI), total cholesterol, HDL cholesterol, LDL cholesterol, Triglyceride, apolipoprotein A-I (apoAI), sex, hypertension and diabetes.

Preparation and purification of human hemoglobin

Fresh human blood collected in 0.1% EDTA was immediately centrifuged at 3000 *g* for 25 min, after that time plasma was removed by aspiration. The remaining red blood cells (RBCs) were washed five times with three volumes of phosphate-buffered saline (PBS) containing 0.12 *M* NaCl and 12 m*M* phosphate, pH 7.2, and then lysed with two volumes of deionized water at 4 ℃. Cell debris was removed by centrifugation at 3500 *g* for 30 min. The supernatant containing mostly hemoglobin was fractionated by 50% saturated ammonium sulfate at 4 ℃ for 30 min followed by a centrifugation at 4500 *g* for 40 min at 4 ℃. The supernatant was dialyzed against 0.02 *M* sodium phosphate, pH 8.0, at 4 ℃ overnight followed by a filtration through a 0.45 μm nylon filter.

Haptoglobin phenotyping

Haptoglobin typing was performed by means of native polyacrylamide gel electrophoresis (native PAGE) of hemoglobin-supplemented serum or plasma. Haptoglobin phenotype was determined from 10 μl of hemoglobin-supplemented plasma by polyacrylamide gel electrophoresis and peroxidase staining. Briefly, serum 9 μl was mixed with 1 μl of a 40mg/ml hemoglobin solution at room temperature to permit the haptoglobin-hemoglobin complexes formation. An equal volume of sample buffer (containing 125 mM Tris base (pH 6.8), 10% (V/V) glycerol, and 0.025 mg/L bromophenol blue) was added to each sample before electrophoresis. The haptoglobin-hemoglobin complex was resolved by polyacrylamide electrophoresis using a buffer containing 25 mM Tris base and 192 mM glycine. The stacking gel was 5.5 % polyacrylamide (26.5:1 acrylamide:bis-acrylamide), pH 6.8, and the separating

gel was 7 % polyacrylamide (26.5:1 acrylamide:bis-acrylamide), pH 8.8. Electrophoresis was performed at an initial voltage of 120 V was maintain until the dye front reach the separating gel. The voltage was increased to 150 V for the duration of the run for 50 minutes. After the electrophoresis was completed, the haptoglobin-hemoglobin complexes were visualized by shaking the gel in freshly prepared staining solution. The staining solution contained 25 mg of 3,3 -Diaminobenzidine dissolved in 0.5 mL of dimethyl sulfoxide, 49 ml of phosphate-buffered saline, and 100µl of 35% hydrogen peroxide added before using. The bands corresponding to the haptoglobin-hemoglobin complex were readily visible within a period 5~10 minutes and were stable. Phenotypes Hp 1-1, Hp 2-2, and Hp 2-1 were distinguished by a characteristic pattern of bands representing the haptoglobin-hemoglobin complex

Analysis of lipids and lipoproteins

Blood sample mixed with 0.1% ethylenediamine tetraacetic acid (EDTA) were drawn after a 12-hour overnight fasting in all study cohorts. Total cholesterol (TC), cholesterol content of high density lipoprotein (HDL) cholesterol and low density lipoprotein (LDL) cholesterol, and triglyceride (TG) were analyzed enzymatically using commercial reagents (CHOP-PAP method, Merck Scientific Corporation, Germany).

Statistical methods

The distribution of the Hp phenotypes was tested against Hardy-Weinberg equilibrium according to a χ^2 test [15]. Among the three groups Hp 1-1, Hp 2-1 and Hp 2-2, the equality of distributions of continuous variables was statistically evaluated according to the Kruskal–Wallis test, while proportions were compared using Fisher's exact test. In this matched case-control study,

the association between the Hp polymorphism and mortality from CHD, independently of classical coronary risk factors, was modeled using conditional logistic regression for matched sets [16]. Multivariately adjusted odds ratios were calculated together with their 95% confidence intervals. Statistical significance of the estimated regression coefficients was judged according to the Wald χ^2 statistic. The global level for statistical significance was taken as $\alpha = 0.05$. Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA).

Results

Hp phenotyping:

Essentially, Hp phenotyping was conducted using Hb supplemented serum samples (2 mg/mL) on native-PAGE followed by a Hb peroxidase staining. Figure 2 shows the distinguished three phenotypes. Hp 2-1, in addition to its polymers, shared a monomeric form of Hp 1-1. Whereas, Hp 2-2 exhibited trimeric and other polymeric forms. Apparently, the developed method was consistent to that using Western blot analysis.

Hp phenotypes in angiographically documented patients with and without CAD

A total of 1132 subjects containing 559 hospital normal control and 573 CAD patients were investigated for their Hp phenotypes. All the hospital normal controls and CAD patients were identified from the hospitalized coronary angiography. The distribution of the haptoglobin phenotypes was 8% (Hp 1-1), 41% (Hp 2-1), and 51% (Hp 2-2) in studied group (Table 1). Less than 8% of the normal population in Taiwan corresponded to Hp 1-1, while Hp 2-1 and 2-2 were about 42% and 50%. Such distribution was similar to other Far East Asian areas: China, Korea, and Japan (Table 3).

As shown in Table 1, the distribution in hospital normal controls was 7.5 %, 42.2 %, and 50.3 % for Hp 1-1, 2-1, and 2-2. The phenotype distribution in CAD was 8.6 %, 40.1 %, and 51.3 % for Hp 1-1, 2-1, and 2-2, respectively (Table 1). The distribution of Hp 2-1 and 2-2 were almost equal between CAD and hospital controls. Statistically, the phenotype difference between patients with and without CAD was not significant (P=0.69).

Table 2 shows that no associations were found between the Hp phenotypes and lipid profiles (plasma total cholesterol, HDL cholesterol, LDL-cholesterol, and triglyceride).

In control group, HDL cholesterol in Hp 1-1 group was mildly higher than that of Hp 2-1 and 2-2, which almost reaching the statistical significance (p = 0.062) (Table 4). However apoAI, a major apolipoprotein of HDL, in Hp 1-1 (138.2±35.0 mg/mL) was significantly higher than that in Hp 2-1 (121.4±29.6 mg/mL) and Hp 2-1 (124.5±29.7 mg/mL) (p = 0.024).

In patients with CAD, the mean apoA-I levels were found to be remarkably lower in all the phenotypes (107.4±28.0 mg/mL) than in controls (125.6±32.6 mg/mL) consistent to our previous finding over the population in Minnesota area (NEJM). However, none of the other lipoid (except HDL) levels were significantly difference from that of control subjects (Table 1). Thus, we demonstrate again that low apoA-I level in plasma is a superior risk factor in patients with CAD (mao, NEJM). Except apoAI, age in CAD group (67.3±9.5) is older than normal control (61.4±12.3) (p=). It matches the reported that the incidence of cardiovascular disease in adults is increase with age of life (52). The proportion of male in CAD (91%) is higher than normal control (70%) (p=). Men had higher risk scores of coronary artery disease risk than

women did (51). In CAD patients (54%) there has higher hypertension susceptibility than normal control (31%).

Hp 1-1 phenotype was associated with CAD patients when major risk factors were adjustment

In the next study, we attempted to only address whether Hp phenotypes was associated with coronary artery disease.

We tested the hypothesis whether or not Hp phenotypes may become a risk factor when and other established risk factors (age, sex, hypertension, and apoAI levels) are adjusted. As shown in Table 2, stepwise conditional logistic regression analysis, confirms that more than doubling in susceptibility of coronary artery disease rates in the Hp 1-1 group compared with the rest, with an adjusted odds ratio of 2.22 in Hp 1-1/ Hp 2-1and 2.19 in Hp 1-1/ Hp 2-2. Thus, the data strongly suggest that Hp 1-1 was associated with CAD.

The overall association between the three Hp phenotypes and CAD was not significant ($p = 0.69$).

As mentioned above, low levels of apoAI was a major factor associated with CAD patients.

Geographic distribution of hp phenotypes:

In the next study by reviewing published reports, we show that the Hp 1-1 phenotype distribution in Taiwan, China, and Southeast Asia are dramatically different from the other countries. Hp 1-1distribution in Africa is more than 20 % and up to 49% in Liberia, between 10~20% in Europe and North America, and less than 10 % in Asia (Table 5). The dominant Hp 1-1 distribution is among New Guinea and Chile (Indians) with values greater than 56%.

Discussion

The method developed in this study using a native-PAGE for Hp phenotyping (Fig.1) was fully agreeable with immunological phenotyping (data not shown). In contrast to those utilizing starch gel, high performance gel permeation chromatography (22, 23), agarose gel electrophoresis (24), and Western blot, the present method supplemented with exogenous Hb to form the Hb-Hp complex was sensitive, simple, rapid and data can be preserved forever. It could virtually be finished within 1 h. The procedure represents a definite improvement for Hp typing in clinical diagnoses.

Differential susceptibility to atherosclerotic coronary artery disease cannot be explained entirely by conventional cardiac risk factors. There exists a growing awareness of the existence of polymorphic genetic loci that may act to modulate susceptibility to coronary heart disease (CHD).

Presumably, this was due to the marked complicated structure of Hp 2-1 and 2-2 as one extra-disulfide bond in the α2 subunit forms heterogeneous polymers, in which some of the biologically functional groups are not fully expressed on the surface. This apparent paradox might be explained by differences in the frequency of diabetes in the two populations and by separately analyzing the risk of CHD in non-diabetics and diabetics in the two studies. In the Belgian study the increased risk of Hp 1-1 was seen only in non-diabetic individuals whereas in the Strong Heart Study the decreased risk of Hp 1-1 was seen only in individuals with diabetes (25).

Less than 8% of the normal population in Taiwan corresponded to Hp 1-1, while Hp 2-1 and 2-2 were about 42% and 50%. Such distribution was similar to other Far East Asian areas: China, Korea, and Japan (Table 3). Hp 1-1 or

2-2 represents the homozygous individuals from human allele-1 or allele-2, respectively, located at chromosome 16q22.1. It is puzzling, however, that the heterozygous Hp 2-1 individuals generally account for 50% among all the populations (5-12,26-46), even though the frequency of Hp 1-1 is as low as 8% in Southeast Asia (Table 3). Genetically, since α 2 structure of Hp 2-2 contains one complete α 1 copy and a partial repeat of α 1 (as a result of α 1 insertion) and the α 2 structure has not been found in non-human subjects, as such human phenotype 2-2 may be evolved later than Hp 1-1. Hp 2-2 homozygous is then reproduced from heterozygous 2-1.

Haptoglobin genotype is an independent risk factor for cardiovascular disease and that this relationship is specific for diabetes.

Hp 2-2 molecule was significantly less than Hp 2-1 and 1-1 in protection from Cu2+-induced LDL oxidation. We respected to the mechanism involved in the pathogenesis of Hp phenotypes in CAD, The present study demonstrates that Hp phenotype was associated with CAD. The data was reported by Melamed-Frank et al, 2002 (Blood, 2001), in which they demonstrate that the antioxidant effect of Hp 1-1 is differentially superior to Hp 2-1 and 2-2 using hemoglobin-induced LDL oxidation, a protection mechanism thought to be mediated through Hp-Hb complex formation. In patients with diabetes mellitus developing nephropathy, Nakhoul et al (2001, Diabetologia) have postulated that the differences in the molecular shape and size between the Hp 1-1 and 2-2 are involved. Recently, we have shown (bills purification paper) that a significant overall difference in structure between Hp 1-1 and Hp 2-1 or 2-2 using a circular dichroic spectrum analysis (bills paper). We suggest that the availability of "functional surfaces" of Hp 1-1 and 2-1 may be

greater than that of Hp 2-2. This is because that only the Hp 2-2 can form heterogeneous cyclic polymers (Fig. 1) with some of the functional domains "buried" inside the bulky arrangement. Such structural diversity may account, in part, for the clinical outcome by which Hp 2-2 phenotype is associated with susceptibility to CAD and other related diseases. Nevertheless, identification and understanding of the biochemical basis for the differences among Hp phenotypes may lead to a rational design in intervening new pharmacological agents, such as mini-Hp, which have been recently proposed [9 and Tolosano 2002, Blood].

In control group, Hp 1-1 has higher concentrate of apoAI (Table 1). It was readily known that apo AI concentration was a marker of atherosclerosis (47). Plasma level of apoAI is an important determinant and is more useful than HDL cholesterol for identifying patients with coronary artery disease (47, 48). So to estimate other risk factors for CAD, it must adjust the apoAI level before statistic. The higher concentration of apo AI is, the lower of atherosclerosis frequency. According to above, Hp 1-1 seems a protection role in control group. Apo AI is thought to be better predictor of coronary artery disease than HDL cholesterol. Apo AI is the major component of HDL, and it displays anti-atherogenic properties (49). Michelle C. et al reported that apoAI concentration was an independent predictor of CAD (50, mao).

In the other hand, the increase risk of coronary artery disease with the increase in age occurred due to a combination of factors (51). According to Kannel and Wilson (52), the incidence of cardiovascular disease in adults doubles approximately at each decade of life. Men had higher risk scores and were more concentrated in the highest positions of the ranges of coronary risk

than women did (51). The biological explanation for this fact could be the female protection provided by estrogen, which has a direct effect on the circulatory system, causing vasodilatation and inhibiting the progression of the atherosclerotic processes, avoiding, therefore, ischemic events (53). Hypertension and coronary artery disease (CAD) are often associated, reflecting common origins in lifestyle and the role of hypertension as a risk factor for CAD (54, 55).

To estimate the really effect of Hp phenotypes on CAD, these existed conventional risk factors should be considered to modulate and predict the susceptibility in the general population to atherosclerotic coronary artery disease. Therefore, we used the conditional stepwise logical regression to export the masked effects.

Compare the phenotypes distribution of Hp show dramatic geographical differences. Table 3 collects Hp phenotypes distribution among geographical population. Haptoglobin polymorphism is associated the prevalence and clinical evolution of many inflammatory diseases, including infections, atherosclerosis, and autoimmune disorders. The strong genetic pressure favoring the Hp 2-2 phenotype suggests an important role of Hp in human pathology (56). The Phenotype distribution difference of various populations is probability programmed in geographic differences in diseases. Such as, Diabetes, cardiovascular disease, certain cancers, and infection diseases. For example, Hp 1-1 is associated with susceptibility to falciparum malaria and the development of severe complications; alternatively, the other phenotypes may confer resistance (57). It had been reported that women with the Hp 1-1 reproduce at an earlier age and have higher natural fertility

potential than women with other Hp genotype (58). There exists the highest distribution of Hp 1-1 in the world, it could partially explain the earlier reproduce in Africa than other area.

It has been proposed that the Hp-2 allele was originated at about 2 million years ago (59) and had since spread over the world under a strong genetic pressure, which favors the Hp 2-2 phenotype displacing the monopoly of Hp 1 allele gradually. As shown in Table 3, the greatest value of Hp 1-1 distribution is being in the West Africa (49%) (31) and Indians of Chile (56-63%) (42), with the Hp 2-2 distribution less than 10%. Its distribution decreases from West Africa (49%) in the direction of East Africa (25%), Northwest Europe (16%), Southeast Europe (15%) to Asia (2-8%) (5-12,26-46). Thus, the data suggest that Hp phenotype distribution variance might be regarded as an index factor in relation to ancient human population migration.

In the patient with diabetes increased oxidative stress, (20) differences in genetically determined endogenous antioxidant protection may have exaggerated importance.

Finally, since Hp phenotype is an independent risk factor in developing CAD and its provocative antioxidant role in protecting atherosclerosis, determination of Hp concentrations in human plasma would be essential. Although methods using immunodiffsion and ELISA have been developed (----), our preliminary study indicated that the immunoreactivity of each type of Hp was diverse and heterogeneous depending on the epitope specificity of the molecular orientation of Hp. Thus, it is somewhat difficult to accurately determine the concentrations of Hp using immunoassays. For example, at the

equal concentrations of each Hp phenotype, we show that the immunoreactivity of Hp 1-1 was significantly higher than the other two types (data not shown). Regardless, we are currently in progress to determine the plasma Hp using a "surface specific" monoclonal antibody that could fully recognize the epitope of Hp in each phenotype.

Acknowledgements

This work was supported by the grant NHRI-EX92-9229SI (S.J.T.M.) from National Health Research Institute, Taiwan, ROC.

也放**Dr. Pan** 的計畫

Reference:

1. McCormick DJ, Atassi MZ: Hemoglobin binding with haptoglobin: Delineation of the haptoglobin binding site on alpha-chain of human hemoglobin. J protein chem. 9:735-742, 1990
2. Hess JR: Review of modified hemoglobin research at Letterman: Attemped to delineate the toxicity of cell-free tetrameric hemoglobin. Artif Cells Blood Substit Immobil Biotechnol 23:277-289, 1995
3. Ogden JE, Parry ES: The development of hemoglobin solutions as red blood cell substitutes. Int Anestrhesiol Clin 33:115-129, 1995
4. Meira MF, Orit L, Benjamin IE, Tal S, Nina SL, Rebeca MR Andrew PL. Structure-function analysis of the antioxidant properties of haptoglobin. Blood 2001;98:3693-3698
5. Kasvosve I, Gordeuk VR, Delanghe JR, Gomo ZA, Gangaidzo IT, Khumalo H, Moyo VM, Saungweme T, Mvundura E, Boelaert JR. Iron status in black persons is not influenced by haptoglobin polymorphism. Clin Chem Lab Med 2002;40(8):810-2
6. Buettner JJ., Buettner JV. Hemoglobins, haptoglobins, and transferrins in indigenous populations of Kenya. Am J Phys Anthropol. 1970;31(1):27-32
7. Kasvosve I, Gomo ZA, Gangaidzo IT, Mvundura E, Saungweme T, Moyo VM, Khumalo H, Boelaert JR, Gordeuk VR, Delanghe JR. Reference range of serum haptoglobin is haptoglobin phenotype-dependent in blacks. Clin Chim Acta 2000;296(1-2):163-70.
8. Quaye IK, Ekuban FA, Brandful JA, Gyan BA, Akanmori BD, Ankrah NA. Haptoglobin phenotypes in HIV-1-seropositive patients in Ghana: decreased risk for Hp0 individuals. Hum Hered 2000;50(6):382-3
9. Van Sande M, Van Ros G, Druet R. Determination of Haptoglobin-groups frequencies by starch-gel and ager-gel electrophoresis: application to Belgian and Barundi populations. Nature 1963:197:603-4.

10. Allison AC, Blumberg BS, Rees W. Haptoglobin types in Britis, Spanish Basque and Nigerian African population [Letter]. Nature 1959;183:192
11. Delanghe JR, Langlois MR, Duprez DA, De Buyzere ML, Clement DL. Haptoglobin polymorphism and peripheral arterial occlusive disease. Atherosclerosis 1999;145: 287-292.
12. Lange V. der haptoglobin Polymorphismus – nicht nut eine genetische markierungsfife [Review]. Anthropol Anz 1992;50:281-302.
13. Cerda S, Oh SK. Methods to quantitate human haptoglobin by complexation with hemoglobin. J Immunol Methods. 1990;134:51-59.
14. Delanghe JR, Duprez DA, De Buyzere ML, Bergez BM, Claeys LR, Leroux-Roels GG, Clement DL. Refractory hypertension is associated with the haptoglobin 2-2 phenotype. J Cardiovasc Risk 1995;2(2):131-6
15. Langlois RL, Delanghe JR. Biologival and clinical significance of Haptoglobin polymorphism in humans. Clin Chem. 1996; 42(10): 1589-600.
16. n
17. n
18. n
19. De Bacquer D, De Backer G, Langlois M, Delanghe J, Kasteloot H, Kornitzer M. Haptoglobin polymorphism as a risk factor for coronary heart disease mortality. Atherosclerosis 2001;157:161 6.
20. Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SKA Moestrup SK. Identification of the haemoglobin scanvenger receptor. Nature 2001: 409:198-201
21. Levy AP, Hochberg I, Jablonski K, Resnick HE, Lee ET, Best L. Haptoglobin phenotype is an independent risk factor for cardiovascular disease in individuals with diabetes: the Strong Heart Study. J Am Coll Card 2002;40:1984 90.
22. Smithies O. Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. Biochem J 1955;61:629-41
23. Delanghe J Allcock K, Langlois M, Claeys L, De Buyzere M. Fast determination of haptoblobin phenotype and caculation of Hb binding capacity using high press gel permeation chromatography. Clin Chim Acta 200;291:43-51.
24. Wassel J, Keevil B. A new method for haptoglobin phenotyping. Ann Clin Biochem 1999;11:609-12
25. Andrew P. Levy, Martin G. Larson, Diane Corey, Rachel Lotan, Joseph A. Vita, Emelia J. Benjamin. Haptoglobin phenotype and prevalent coronary heart disease in the Framingham offspring cohort Atherosclerosis 172 (2004) 361 365
26. Blackwell RQ, Lin T-Y, Shiao D. Distribution of Haptoglobin amongchinese in Taiwan. Nature 1963;193:284-5.
27. Maes M, Delanghe J, Bocchio Chiavetto L, Bignotti S, Tura GB, Pioli R, Zanardini R, Altamura CA. Haptoglobin polymorphism and schizophrenia: genetic variation on chromosome 16. Psychiatry Res 2001;104(1):1-9
28. Kasenov KU, Sundetov ZhS. Haptoglobin types among the population of western Kazakhstan and their relation to humoral factors of body resistance. Genetika 1985;21(2):347-9
29. Louagie H, Delanghe J, De Bombere I, De Buyzere M, Hauser P, Leroux-roels G. Haptoglobin polymorphism and immune response after hepatitis

B vaccination. Vaccine 1993;11:1188-90.
30. n
31. Thomas L. Haptoglobin/Hämopexin. In: Thomas L, ed. Labor und Diagnose, 4th ed. Marurg: Medizinische Verlags., 1992:813-20.
32. Fröhlander N, Jhonson O. Haptoglobin groups in acute myocardial infraction. Hum Hered 1989;39:345-50.
33. Hever O. Relations entre les phénotypes d'haptoglobine dansdiverses maladies. Press Méd 1969;30:1081-2.
34. n
35. Xu JJ, Cui MY, Li SZ, Chen LZ, Du RF, Goedde HW, Benkmann HG, Kriese L, Bogdanski P. Polymorphisms of Pi, Hp, ADA and AK in Mongolian, Korean and Zhuang populations of China. Ann Hum Biol 1986;13(3):245-51.
36. Kirk RL. A haptoglobin mating frequency, segregation and ABO blood-group intraction analysis for additional series of families. Ann Hum Genet. 1971;34(3):329-37.
37. Bandopadhaya AR. Interaction between ABO and haptoglobin systems in a Bengalee population. Hum Hered 1992;42(3):201-3.
38. Bowman JE. Haptoglobin and transferring differences in some Iranian populations. Nature 1964;201:88
39. Roy R, Roy IC. Haptoglobin phenotyping from older bloodstains by enzyme immunoassay and haptoglobin phenotypes within a Nebraska Caucasian population. J Forensic Sci 1991;36(2):571-5.
40. Spitsyn VA, Titenko NV, Gokhman II, Bogdanova VI, Chistov YuK, Makarov SV. Serum protein polymorphisms in the population of south Yemen. Hum Hered 1991;41(1):43-6.
41. Scutton HE, Matson GA, Robinson AR, Kouchy RW. Distribution of Haptoglobin, transferrin, and Haptoglobin types among Indians of Southern Mexico and Guatemala. Am J Hum Genet 1960;12:338
42. Nagel R, Etcheverry R, Types of Haptoglobin in Araucanian Indians of Cile. Nature 1963;197:187-8
43. n
44. Flory LL. Serum factors of Australian aborigines from north Queensland. Nature 1964;201:508-9.
45. Hamad M, Awadallah S. Age group-associated variations in the pattern of Hp type distribution in Jordanians. Clin Chim Acta 2000;300(1-2):75-81.
46. Benhamou JP. Diagnostic approach to a liver mass: diagnosis of an asymptomatic liver tumor in a young woman. Hepatol 1996;25(1):10-4.
47. Maciejko JJ, Holmes DR, Kottke BA, Dinh DM, Mao SJT. Apolipoprotein A-I as a marker of coronary artery disease. N. Eng. J. Med. 1983; 309:385-389.
48. Wu JH, Kao JT, Wen MS, Wu D. Coronary artery disease risk predicted by plasma concentrations of high-density lipoprotein cholesterol, apolipoprotein AI, apolipoprotein B, and lipoprotein(a) in a general Chinese population. Clin Chem. 1993 Feb;39(2):209-12.
49. Patrick Benoit; Florence Emmanuel; Jean Michel Caillaud; Laurent Bassinet; Graciela Castro; Pierre Gallix; Jean Charles Fruchart; Didier Branellec,; Patrice Dene`fle; Nicolas Duverger. Somatic Gene Transfer of Human ApoA-I Inhibits Atherosclerosis Progression in Mouse Models Circulation. 1999; 99: 105-110.

50. Michelle C. Francis, Jiri J. Frohlich. Coronary artery disease in patients at low risk — apolipoprotein AI as an independent risk factor. Atherosclerosis 155 (2001) 165 170
51. Sandhi Maria Barreto, Valéria Maria Azeredo Passos, Alisson Renê Aquino Cardoso, Maria Fernanda Lima-Costa. Quantifying the Risk of Coronary Artery Disease in a Community. The Bambuí Project. Arq Bras Cardiol, volume 81 (nº 6), 556-61, 2003
52. Kannel WB, Wilson PW. Comparison of risk profiles for cardiovascular events: implications for prevention. Arc Intern Med 1997;42:39-66.
53. Mendelsohn ME, Karas RH. The protective effects of oestrogen on the cardiovascularsystem. N Engl J Med 1999;340:1801-11.
54. Cortigiani L, Paolini EA, Nannini E. Dipyridamole stress echocardiography for risk stratification in hypertensive patients with chest pain. Circulation. 1998;98:2855 2859.
55. Thomas H. Marwick, Colin Case, Stephen Sawada, Charles Vasey, James D. Thomas. Prediction of Outcomes in Hypertensive Patients With Suspected Coronary Disease. Hypertension. 2002;39:1113-1118.
56. Langlois MR, Delanghe JR, Biological and clinical significance of Haptoglobin polymorphism in humans. Clin Chem. 1996;42:1589-1600
57. Elagib AA, Kider Ao, Akerstrom B, Elbashir MI. Association of the Haptoglobin phenotype (1-1) with falciparum malaria in Sudan. Trans R Soc Trop Med Hyg. 1998 May-Jun;92(3):309-11.
58. Zhao H, Zhang G, Duan Y, Yu S. Haptoglobin types in Chinese ethnic groups. Hum Hered 1993;43:131-3.
59. Schultze HE, Heremans JF, eds. Molecular biology of human proteins, Vol 1: nature and metabolism of extracllular proteins. Amsterdam: Elservier, 1966:384-402.

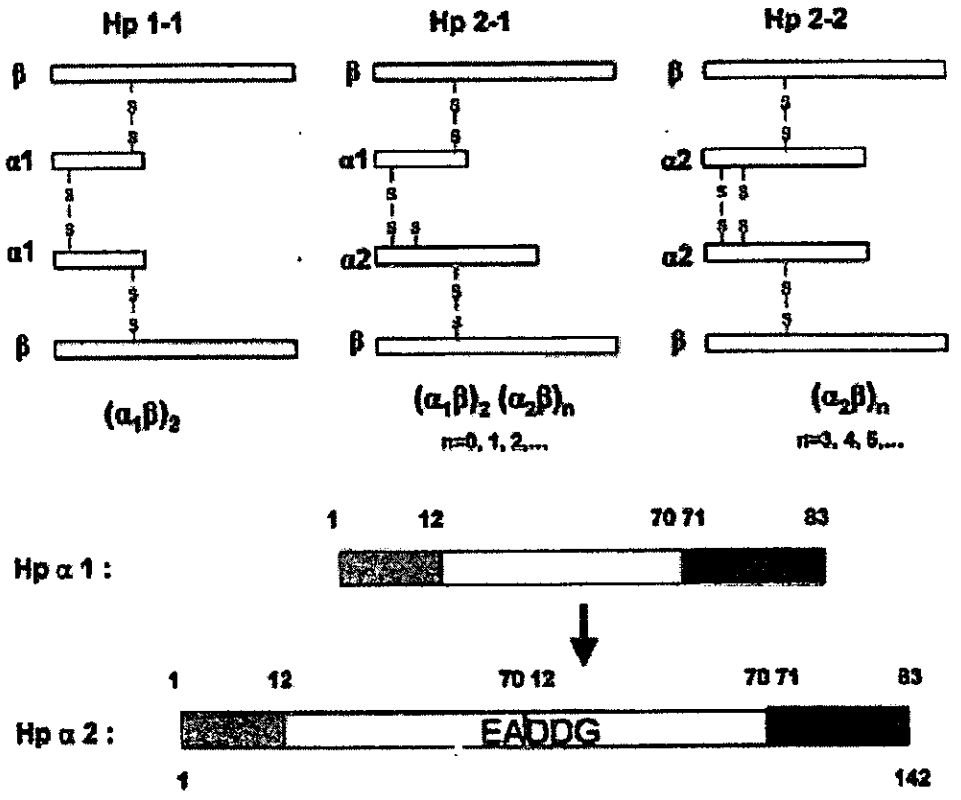

Fig. 1. Schematic drawing of the phenotype structure of human Hp 1-1, 2-1, and 2-2. All three phenotypes share a common structure of β chains. The degree of polymerization within the inter-molecular arrangement is shown. The difference between α1 and α2 chains and their combinations determine the Hp phenotype (bottom). α2 is a duplicate of α1 with a repeat insert of residues 12 70. Making it simple, α2 (142 amino acids) contains two repeated domains showing a unique sequence of EADDG (residues 69 73) at the splicing site.

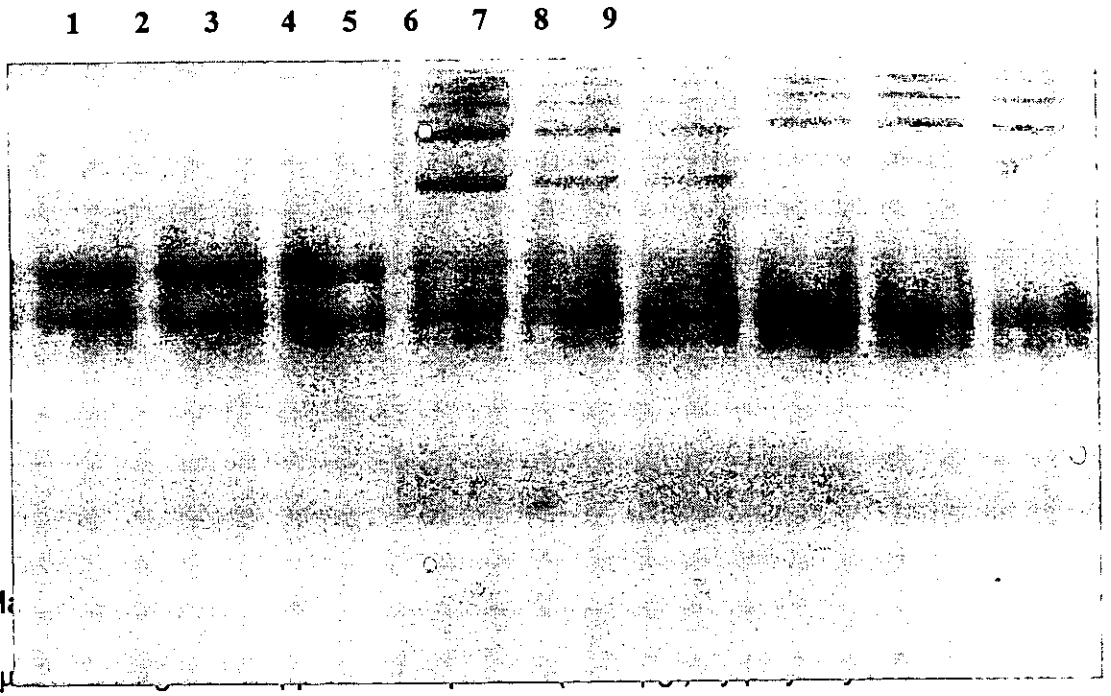

Fig.2 Ha

from 10 μ

gel electrophoresis and peroxidase staining. Lane 1~3, Hp 1-1; Lane 4~6, Hp 2-1; Lane 7~9, Hp 2-2.

TABLE 1. Hp phenotypes distribution of all subjects and subjects with and without coronary artery disease.

	Total	Hp 1-1	Hp 2-1	Hp 2-2	p-value
All subjects, % (n)	100% (1132)	8% (91)	41% (466)	51% (575)	
CAD, % (n)	100% (573)	9% (49)	40% (230)	51% (294)	
Control, % (n)	100% (559)	8% (42)	42% (236)	50% (281)	0.69

*The *p*-value is from a two degree of freedom test comparing all haptoglobin types.

TABLE 1. Hp phenotypes distribution of all subjects and subjects with and without coronary artery disease.

	Total	Hp 1-1	Hp 2-1	Hp 2-2	p-value
All subjects	100% (1132)	8% (91)	41% (466)	51% (575)	
CAD	100% (573)	9% (49)	40% (230)	51% (294)	
Control	100% (559)	8% (42)	42% (236)	50% (281)	0.69

Clinical and biochemical parameters for **overall population**					
Phenotypes	1-1	2-1	2-2	Total	p-value
All subjects					
Age (yrs)	63.4±11.7	63.5±12.1	64.7±11.0	64.4±11.3	0.23
BMI	24.4±3.0	24.5±3.4	25.3±8.2	24.9±6.1	0.15
Total cholesterol	201.9±51.3	202.3±54.6	205.4±60.7	204.1±56.9	0.67
HDL cholesterol	39.0±13.7	38.2±12.5	39.9±17.0	39.7±15.0	0.23
LDL cholesterol	142.7±48.7	139.4±55.8	139.4±59.4	139.6±56.8	0.89
Triglycerides	149.0±84.0	166.8±162.8	165.4±125.3	165.2±149.1	0.55
apoA1*	118.2±35.9	114.5±29.1	116.7±30.5	116.9±31.8	0.48
apoB	92.8±30.8	93.4±31.8	95.6±31.5	95.0±31.6	0.57
Male (%)	73%	81%	81%	81%	0.20
Hypertension (%)	40%	41%	44%	43%	0.64
Diabetes (%)	11%	13%	15%	14%	0.45
Smoke (%)	60%	48%	47%	49%	0.10
Drink (%)	29%	36%	34%	34%	0.54

* The p-value comparing apoA1 between 2-1 and 1-1 is 0.37
The p-value comparing apoA1 between 2-2 and 1-1 is 0.71
The p-value comparing apoA1 between 2-1 and 2-2 is 0.30

Clinical and biochemical parameters according to **diabetes status**					
Phenotypes	1-1	2-1	2-2	Total	p-value
Non-diabetes					
Age (yrs)	63.4±12.0	62.5±12.5	64.1±11.5	63.7±11.7	0.15
BMI	24.2±3.0	24.4±3.4	25.2±8.8	24.7±6.5	0.23
Total cholesterol	200.8±53.1	205.1±56.2	208.5±62.4	206.7±58.3	0.49
HDL cholesterol	39.8±14.2	38.5±12.8	41.2±17.9	40.4±15.6	0.062
LDL cholesterol	142.8±50.8	141.7±57.4	141.2±61.1	141.6±58.1	0.98
Triglycerides	139.5±63.6	166.3±167.4	159.8±127.4	161.4±151.1	0.32
apoA1	119.6±36.0	115.3±29.5	118.2±30.9	118.1±32.3	0.38
apoB	91.9±31.1	94.1±32.9	95.9±32.4	95.5±32.5	0.60
Male (%)	73%	80%	81%	80%	0.29
Hypertension (%)	41%	37%	40%	39%	0.67
CAD (%)	51%	45%	45%	46%	0.66
Smoke (%)	59%	49%	47%	49%	0.15
Drink (%)	32%	35%	34%	35%	0.88
Diabetes					
Age (yrs)	61.3±10.0	69.4±7.8	67.3±7.5	68.3±7.8	0.013
BMI	24.7±3.4	25.6±2.9	26.3±3.5	25.8±3.2	0.38
Total cholesterol	199.1±34.2	185.0±39.7	188.3±47.2	188.4±44.9	0.66
HDL cholesterol	33.6±8.7	36.3±10.4	33.8±8.0	36.0±10.7	0.35
LDL cholesterol	135.4±27.6	122.1±43.0	129.3±45.9	125.7±45.3	0.59
Triglycerides	191.9±144.8	175.8±148.3	198.4±114.0	188.5±143.6	0.61
apoA1	103.4±36.0	107.7±27.0	107.5±28.1	108.1±27.8	0.93
apoB	100.1±31.9	87.8±23.3	95.4±25.8	92.6±25.6	0.28
Male (%)	67%	88%	81	83%	0.23
Hypertension (%)	33%	68%	67%	65%	0.13
CAD (%)	89%	84%	79%	82%	0.69
Smoke (%)	67%	42%	51%	48%	0.35
Drink (%)	0%	42%	36%	36%	0.060

Table1. Clinical and biochemical parameters according to Hp phenotypes

Phenotypes	1-1	2-1	2-2	Total	p-value
Control					
n (%)	42 (7.5%)	236 (42%)	281 (50 %)	559	
Age (yrs)	60.6±12.5	60.1±13.3	62.6±11.4	61.4±12.3	0.074
BMI	24.2±2.8	24.5±3.6	24.9±3.3	24.6±3.4	0.34
Total cholesterol	214.1±55.6	213.8±62.6	214.9±65.8	214.1±63.3	0.98
HDL cholesterol	45.9±15.2	40.1±13.8	43.3±19.1	42.6±16.7	0.062
LDL cholesterol	150.8±53.6	148.3±63.0	144.2±64.9	145.2±62.3	0.73
Triglycerides	156.7±102.7	179.4±200.9	164.6±147.0	173.7±186.8	0.55
apoA1	138.2±35.0	121.4±29.6	124.5±29.7	125.6±32.6	0.024
Male, *n* (%)	26 (62%)	165 (70%)	200 (71%)	391 (70%)	0.50
Hypertension, *n* (%)	12 (29%)	66 (28%)	95 (34%)	173 (31%)	0.39
CAD					
n (%)	49 (8.6%)	230(40%)	294(51%)	557	
Age (yrs)	65.8±10.4	67.0±9.7	66.7±10.1	67.3±9.5	0.74
BMI	24.4±3.2	24.5±3.1	25.7±11.2	25.1±8.0	0.32
Total cholesterol	191.7±45.6	190.8±42.3	196.4±54.1	194.8±48.2	0.42
HDL cholesterol	33.8±9.9	36.3±10.8	36.7±13.9	36.9±12.5	0.37
LDL cholesterol	136.5±44.4	130.1±45.7	134.7±53.3	134.0±50.0	0.56
Triglycerides	142.6±65.1	154.3±112.0	166.2±100.8	157.1±100.9	0.23
apoA1	103.6±29.2	106.8±26.6	108.4±29.2	107.4±28.0	0.60
Male, *n* (%)	41 (83%)	212 (92%)	267 (91%)	506 (91%)	0.11
Hypertension, *n* (%)	25 (50%)	126 (55%)	159 (54%)	300 (54%)	0.84

Data are mean ± SD given in mg/dL

* The p-value comparing apoA1 (control) between 2-1 and 1-1 is 0.0066
The p-value comparing apoA1 (control) between 2-2 and 1-1 is 0.026
The p-value comparing apoA1 (control) between 2-1 and 2-2 is 0.29

TABLE 5. Impact of Hp 1-1 on coronary artery disease adjusted for classical risk factors

	OR for stepwise multivariable adjusted		
*Adjust Risk factors	Hp 1-1/Hp 2-1	Hp 1-1/Hp 2-2	*p*
Non	1.20	1.12	0.69
Age	1.18	1.17	0.78
Age + Gender	1.43	1.40	0.36
Age + Gender + Hypertension	1.49	1.50	0.28
Age + Gender + Hypertension + Apo AI	2.22	2.19	0.044

*** Multivariately adjusted according to conditional logistic regression.**

在 **Diabetes group** 我們沒有得到相同結果
在 **control/CAD** 我們發現 **apoAI, Age, gender, Hypertension** 有 **difference**

For non-diabetes

CAD versus new_Hp_type
OR of having CAD comparing 1-1 and 2-1 = 1.25
OR of having CAD comparing 1-1 and 2-2 = 1.23
OR of having CAD comparing 2-1 and 2-2 = 1.02
p-value for new_Hp_type = 0.66

CAD versus new_Hp_type, adjusting for age
OR of having CAD comparing 1-1 and 2-1 = 1.22
OR of having CAD comparing 1-1 and 2-2 = 1.26
OR of having CAD comparing 2-1 and 2-2 = 0.97
p-value for new_Hp_type = 0.67

CAD versus new_Hp_type, adjusting for age and sex

OR of having CAD comparing 1-1 and 2-1 = 1.39
OR of having CAD comparing 1-1 and 2-2 = 1.42
OR of having CAD comparing 2-1 and 2-2 = 0.98
p-value for new_Hp_type = 0.41

CAD versus new_Hp_type, adjusting for age, sex and hypertension
OR of having CAD comparing 1-1 and 2-1 = 1.37
OR of having CAD comparing 1-1 and 2-2 = 1.43
OR of having CAD comparing 2-1 and 2-2 = 0.96
p-value for new_Hp_type = 0.43

CAD versus new_Hp_type, adjusting for age, sex, hypertension and apoA1
OR of having CAD comparing 1-1 and 2-1 = 2.04
OR of having CAD comparing 1-1 and 2-2 = 1.97
OR of having CAD comparing 2-1 and 2-2 = 1.04
p-value for new_Hp_type = 0.12

For diabetes

CAD versus new_Hp_type
OR of having CAD comparing 1-1 and 2-1 = 1.50
OR of having CAD comparing 1-1 and 2-2 = 2.12
OR of having CAD comparing 2-1 and 2-2 = 0.71
p-value for new_Hp_type = 0.61

CAD versus new_Hp_type, adjusting for age
OR of having CAD comparing 1-1 and 2-1 = 2.48
OR of having CAD comparing 1-1 and 2-2 = 3.17
OR of having CAD comparing 2-1 and 2-2 = 0.78
p-value for new_Hp_type = 0.49

CAD versus new_Hp_type, adjusting for age and sex
OR of having CAD comparing 1-1 and 2-1 = 3.21
OR of having CAD comparing 1-1 and 2-2 = 3.80
OR of having CAD comparing 2-1 and 2-2 = 0.84
p-value for new_Hp_type = 0.44

CAD versus new_Hp_type, adjusting for age, sex and hypertension
OR of having CAD comparing 1-1 and 2-1 = 3.13
OR of having CAD comparing 1-1 and 2-2 = 3.65
OR of having CAD comparing 2-1 and 2-2 = 0.86
p-value for new_Hp_type = 0.48

CAD versus new_Hp_type, adjusting for age, sex, hypertension and apoA1
OR of having CAD comparing 1-1 and 2-1 = 2.90
OR of having CAD comparing 1-1 and 2-2 = 4.82
OR of having CAD comparing 2-1 and 2-2 = 0.60
p-value for new_Hp_type = 0.34

● **Table 3. Geographical distribution of Hp phenotypes. (% of population)**

Population	Hp 1-1	Hp 2-1	Hp 2-2	Ref.
Africa	31	38	18	26
Kenya	27.5	59.5	13.6	27
Black Zimbabwean	29.1	39.6	22.3	28
Ghana	21.5	27.9	7.6	29
Burundi	28	48	20	30
Liberia	48.7	42.3	9.1	31
Europe				
Northwestern Europe	15.7	47.8	36.5	32
Northwestern Italian	17.0	51.3	38.5	34
Russian	20.4	50.0	29.6	35
Ukraine	12.7	48.1	36.5	53
Kazakh	18.1	49.3	32.6	35
Caucasians	15.9	48.2	35.9	53
Belgium	13.0	53.0	34.0	36
France	15.3	49.7	35.0	30
Germany	14.0	48.0	38.0	38
Sweden	13.5	47.5	39.0	39
Hungary	12.6	47.2	40.2	40
UK	10.1	55.5	31.7	31
Gypsies	0.0~5.3	21.2~32.2	65.5~77.9	33
Asia				
China	9.4	35.5	55.4	57
Taiwan	7.5	44.5	48.0	*
Korea	7.5	47.2	43.9	42
Mongolian	7.5	35.5	54.3	42
Japan	7.6	46.5	45.9	43
Iran	8.2	40.8	51.3	45
Jordanian	8.6	41.6	49.8	50
Bengal	2.6	26.8	70.6	44
India Madras	1.9	21.3	76.3	43
South Yemen	14.7	41.4	44.0	47
America				
Nebraska	17.6	51.6	30.2	46
U.S. Hutterites	20.1	51.2	23.3	43
U.S.Tecumseh	17.7	48.7	33.4	43
Guatemala	33.4	49.6	15.6	48
Chile (Mapucho Indians)	56.0	33.6	10.3	49
Chile (Pehuenche Indians)	62.8	34.5	2.7	49
Pacific				
New Guinea	61.3	31.5	6.45	43
Australia (North Queensland)	2.0	31.7	66.3	51
Australia (Bushmen)	10.6	35.4	52.2	19

*From the present study

Molecular properties of human plasma haptoglobin as probed by monoclonal antibodies

Yuan Ting Yang, Siew Mumn Lai, Chi Feng Tseng, Chi Fang Wu, and Simon J. T. Mao

Research Institute of Biochemical Engineering, Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan, ROC

*Correspondence to:

Simon J. T. Mao, Ph.D.

Professor and Director

Research Institute of Biochemical Engineering

Department of Biological Science and Technology

National Chiao Tung University

75 Po-Ai Street

Hsinchu, Taiwan, ROC

Phone: 886-3-571-2121 ext. 56934

FAX: 886-3-572-9288

E-mail: mao1010@ms7.hinet.net

Human plasma haptoglobin (Hp) is known as an acute phase protein, its plasma level elevates in response to inflection or inflammation. One of the key functions of Hp is to capture the hemoglobin by forming a high-affinity Hp-hemoglobin complex. Similar to blood types, there are three major Hp phenotypes 1-1, 2-1, and 2-2 in human plasma attributed by two common alleles (1 and 2). Clinically, patients with polymeric form of Hp 2-1 or 2-2 are associated with the complications of myocardial infarction, kidney failure, and diabetics. Presumably, this was due to the marked complicated structure of Hp 2-1 and 2-2 as the extra-disulfide bond forms heterogeneous polymers, in which some of the biologically functional groups are not fully expressed on the surface. Owing to the surface availability that might be attenuated in polymeric Hp 2-1 and 2-2, we tested the hypothesis whether or not the antigenic domains located at the Hp 1-1 surface were more susceptible than that of Hp 2-1 and 2-2. Monoclonal antibodies (mAb) against human Hp were prepared to address this possibility. From 6 mAb produced, one of them (W1) was specific to Hp α-chains, while the others were to β-chain. Unlike to the other mAb, one β-chain mAb (G2D) possessed the immunoreactivity that was not largely affected by the blockage of disulfide linkages (carboxymethyl modification on cysteine) and trypsin treatment of Hp. The immunoreactivity of Hp 1-1 against all the 6 mAb were significantly higher than that of 2-1 and 2-2. However, the immunoreactivity of disulfide-blocked Hp among the phenotypes was identical. Thus, the data suggest that the surface areas of polymeric forms of Hp 2-1 and 2-1 are not fully exposed.
Supported by the grant NHRI-EX92-9229SI (S.J.T.M.)

Introduction

Hemoglobin is the most abundant and functionally important protein in erythrocytes and blood. However, once released from red blood cells, it becomes highly toxic because of the oxidative properties of iron-containing heme, which participates in the Fenton reaction to produce reactive oxygen species causing cell injury (mao thrombosis paper and 3, from Tolosano paper).

On the other hand, human haptoglobin (Hp) is known as an acute phase protein, and its plasma level elevates in response to inflection or inflammation [1-3]. One of the key functions of Hp is to capture the hemoglobin by forming a high-affinity Hp-hemoglobin complex (1, 2 of Tolosano paper). The complex is then metabolized through receptor-mediated process (3-8 of Kristiansen of Nature paper) including a recent report showing the CD 163 receptor of macrophages (Kristiansen).

Similar to blood types, there are three major Hp phenotypes 1-1, 2-1, and 2-2 in human plasma attributed by two common alleles (1 and 2). Structurally, Hp is "tetrameric" or $(\alpha\beta)_2$ joined by disulfide linkages among the 2 α and 2 β chains [4-6]. Fig. 1 shows that all the phenotypes share the same 2 β chains (each with about Mr 40,000 kDa containing 243 amino acids and approximately 30% carbohydrate). A typical structure of homozygous Hp 1-1 is composed of two identical α1 chains (each with about Mr 9,000 kDa containing 83 amino acids). Homozygous Hp 2-2 is composed of two identical α2 chains (each with about Mr 16,500 kDa containing 142 amino acids) as compared to that of heterozygous Hp 2-1 containing one each of α2 and α1 (Fig. 1). Likewise, the tetrameric arrangement is also found in other animal species such as rat, rabbit, and pig [7-12]. However the 2 identical αβ units (Hp 1-1),

joined by a non-covalent interaction rather than a disulfide bridge, are found in dog, cat, and bear [13-14].

Clinically, patients with polymeric form of Hp 2-1 or 2-2 is associated with the complications of myocardial infarction [15], kidney failure [16], and diabetics [17]. Presumably, this was due to the marked complicated structure of Hp 2-1 and 2-2 as the extra-disulfide bond forms heterogeneous polymers, in which some of the biologically functional groups are not fully expressed on the surface. The assumption, however, has not been tested extensively because the structural and functional studies are hampered by lack of a straightforward isolation procedure in preparing sufficient Hp phenotypes. Recently, we have developed an easy and simple antibody-affinity column technique for the purification of Hp 1-1, 2-1, and 2-2, respectively (). The technique allows us to conduct some structural and functional relationship studies on Hp. Owing to the surface availability might be attenuated in polymeric Hp 2-1 and 2-2, we tested the hypothesis whether or not the antigenic domains located at the Hp 1-1 surface were more susceptible than that of Hp 2-1 and 2-2 in the present study. For this reason, monoclonal antibodies (mAb) against human Hp were prepared to address this possibility. From 6 mAb produced, one of them (W1) was specific to Hp α-chains, while the others were to β-chain. One β-chain mAb (G2D) possessed the immunoreactivity that was not largely affected by the blockage of disulfide linkages (carboxymethyl modification on cysteine) and trypsin treatment of Hp. The binding affinity of all the 6 mAb exhibited significantly higher to Hp 1-1 than that to 2-1 and 2-2. The immunochemical structure of Hp as probed by mAb and its clinical implication are discussed.

Material and Methods

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

Hp were purified using an antibody affinity-column according to the standard procedures recently described by us ( ). Briefly, mAb was first coupled to CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's procedures. Approximately 3 g of freeze-dried Sepharose (1 g of freeze-dried powder gave about 3.5 ml final volume of gel) were swollen and suspended in 1 mM HCl and immediately washed with 20 x volume of the same solution within 15 min on a sintered glass filter [24-26]. The gel was then washed with coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3, and degassed. About 10 ml (18.7 mg/ml) of ammonium-sulfate fraction of mAb in coupling buffer were slowly added into the gel (in 15 ml), while gently stirring by a magnetic bar for 1 h at room temperature. After coupling, the gel was washed with 10 x volume of PBS to remove unbound materials via a sintered glass filter. The gel was then treated with a blocking solution containing 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0, for 2 h at room temperature to saturate the remaining reactive-sites. The degassed gel was then washed with 3 cycles of blocking buffer and a 0.15 M NaCl solution of pH 11.0 (adjusted by ammonium) according to the procedures previously described by us [26]. Finally, the gel was equilibrated in PBS and packed onto a 1.0 x 10 cm column. Filtered human plasma of each Hp-phenotype batch was loaded onto the antibody affinity-column (10 ml in bed volume) at room temperature. The column was then washed with 50 ml of PBS. 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 with pH 11 adjusted by ammonium [26]. Each eluted fraction was collected in a tube containing 0.25 ml of 1

M Tris-HCl buffer, pH 6.8, to immediately neutralize the pH value. Pooled fractions containing Hp were then concentrated using an Amicon centrifugal filter (Millipore), and loaded onto a HPLC Superose 12 column (1 x 30 cm) (Pharmacia) pre-equilibrated with 50 mM ammonium bicarbonate. The HPLC was run at a flow rate of 0.5 ml/min (). Fractions containing Hp were pooled and directly lyophilized followed by storage at –80℃ until analyzing.

Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis (SDS-PAGE) was performed according to the Laemmli's method [27] with some modification in using 5% polyacrylamide on the stacking gel as previously described [7]. Samples (typically 5 μg) for SDS-PAGE were preheated at 100 ℃ for 10 minutes in a loading buffer [12 mM Tris-HCl, pH 6.8, 0.4% (w/v) SDS, 5% glycerol, 0.02% bromphenol blue] containing 2.88 mM 2-mercaptoethanol as a reducing reagent. For molecular weight calibration, a subset of the following standards was included in each gel: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). The samples were run for about 1.5 h at 100 V and stained using Coomassie brilliant blue R-250.

Production of Hp mAb

Hp 2-1, containing all the α 1 and α 2 and β chains, was chosen as an antigen for immunizing BALB/C mice. Hp (50 ug) in 0.4 ml of 50% complete Freund's adjuvant was injected intradermally and intraperitoneally into the animals. The mice were boosted by intraperitoneal injection with 25 ug of antigen in adjuvant at day 14. A

final injection with 25 ug of antigen without adjuvant was given at day 21. Mice were sacrificed on the day 24 for the fusion. Somatic cell fusion using FO cell line was performed in a manner similar to that described previously by us (). Briefly, primary cultures seeded in 96-well microtiter plates were screened for the presence of IgG antibodies using an ELISA with goat anti-mouse IgG as a secondary antibody. The positive clones recognizing Hp were expanded and the culture media giving the high immunoreactivity on an ELISA were tested for their ability to recognize Hp on a Western blot. After limiting dilution and subcloning, 6 clones (derived from the different primary culture wells), designated as 2H3, G2D, 12B1, 8B1, 4A2, and W1 were established and used in this study.

Purification of Hp monoclonal antibodies

Tissue culture medium containing the anti-Hp antibody was first precipitated by 50% of saturated ammonia sulfate (SAS). The SAS fraction was concentrated by 10 x and then dialyzed extensively in a 0.02 M Tris-HCl buffer, pH 8.5. About 20ml of dialyzed fraction was then loaded onto a DEAE column (1 x 20 cm) pre-equilibrated with 0.02 M Tris-HCl, pH 8.5, followed by an elution with the same buffer containing 0.02 M NaCl. Fractions corresponding to IgG were pooled, desalted, and lyophilized.

Western blot analysis

Each mAb was initially characterized using whole human plasma (Hp 2-1) for the identification of α or β chain specificity and to role out its possible cross-reactivity against other plasma proteins on a Western blot. Following the separation on SDS-PAGE, the gel and was then electrotransferred to a nitrocellulose membrane (Pharmacia) at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad) containing a

transfer buffer. The transferred membrane was then immersed in 5% skim milk in PBS for 1 hour at room temperature, while shaking gently. After 3 x washing with PBS for 5 min, the membrane was incubated with mouse mAb against human Hp in PBS buffer containing 1% (w/v) skim milk and 0.05% Tween-20 for 1 hour at room temperature and washed 3 x for 5 min. The membrane was then incubated with 1:10,000 diluted goat anti-mouse IgG conjugated with horseradish peroxidase in the same buffer for 1 h. In addition, the membrane was washed 2 x with washing buffer and further washed 1 x with PBS. Finally, the membrane was developed using 3,3 - diaminobenzidine (DAB) as a substrate for horseradish peroxidase [7, 25].

Enzyme linked immunosorbent assay

Briefly, approximate 1 ug of Hp 2-1 in 50 uL of PBS was coated on a 96-well microtiter plate (Nunc, Roskilde, Denmark) for screening against of hybridoma culture media or Hp mAb to be tested using the method previously established in our laboratory (). Unbound proteins were washed with PBS 1 x and subsequently blocked by an addition of 350 uL of PBS containig 1% skim milk (v/v) (PBSS) (21). Following 2 x washes with PBSS, 50 uL of hybridoma culture medium (2 weeks following the fusion) or Hp mAb were added and incubated at room temperature for 60-90 min. Each well was washed 2 x with PBSS containing 0.05% Tween-20. Bound antibodies were detected using a goat anti-mouse IgG conjugated with horseradish peroxidase for 60 min in PBSS. Finally, each well was washed and developed with 0.04% 2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) containing 0.01% H_2O_2 in PBS.

Determination of Binding affinity of mAb between Hp 1-1, 2-1, and 2-2

The binding affinity (Ka) was determined using an ELISA according to the method previously described by Beatly et al (). In brief, the determination was based upon the Law and Mass Action and using serial dilution of both Hp (coating the plate) and purified Ig G fraction of mAb. After the incubation, the plate was sequentially incubated with a second peroxidase-labelled anti-mouse IgG. The amount of mAb bound to Hp on the plate was measured by optical density (). The final Ka was measured according to the method similar to our previous reports (Mao et, clinical chem, and Kreistanasky and mao, Febs letter).

Reduction and carboxylmethylation of Hp 1-1 and 2-1

The reaction was conducted according to the procedures previously reported by Mao et al (BBA). To 5 mg Hp were added 4 ml of 0.1 M Tris-HCl buffer (pH 8.6), 5 M urea, 0.02 M dithioerythritol. The reaction mixture was flushed with nitrogen and incubated at 23 C. After 2h, 10 mg iodoacetic acid were added and the reaction mixture was maintained at pH 8.6 by the addition of 1M NaOH. After 30 min, carboxymethylated Hp was dialyzed against 0.05 M ammonium bicarbonate and lyophlized

Other methods

Trypsin treatment of Hp was conducted using 4 μg of Hp 2-1, in which 0.2 ul of trypsin (2 mg/ml) were added and incubated at 37℃ for 12 hours. The reaction mixture was immediately applied into 15% SDS-PAGE followed by a Western blot analysis.

For the determination of circular dichroic spectra, the lyophilized Hp 1-1, 2-1, or 2-2 was dissolved in 10 mM phosphate buffer at pH 7.4 with a final concentration of 0.2 mg/ml. About 300 μl of Hp solution was used to analyze within a cuvette of 1-mm path length. Circular dichroic spectrum was conducted between 190 and 300 nm in a Jasco J-715 spectropolarimetry. The obtained spectrum of each type of Hp was accumulated for 20 times at a scanning rate of 50 nm/min and the % α-helical content was estimated from the mean residue molar elliplicity (θ_{222}). % α-helix = [(θ_{222} + 3000)/(36000+3000)] X 100 [28].

Results

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

Each Hp phenotype of human plasma was first identified and then subjected to purification by an antibody-affinity column followed by a HPLC Superose 12 (). As shown in Fig. 2, the homogeneity of each phenotype in the presence of reducing reagent with greater than 95% on SDS-PAGE. Meanwhile, the presence of multimeric forms in Hp 2-1 or 2-2 in the absence of reducing reagent is revealed (Fig. 2)

Production of Hp monoclonal antibodies and its purification

Hp 2-1 was chosen as antigen for immunizing mice due to its subunits (β, α 1, and α 2) are commonly shared in either Hp 1-1 or 2-2 (Fig 1). There were about 20 positive clones reacting with Hp in initial screenings from 594 hybridomas. Following the selection (high titers on ELISA and positive on Western blot), limiting dilution, and subcloning; 6 monoclonals against Hp were established. To avoid each mAb derived from the culture medium might contain hemoglobin, each mAb was then purified on a

DEAE-column. Notably, the purity of each mAb was greater than 90% (Fig. 3). Subtyping of each mAb using an ELISA indicated that they were all IgG1 subclass (Table 1).

Specificity and characterization of the mAb

Specificity of each mAb was evaluated on Western blot using whole human plasma or purified Hp 2-1. Fig. 4 shows that 5 of the mAb (clones 2H3, G2D, 12B1, 8B1, and 4A2) were specific to the Hp β subunit, while only one (clone W1) recognized α subunits α 1 and α 2 (Table 1). Because α 2 represents a repeat domain of α 1, clone W1 thus reacted with both. It also indicates that clone W1 recognized an epitope between residues 1-83 of α 1 chain. None of the mAb reacted with human plasma proteins other than Hp (Fig. 4). To further explore the epitope specificity, Hp was treated with trypsin in a limited digestion. Only mAb G2D and W1 were still able to react with trypsinized fragments (Fig. 5). Others were sensitive for the treatment. The limited treatment seems not to alter the α chain in great extent, since both immunoreactivity and molecular size were retained as assessed by mAb W1 (Fig. 5).

Binding affinity of mAb to Hp 1-1, 2-1, and 2-2

In the next experiment using our mAb as a probe, we tested hypothesis whether or not the surface structure of Hp 2-1 and 2-2 is different from that of Hp 1-1 due to their complicated polymeric forms. We determined the binding affinity (Ka) of all the mAb against each respective Hp 1-1, 2-1, and 2-2 using an non-competitive ELISA previously established by Beaty et al (). Typical examples using mAb G2D and 12B1 with respective to the binding of Hp 1-1, 2-1 and 2-2 are shown in Fig. 6. The Ka values of all the mAb to each Hp phenotype are given in Table 2. The binding

affinity of all the mAb directed against Hp 1-1 was significantly higher (between 2-4 folds) than that against Hp 2-1 and 2-2. Thus, the data suggest that Hp 1-1 was more susceptible for the antibody binding than Hp 2-1 and 2-2.

Chemical modification of cysteine groups on Hp

Since the extra-cysteines (refer to Fig. 1) are responsible for the polymerization of Hp 2-1 or 2-2, we than tested whether or not the blockage of disulfide linkages might equalize the immunoreactivity between Hp 1-1 and 2-2 (the most distinct phenotypes) using a competitive ELISA. First, Hp 2-2 were irreversibly modified by reduced carboxymethylation in the presence of 5 M urea () to block all the cross linkages between the α- and β-chains as well as the linkages within the Hp molecules. SDS-PAGE analysis without the addition of reducing reagent (mercaptoethanol) demonstrated that α- and β-chains were completely separated and migrated to each expected molecular weight of α and β chains, respectively, as that shown previously (Bills paper). Chemical reagent _____() also demonstrated that essentially there were no free thio groups left over the modified cysteines. Because the overall structure of Hp underwent drastic changes upon the disruption of disulfide linking (even within each monomer), the antibodies (2H3, 12B1, 8B1, 4A2, and W1) could not recognize chemically modified Hp as shown in Fig. 7. Fortunately, mAb G2D was still able to reacted with reduced Hp 2-2 (Fig. 7). In the next experiment, we show that immunoreactivity of carboxymethylated Hp 2-2 was almost completely identical to that carboxymethylated Hp 1-1 in a dose-dependent manner (Fig. 7). It indicates that the immunoreactivity (assessed by mAb G2D) between Hp 1-1 and 2-2 was identical when they were chemically reduced. Thus, the data are consistent with

our notion that the surface availability of Hp 2-2 are attenuated by its polymerization, in which disulfide-linkages are responsible.

Discussion

Purification of human Hp has been hampered for years due to its structural diversity [7, 18-21]. In the present study, plasma without any additional manipulations (e.g., ammonium-sulfate precipitation) was utilized for Hp isolation. The solution property of each Hp phenotype on Superose column was consistent with its molecular forms, of which Hp 1-1 was more homogeneous than the other two species in size (). Using 6% SDS-PAGE without reducing reagent, Fig. 2 revealed the monermeric nature of Hp 1-1; and confirmed that the isolated Hp 2-1 was heterogeneous containing Hp monomer and dimer, while the polymeric Hp 2-2 contained neither.

Since Hp 1-1 represents a monomeric unit of Hp (β-α-α-β), we speculated that the molecular hindrance for the "functional surface" is minimal and the antigenic epitopes might be more accessible for the antibody binding than that of polymeric Hp 2-1 or 2-2. To test this hypothesis, we determined the immunoreactivity of each phenotype of Hp. Fig. 6 and Table 2 demonstrated that the immunoreactivity of Hp 1-1 tested against all the mAb was indeed significantly greater than that of Hp 2-1 and 2-2. Thus, it proved the evidence that the antigenic domains located at monomeric Hp were well expressed and the immunoreactivity of Hp 1-1 was in general differentially higher than that of Hp 2-1 and 2-2. The immunoreactivity, however, was indistinguishable between the reduced Hp 1-1 and reduced 2-1, when using mAb G2D (Fig. 7). It suggests that disulfide linkages play an essential role in molecular hindrance and overall conformation of Hp. To test this hypothesis, we characterized the secondary structure of each Hp phenotype by determining the conformation of Hp

by CD (Fig. 8A). The estimated α-helical content was about 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively. On the other hand, the UV spectra among the phenotypes were indistinguishable (Fig. 8B). Statistically, the α-helical content in Hp 1-1 was significantly higher than that in Hp 2-1 and 2-2 ($P < 0.001$). The Hp 1-1 gave a more ordered structure than that of Hp 2-1 and 2-2. In addition, we show that the ordered structure was markedly abolished when the disulfide linkages were truncated (Fig. 8). It also explains why all the mAb (excepted G2D) could not recognize chemically reduced Hp. On the contrary, G2D antibody seemed to be "sequence dependent" since it resisted to both carboxylmethylation (Fig. 7) and trypsin treatment (Fig. 4) on Hp.

Taking together, we identified that the immunoreactivity of Hp 1-1 was greater than that of Hp 2-1 and 2-2 suggesting that the availability of "functional surfaces" of Hp 1-1 may be greater as compared to Hp 2-1 and 2-2. The circular dichroic spectra also revealed a significant overall difference in structure between Hp 1-1 and Hp 2-1 or 2-2, although not much difference was found between Hp 2-1 and 2-2. Such structural diversity may explain, in part, the clinical outcome by which Hp phenotype is associated with differential susceptibility to infections, atherosclerosis, and autoimmune disorders [3, 29]. These effects are correlated with a phenotype-dependent modulation of oxidative stress and prostaglandin synthesis. In general, patients with Hp 2-2 are more susceptible in developing the severity of the diseases mentioned above. For example, Hp 2-2 and 2-1 are associated with an increased risk for the development of nephropathy in patients with diabetes mellitus. Nakhoul et al (2001, Diabetologia) have postulated that the differences in the molecular shape and size between the Hp 1-1 and 2-2 are involved. Melamed-Frank et al (Blood, 2001) further demonstrate that the antioxidant effect of Hp 1-1 is superior to Hp 2-1 and 2-2

in hemoglobin-induced LDL oxidation by a mechanism of higher biniding affinity between Hp 1-1 to hemoglobin. The present study provides an additional evidence that the epitopes of Hp 1-1 were even more accessible for the antibody recognition than the other phenotypes. Nevertheless, identification and understanding of the biochemical basis for the differences among Hp phenotypes may lead to a rational design in intervening new pharmacological agents, such as mini-Hp, which have been recently proposed [30 and Tolosano 2002, Blood].

Finally, our data indicate that the immunoreactivity of each type of Hp was diverse and heterogeneous depending on the epitope specificity of the molecular orientation of Hp. Thus, it is somewhat difficult to accurately determine the concentrations of Hp in human plasma samples using immunoassays. In light of the recent clinical studies indicating that human plasma concentration of Hp 1-1 is significantly and differentially higher than polymeric forms of Hp 2-1 and 2-2 using immunoassays (). Whether or not these were due to the high immunoreactivity of Hp 1-1 remains to be clarified. If this was the case, the phenotype of individual's plasma should be first classified prior to the determination of Hp concentration, and used each respective Hp phenotype as a calibration curve to correct the true values. We are currently in progress to address this possibility.

Acknowledgements

This work was supported by the grant NHRI-EX92-9229SI (S.J.T.M.) from National Health Research Institute, Taiwan, ROC.

References

拾、九十三年度計畫執行情形

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於: ■子計畫； 或 □總計畫(請自行整合)

一、請簡述原計畫書中，九十三年預計達成之研究內容

Specific aims:

1. Develop a new and fast approach for human Hp phenotping (Hp 1-1, 2-2, and 2-1) using monoclonal antibodies, and to test the hypothesis whether or not Hp phenotypes are correlated to the patients with coronary artery disease in Taiwan (2003-2005).

2. Study the Hp mRNA expression in macrophages and SMC in the presence of oxidized LDL and lipoproteins VLDL, LDL, and HDL (2003-2004).

3. Test the hypothesis that Hp may exert an antioxidant role in macrophages and SMC transfected with sense and antisense Hp cDNA; to define the antioxidant activity of Hp phenotypes *in vitro* (2003-2004).

4. Investigate the functional role of Hp in the progression of atherosclerosis using cholesterol-fed rabbits and antiatherogenic effect (if any) using Hp transgenic mice (2004-2005).

GENTT CHART (TIME-TABLE) FOR PROPOSED PLAN

Task Force and Experiments	Year 1 (2003) Half		Year 2 (2004) Half		Year 3 (2005) Half	
AIM 1:						
Determination of human Hp phenotypes:						
Monoclonal antibody production						
Immunoassay using ELISA for phenotyping						
Immunoassay using immunogold for phenotyping						
Using Protein/Antibody chips for Hp phenotyping						
Correlation of plasma Hp phenotypes to the domestic patients with atherosclerosis:						
Patients selection						
Hypothesis to be tested and statistics						
AIM 2:						
Macrophage experiment using oxidized LDL						
Macrophage experiment using VLDL and HDL						
SMC experiment						
AIM 3						
Human Hp cDNA cloning and plasmid construct						
Cloning of green fluorescent protein (GFPemd) as the reporter gene						
Cloning of Hp1-1 gene at sense and antisense orientation						
Antioxidant role experiments						
Transfection experiment (ex vivo)						
AIM 4						
Evaluation of atherosclerosis in rabbit model						
Transgenic Hp mouse model						
Evaluation of atherosclerosis in transgenic model						
Publications						
Possible Patent Application						

二、 請詳述九十三年度計畫執行情形，並評估是否已達到原預期目標（請註明達成率）

Aim 1: (2003-2005) 達成率：90%

We have successfully established a rapid diagnostic method on Hp phenotyping without performing such a complicated, traditional starch gel. Human plasma was mixed with hemoglobin (Hb) first. By such a high affinity between Hp and Hb, they will form a complex. A native PAGE was subsequently preformed, and then the gel was immersed in 0.05% H2O2 solution containing diaminobenzidine which was a chromogenic substrate. By the pseudo-peroxidase activity from Hb, the pattern of Hp-Hb complex would be represented. Therefore, the phenotype of Hp will be easily determined. In addition, polyclonal and monoclonal antibodies against Hp have also been produced. And two Hp purification methods have also been established and published in well peer-reviewed journals. As regard to the relationship between Hp phenotype and coronary artery disease, we are in collaboration with Cardiology Division of Veterans General Hospital-Taipei. About 1,200 subjects have been investigated with the statistics analyzed. The manuscript will be submitted by the end of 2004.

Aim 2: (2003-2004) 達成率：90%

With respect to study on the Hp mRNA expression in macrophages and SMC in the presence of oxidized LDL and lipoproteins VLDL, LDL, and HDL, the results were under analyzed. Moreover, in previous reports, people belonging Hp 1-1 expresses Hp allele 1 mRNA only; Hp 2-2 expresses Hp allele 2 mRNA only and both of Hp allele 1 and allele 2 mRNA would be

expressed in people belongs Hp 2-1. However, we found all phenotypes have a truncated Hp allele 1 mRNA because a deletion mutation appeared and resulted in a stop codon. So far, we are confirming whether the truncated mRNA may translate into a protein fragment.

Aim 3: (2003-2004) 達成率：90%

According to our first-year report, we have found either in Cu^{2+}- or AAPH -induced lipid oxidation, Hp exerted a strong *in vitro* antioxidant activity. Its potency was markedly superior to probucol: one of the most potent antioxidants. The IC50 of antioxidant activity in ranking was: Hp 1-1> Hp 2-1> Hp 2-2> probucol> vitamin E. Blockage of disulfide linkages between Hp subunits, not only abolished the α-helical content but also diminished its ability to form a complex with hemoglobin (Hb). It exerted almost 4 x greater antioxidant activity than that of native Hp. To investigate antioxidant role of Hp on the cellular level, the cDNA of Hp 1-1 was cloned, constructed (containing the pcDNA3.0 vector with CMV promoter) and transfected to CHO-K1 cells. Following the transfection, these CHO cells were able to express Hp 1-1 protein and significantly ($P<0.001$) elevated the tolerance against the oxidative stress. The elevation was about twice-higher than that normal CHO cells when challenged with hydrogen peroxide for 24 h. Thus, Hp plays a provocative antioxidant role as demonstrated in our *in vitro* and *ex vivo* studies. In addition, carboxymethylated Hp exerted a higher antioxidant activity than native Hp. This result made us suggesting Hp might possess a antioxidant domain and it might be exposed after such a chemical modification. The detailed results have been published in 2004 *PROTEOMICS*. So far, in the following research progress, we found a small fragment of β subunit also possessed its antioxidant activity. Both synthetic

peptides and recombinant protein of truncated Hp will be to explore its minimal functional domains.

Aim 4: (2004-2005) 達成率：50%

To investigate the functional role of Hp in the progression of atherosclerosis, cholesterol-fed rabbits and transgenic mice are used to evaluate the antiatherogenic effect of Hp. The rabbit experiment has been completed, while the construct for preparing transgenic mice is underway.

附錄(一)　九十三年度計畫著作一覽表

[期刊論文]

1. Liau CY, Chang TM, Pan JP, Chen WL, **Mao SJT.** Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography. *J. Chromatogr. B Analyt. Technol. Biomed Life Sci.* 2003, 790(1-2):209-16 (IF=2.085)

2. Tseng CF, Huang HY, Yang YT, **Mao SJT**. Purification of human haptoglobin 1-1, 2-1, and 2-2 using monoclonal antibody affinity chromatography. *Protein Expr Purif.* 2004 Feb;33(2):265-73 (IF=1.470)

3. Tseng CF, Lin CC, Huang HY, Liu HC, **Mao SJT**. Antioxidant role of human haptoglobin. *Proteomics*. 2004 Aug;4(8):2221-8 (IF=5.766)

4. Wang SS, Yueh CH, Yang MC, **Mao SJT**. Epitope mapping of a haptoglobin monoclonal antibody that blocks the haptoglobin-hemoglobin interaction: Implication of the involvement of residues 105-116 of haptoglobin in hemoglobin binding. **To be submitted**

5. Cheng TM, **Mao SJT.** Association of Haptoglobin 1-1 phenotype with angiographically documented coronary artery disease patients. **To be submitted**

6. Yang YT, Lai SM, Tseng CF, Wu CF, **Mao SJT.** Molecular properties of human plasma haptoglobin as probed by monoclonal antibodies. **To be submitted**

[國外研討會論文]

1. **Mao SJT**, Liau CY, Chang TM, Pan RB, Chen WH. (2003) Purification of human plasma haptoglobin by hemoglobin affinity chromatography. **Experimental Biology 2003 San Diego, USA.** FASEB J. 17:A999.

2. Cheng TM, **Mao SJT.** (2003) Epidemiology of haptoglobin phenotypes in chronic diseases. **Experimental Biology 2003 San Diego, USA.** FASEB J. 17:A1000.

3. Tseng CF, Lin CC, Huang HY, Liu HC, **Mao SJT**. (2003) Antioxidant role of human plasma haptoglobin. **Congress understanding Biological systems through Proteomics, Switzerland.** SPS'03 (p81-83)

4. **Mao SJT**, Yang YT, Lai SM, Wu CF, Chang TM, Tseng CF. (2004) Molecular properties of human haptoglobin as probed by monoclonal antibodies. **Experimental Biology 2004 Washington, DC, USA.** FASEB. 18:A1150.

5. Tseng CF, Huang HY, Yang YT, **Mao SJT**. (2004) Purification of human haptoglobin 1-1, 2-1, and 2-2 using monoclonal antibody affinity chromatography. **Experimental Biology 2004 Washington, DC, USA.** FASEB. 18:A1150.

[國內研討會論文]

1. Cheng TM, Pan JP, Wang JS, Chou SC, **Mao SJT.** (2003) Epidemiology of haptoglobin phenotypes in Taiwan. The eighteenth joint annual conference of biomedical sciences.

2. Yang YT, **Mao SJT.** (2003) Functional studies of haptoglobin in rat smooth muscle cell A-10 and production of monoclonal antibody against human hp2-1. The eighteenth joint annual conference of biomedical sciences.

3. Tseng CF, **Mao SJT.** (2003) Antioxidant activity of acute phase protein haptoglobin. The eighteenth joint annual conference of biomedical sciences. Oral presentation.

4. Wu IJ, Wen SL, Tsai TI, **Mao SJT.** (2003) Development of a rapid one-step immunochromatographic assay to for patients with coronary heart disease using monoclonal antibodies against low-density lipoprotein. The eighteenth joint annual conference of biomedical sciences. Oral presentation.

5. Yueh CC, Liu TY, Tseng CF, Wang YH, Wang SS, **Mao SJT**. (2004) Structure and role of haptoglobin. Identification of its deficient in minipigs. Twelfth Symposium on Recent Advances in Cellular and Molecular Biology, R.O.C.

6. Tseng CF, Lin CC, Huang HY, Liu HC, **Mao SJT**. (2004) Antioxidant role of human haptoglobin. Twelfth Symposium on Recent Advances in Cellular and Molecular Biology, R.O.C.

7. Tsai TY, **Mao SJT**. (2004) Structural-functional studies on the antioxidant activity of haptoglobin. Twelfth Symposium on Recent Advances in Cellular and Molecular Biology, R.O.C.

8. Liu HC, **Mao SJT**. (2004) Haptoglobin inhibited vascular smooth muscle cell proliferation and migration. The nineteenth joint annual conference of biomedical sciences.

9. Wang SS, Liu HC, **Mao SJT**. (2004) Structural and activity relationship between haptoglobin and hemoglobin as probed by monoclonal antibodies. The nineteenth joint annual conference of biomedical sciences.

10. Yueh CC, Liu TY, Tseng CF, Wang YH, Wang SS, **Mao SJT**. (2004) Structure and role of haptoglobin. Identification of its deficient in minipigs. The nineteenth joint annual conference of biomedical sciences.

11. Tsai TY, **Mao SJT**. (2004) Structural-functional studies on the antioxidant activity of haptoglobin. The nineteenth joint annual conference of biomedical sciences. Oral presentation.