SNPs are mostly intronic and did not overlap with our SNP markers (<20%).

For genotype-phenotype association studies on the *ABCB1* gene, genotyping of the major functional key SNPs in Blocks 1 and 2 (Table 6) would be useful. Further studies on the clinical significance of the haplotypes described in the present study and elucidation of the haplotype-combinations across blocks, will be required to achieve the goal of personalized drug therapy.

Conclusions

We re-established *ABCB1* haplotypes in the Japanese population based on novel polymorphisms found in a large number of subjects, expanding the promoter region. Our current data added more detailed information on functionally-important haplotypes in Blocks 1 and 2 in the Japanese population, and identified differences in haplotype profiles between ethnic groups. The information provided in this study will be of use in further studies investigating the relationship between genetic markers and functional changes.

Acknowledgements

This study was supported in part by the Program for the Promotion of Fundamental Studies in Health Sciences and in part by the Program for the Promotion of Studies in Health Sciences of the Ministry of Health, Labor and Welfare of Japan.

References

Chowbay, B., Cumaraswamy, S., Cheung, Y. B., Zhou, Q. & Lee, E. J. (2003) Genetic polymorphisms in MDR1 and CYP3A4 genes in Asians and the influence of MDR1 haplotypes on cyclosporin disposition in heart transplant recipients. *Pharmacogenetics* **13**, 89–95.

Cordon-Cardo, C., O'Brien, J. P., Casals, D., Rittman-Grauer, L., Biedler, J. L., Melamed, M. R. & Bertino, J. R. (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci U S A* **86**, 695–698.

Debry, P., Nash, E. A., Neklason, D. W. & Metherall, J. E. (1997) Role of multidrug resistance P-glycoproteins in cholesterol esterification. *J Biol Chem* **272**, 1026–1031.

Drach, J., Gsur, A., Hamilton, G., Zhao, S., Angerler, J., Fiegl, M., Zojer, N., Raderer, M., Haberl, I., Andreeff, M. & Huber, H. (1996) Involvement of P-glycoprotein in the transmembrane transport of interleukin-2 (IL-2), IL-4, and interferon-gamma in normal human T lymphocytes. *Blood* **88**, 1747–1754.

Fojo, A. T., Ueda, K., Slamon, D. J., Poplack, D. G., Gottesman, M. M. & Pastan, I. (1987) Expression of a multidrug-resistance gene in human tumors and tissues. *Proc Natl Acad Sci USA* **84**, 265–269.

Furuno, T., Landi, M. T., Ceroni, M., Caporaso, N., Bernucci, I., Napp, G., Martignoni, E., Schaeffeler, E., Eichelbaum, M., Schwab, M. & Zanger, U. M. (2002) Expression polymorphism of the blood-brain barrier component P-glycoprotein (MDR1) in relation to Parkinson's disease. *Pharmacogenetics* **12**, 529–534.

Geick, A., Eichelbaum, M. & Burk, O. (2001) Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* **276**, 14581–14587.

Goto, M., Masuda, S., Saito, H., Uemoto, S., Kiuchi, T., Tanaka, K. & Inui, K. (2002) C3435T polymorphism in the MDR1 gene affects the enterocyte expression level of CYP3A4 rather than Pgp in recipients of living-donor liver transplantation. *Pharmacogenetics* **12**, 451–457.

Hinds, D. A., Stuve, L. L., Nilsen, G. B., Halperin, E., Eskin, E., Ballinger, D. G., Frazer, K. A. & Cox, D. R. (2005) Whole-genome patterns of common DNA variation in three human populations. *Science* **307**, 1072–1079.

Hoffmann, U. & Kroemer, H. K. (2004) The ABC transporters MDR1 and MRP2: multiple functions in disposition of xenobiotics and drug resistance. *Drug Metab Rev* **36**, 669–701.

Hoffmeyer, S., Burk, O., von Richter, O., Arnold, H. P., Brockmoller, J., Johne, A., Cascorbi, I., Gerloff, T., Roots, I., Eichelbaum, M. & Brinkmann, U. (2000) Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. *Proc Natl Acad Sci USA* **97**, 3473–3478.

Ieiri, I., Takane, H. & Otsubo, K. (2004) The MDR1 (ABCB1) gene polymorphism and its clinical implications. *Clin Pharmacokinet* **43**, 553–576.

Itoda, M., Saito, Y., Komamura, K., Ueno, K., Kamakura, S., Ozawa, S. & Sawada, J. (2002) Twelve Novel Single Nucleotide Polymorphisms in ABCB1/MDR1 among Japanese Patients with Ventricular Tachycardia who were Administered Amiodarone. *Drug Metab Pharmacokinet* **17**, 566–571.

Johne, A., Kopk, K., Gerloff, T., Mai, I., Rietbrock, S., Meisel, C., Hoffmeyer, S., Kerb, R., Fromm, M. F., Brinkmann, U., Eichelbaum, M., Brockmoller, J., Cascorbi, I. & Roots, I. (2002) Modulation of steady-state kinetics of digoxin by haplotypes of the P-glycoprotein MDR1 gene. *Clin Pharmacol Ther* **72**, 584–594.

Kim, R. B., Leake, B. F., Choo, E. F., Dresser, G. K., Kubba, S. V., Schwarz, U. I., Taylor, A., Xie, H. G., McKinsey, J., Zhou, S., Lan, L. B., Schuetz, J. D., Schuetz, E. G. & Wilkinson, G. R.. (2001) Identification of functionally

variant MDR1 alleles among European Americans and African Americans. *Clin Pharmacol Ther* **70**, 189–199.

Kim, R. B. (2002) MDR1 single nucleotide polymorphisms: multiplicity of haplotypes and functional consequences. *Pharmacogenetics* **12**, 425–427.

Kimchi-Sarfaty, C., Gribar, J. J. & Gottesman, M. M. (2002) Functional characterization of coding polymorphisms in the human MDR1 gene using a vaccinia virus expression system. *Mol Pharmacol* **62**, 1–6.

Kitamura, Y., Moriguchi, M., Kaneko, H., Morisaki, H., Morisaki, T., Toyama, K. & Kamatani, N. (2002) Determination of probability distribution of diplotype configuration (diplotype distribution) for each subject from genotypic data using the EM algorithm. *Ann Hum Genet* **66**, 183–193.

Kurata, Y., Ieiri, I., Kimura, M., Morita, T., Irie, S., Urae, A., Ohdo, S., Ohtani, H., Sawada, Y., Higuchi, S. & Otsubo, K. (2002) Role of human MDR1 gene polymorphism in bioavailability and interaction of digoxin, a substrate of P-glycoprotein. *Clin Pharmacol Ther* **72**, 209–219.

Kroetz, D. L., Pauli-Magnus, C., Hodges, L. M., Huang, C. C., Kawamoto, M., Johns, S. J., Stryke, D., Ferrin, T.E., DeYoung, J., Taylor, T., Carlson, E. J., Herskowitz, I., Giacomin, i K. M. & Clark, A. G. (2003) Pharmacogenetics of Membrane Transporters Investigators. Sequence diversity and haplotype structure in the human ABCB1 (MDR1, multidrug resistance transporter) gene. *Pharmacogenetics* **13**, 481–494.

Liu, Y. Y., Han, T. Y., Giuliano, A. E. & Cabot, M. C. (2001) Ceramide glycosylation potentiates cellular multidrug resistance. *FASEB J* **15**, 719–730.

Morita, N., Yasumori, T. & Nakayama, K. (2003) Human MDR1 polymorphism: G2677T/A and C3435T have no effect on MDR1 transport activities. *Biochem Pharmacol* **65**, 1843–1852

Riordan, J. R., Deuchars, K., Kartner, N., Alon, N., Trent, J. & Ling, V. (1985) Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature* **316**, 817–819.

Sai, K., Kaniwa, N., Itoda, M., Saito, Y., Hasegawa, R., Komamura, K., Ueno, K., Kamakura, S., Kitakaze, M., Shirao, K., Minami, H., Ohtsu, A., Yoshida, T. Saijo, N., Kitamura, Y., Kamatani, N., Ozawa, S. & Sawada, J. (2003) Haplotype analysis of ABCB1/MDR1 blocks in a Japanese population reveals genotype-dependent renal clearance of irinotecan. *Pharmacogenetics* **13**, 741–757.

Schwab, M., Schaeffeler, E., Marx, C., Fromm, M. F., Kaskas, B., Metzler, J., Stange, E., Herfarth, H., Schoelmerich, J., Gregor, M., Walker, S., Cascorbi, I., Roots, I., Brinkmann, U., Zanger, U. M. & Eichelbaum, M. (2003) Association between the C3435T MDR1 gene polymorphism and susceptibility for ulcerative colitis. *Gastroenterology* **124**, 26–33.

Siegsmund, M., Brinkmann, U., Schaffeler, E., Weirich, G., Schwab, M., Eichelbaum, M., Fritz, P., Burk, O., Decker, J., Alken, P., Rothenpieler, U., Kerb, R. & Hoffmeyer, S. (2002) Association of the P-glycoprotein transporter MDR1(C3435T) polymorphism with the susceptibility to renal epithelial tumors. *J Am Soc Nephrol* **13**, 1847–1854.

Takane, H., Kobayashi, D., Hirota, T., Kigawa, J., Terakawa, N., Otsubo, K. & Ieiri, I. (2004) Haplotype-oriented genetic analysis and functional assessment of promoter variants in the MDR1 (ABCB1) gene. *J Pharmacol Exp Ther* **311**, 1179–1187.

Tanabe, M., Ieiri, I., Nagata, N., Inoue, K., Ito, S., Kanamori, Y., Takahashi, M., Kurata, Y., Kigawa, J., Higuchi, S., Terakawa, N. & Otsubo, K. (2001) Expression of P-glycoprotein in human placenta: relation to genetic polymorphism of the multidrug resistance (MDR)-1 gene. *J Pharmacol Exp Ther* **297**, 1137–1143.

Tang, K., Ngoi, S. M., Gwee, P. C., Chua, J. M., Lee, E. J., Chong, S. S. & Lee, C. G. (2002) Distinct haplotype profiles and strong linkage disequilibrium at the MDR1 multidrug transporter gene locus in three ethnic Asian populations. *Pharmacogenetics* **12**, 437–450.

Tang, K., Wong, L. P., Lee, E. J., Chong, S. S. & Lee, C. G. (2004) Genomic evidence for recent positive selection at the human MDR1 gene locus. *Hum Mol Genet* **13**, 783–797.

Taniguchi, S., Mochida, Y., Uchiumi, T., Tahira, T., Hayashi, K., Takagi, K., Shimada, M., Maehara, Y., Kuwano, H., Kono, S., Nakano, H., Kuwano, M. & Wada, M. (2003) Genetic polymorphism at the 5′ regulatory region of multidrug resistance 1 (MDR1) and its association with interindividual variation of expression level in the colon. *Mol Cancer Ther* **2**, 1351–1359.

van Helvoort, A., Smith, A. J., Sprong, H., Fritzsche, I., Schinkel, A. H., Borst, P. & van Meer, G. (1996) MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* **87**, 507–517.

Wong, M., Evans, S., Rivory, L. P., Hoskins, J. M., Mann, G. J., Farlow, D., Clarke, C. L., Balleine, R. L. & Gurney, H. (2005) Hepatic technetium Tc 99m-labeled sestamibi elimination rate and ABCB1 (MDR1) genotype as indicators of ABCB1 (P-glycoprotein) activity in patients with cancer. *Clin Pharmacol Ther* **77**, 33–42.

Yi, S. Y., Hong, K. S., Lim, H. S., Chung, J. Y., Oh, D. S., Kim, J. R., Jung, H. R., Cho, J. Y., Yu, K. S., Jang, I. J. & Shin, S. G. (2004) A variant 2677A allele of the MDR1 gene affects fexofenadine disposition. *Clin Pharmacol Ther* **76**, 418–427.

Received: 20 April 2005
Accepted: 21 October 2005

Journal of Thrombosis and Haemostasis, 4: 2010–2013

ORIGINAL ARTICLE

Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers

R. KIMURA,* T. SAKATA,† Y. KOKUBO,‡ A. OKAMOTO,† A. OKAYAMA,‡ H. TOMOIKE‡ and T. MIYATA*

**Research Institute; †Laboratory of Clinical Chemistry; and ‡Department of Preventive Cardiology, National Cardiovascular Center, Suita, Osaka, Japan*

To cite this article: Kimura R, Sakata T, Kokubo Y, Okamoto A, Okayama A, Tomoike H, Miyata T. Plasma protein S activity correlates with protein S genotype but is not sensitive to identify K196E mutant carriers. *J Thromb Haemost* 2006; 4: 2010–13.

See also Okada H, Yamazaki T, Takagi A, Murate T, Yamamoto K, Takamatsu J, Matsushita T, Naoe T, Kunishima S, Hamaguchi M, Saito H, Kojima T. *In vitro* characterization of missense mutations associated with quantitative protein S deficiency. This issue, pp 2003–9.

Summary. *Background:* Protein S (PS) is an anticoagulant protein that functions as a cofactor for activated protein C (APC), and congenital PS deficiency is a well-known risk factor for the development of deep vein thrombosis (DVT). Recently, we and others identified the K196E missense mutation in the second epidermal growth factor-like domain of PS as a genetic risk factor for DVT in the Japanese population. The incidence of this mutation is high in the Japanese population. *Objectives:* In the present study, we investigated the relationship between plasma PS activity and the presence of the K196E mutation. *Patients and methods:* We measured PS activity as a cofactor activity for APC in 1862 Japanese individuals and determined the PS K196E genotype in this population. *Results:* Individuals heterozygous for the mutant E-allele had lower plasma PS activity than wildtype subjects (mean ± SD, 71.9 ± 17.6%, $n = 34$ vs. 87.9 ± 19.8%, $n = 1828$, $P < 0.0001$). However, the PS activity of several heterozygous individuals ($n = 8$) was greater than the population average. In contrast, multiple wildtype subjects ($n = 26$) had PS activity less than 2 SD below the population mean, indicating that other genetic or environmental factors affect PS activity. *Conclusions:* Plasma PS activity itself is not suitable for identifying PS 196E carriers and other methods are required for carrier detection.

Keywords: deep vein thrombosis, missense mutation, protein S.

Introduction

Protein S (PS) is an important regulator of coagulation that serves as a cofactor for activated protein C (APC), the anticoagulant protease that proteolytically degrades activated factor (F) V and FVIII [1]. Individuals with homozygous or compound heterozygous deficiency for PS develop disseminated thrombosis after birth, and heterozygosity for PS deficiency increases the risk of deep vein thrombosis (DVT) [2,3].

Recently, we and others identified that a PS missense mutation prevalent in the Japanese population, which causes Lys196 to be replaced by Glu (K196E mutation, formerly known as PS Tokushima, and referred to as K155E mutation), is a genetic risk factor for the development of DVT [4,5]. This mutation lies within the second epidermal growth factor-like domain of PS, and, *in vitro*, K196E mutant PS has decreased APC cofactor activity and poorly accelerates prothrombinase inactivation [6–8]. This missense mutation was originally identified in Japanese patients with PS deficiency suffering from DVT [9,10]. However, the plasma PS activity in individuals with this mutation remained controversial. In one report, PS activity was decreased in carriers of the K196E mutation with normal PS levels [9]. In contrast, another study found PS activity within the normal range in affected individuals [10].

We identified 66 heterozygotes and no homozygotes for the mutant PS 196E-allele from a population of 3651 individuals [5]. Therefore, the frequency of the mutant E-allele in the Japanese population was about 0.009. Extrapolating from these values, we estimated that approximately one out of every 55 Japanese individuals is heterozygous for the E-allele [11]. Thus, a substantial number of Japanese carry the E-allele for PS and are at increased risk for the development of DVT. Given the relatively high frequency of this mutation and its strong correlation with DVT, it may be advisable to screen individuals for the presence of this mutation so that carriers can avoid additional environmental risk factors associated with DVT. An appropriate screening test is lacking, however, and we hypothesized that plasma PS activity levels may directly correlate with PS genotype. If this were the case, genetic testing

Correspondence: Toshiyuki Miyata, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Tel.: +81 6 6833 5012 ext. 2512; fax: +81 6 6835 1176; e-mail: miyata@ri.ncvc.go.jp

Received 27 March 2006, accepted 1 June 2006

would not need to be undertaken to determine the PS genotype of a large population.

In this study, we examined the relationship between PS activity and the presence of the K196E mutation. The mean PS activity of individuals heterozygous for the K196E mutation was significantly less than that of wildtype individuals. However, there was substantial overlap in PS activity between these populations, and, thus, PS activity is not an appropriate method to differentiate K196E carriers from the general population.

Methods

We previously measured the PS activity in a population of Japanese individuals as part of the Suita Study, and we determined their genotype with respect to the PS K196E mutation [5,12]. The ability of PS to act as a cofactor for PC activation was measured on the basis of the activated partial thromboplastin time assay using Staclot PS (Diagnostica Stago, Asnières, France) [12]. The plasma levels of PS activity were expressed as percentages of the levels obtained from commercially available standard human plasma (Behringwerke, Marburg, Germany). The intra-assay coefficient of variation for PS activity was 6.9% ($n = 10$). The PS K196E genotype was determined by the TaqMan genotype discrimination method [5], using the primers 5′-ACCACTGTTCCTGTAAAAATGGTTT/5′-TGTGTTTTAATTCTACCATCCTGCT and the probes 5′-VIC-CAAATGAGAAAGATTGTAAAG-MGB (the mutant E-allele)/5′-FAM-CAAATAAGAAAGATTGTAAAG-MGB (the wild-type allele). The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. PS activity was measured in 2690 population individuals [12] and the genotype was determined in 3651 individuals [5]. The 1862 individuals with both known PS activity and genotype were used for analysis in this study. Plasminogen activity was previously measured using the chromogenic assay method with streptokinase as the activator and the specific substrate S-2251 (Chromogenix AB, Stockholm, Sweden) [13]. Plasminogen activity was determined in 4517 individuals [13], and the plasminogen A620T mutation genotype was determined in 3295 out of 4517 individuals by the TaqMan method using the primers 5′-TGTGGAGGCACCTTGATATCC/5′-TGTCAATTGTCCCCTAAACATACTTC and the probes 5′-VIC-TGTTGACTACTGCCCACT-MGB (the mutant T-allele)/5′-FAM- TGTTGACTGCTGCCCACT-MGB (the wild-type allele). Analysis of variance was used to compare mean values between groups by Student's *t*-test using JMP v 5.1 software (SAS Institute Inc., Cary, NC, USA).

Results

We measured the PS activity in 1862 individuals of known PS genotype, and we compared the activity of wildtype and heterozygous individuals. Within this population, 1828 subjects harbored the wildtype allele while 34 were heterozygous for the K196E mutation. No individuals were homozygous for the mutant E-allele. Within the total population, the mean ± SD PS activity was 87.6% ± 19.9%.

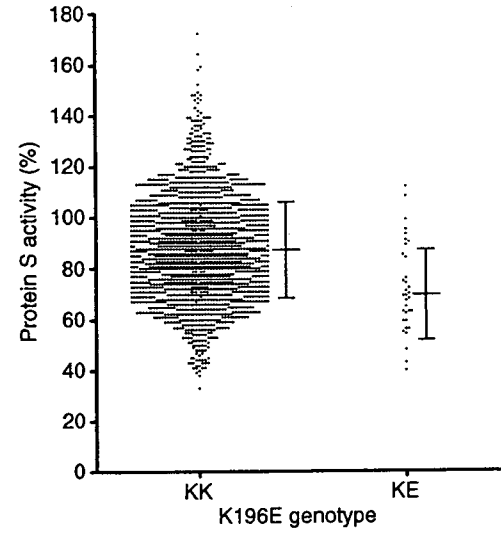

Fig. 1. Protein S (PS) activity in wild-type and K196E heterozygous individuals. Mean ± SD PS activity in heterozygous and wild-type individuals was 71.9% ± 17.6% ($n = 34$) and 87.9% ± 19.8% ($n = 1828$) ($P < 0.0001$), respectively.

Individuals heterozygous for the K196E mutation had reduced plasma PS activity compared to individuals with the KK genotype (mean ± SD, 71.9% ± 17.6%, $n = 34$ vs. 87.9% ± 19.8%, $n = 1828$, $P < 0.0001$) (Fig. 1). However, several heterozygous individuals with the mutant E-allele ($n = 8$) had measured PS activity greater than the total population average, while 26 wildtype subjects had PS activity at least 2SD less than the population mean (47.8%). Thus, PS activity does not appear to be a useful surrogate marker for PS genotype.

To determine whether an individual's genotype for any coagulation related protein could be determined by measuring the activity of the respective factor, we further examined the genotype and plasma activity of plasminogen in 3295 subjects. We identified 92 individuals heterozygous for the plasminogen A620T mutation, and the plasma plasminogen activity of these individuals was significantly less than wildtype individuals. Furthermore, there was little to no overlap between the measured plasminogen activities of wildtype and heterozygous individuals. Thus, the concept we originally wished to test was validated (Fig. 2).

There are well-documented gender- and age-related differences in PS activity [14], and this was true for our study population as reported [11] (Fig. 3A). When we examined the relationship between PS activity, genotype, and age, we observed decreased PS activity across all ages for individuals with the KE-genotype (Fig. 3B).

Discussion

DVT is a multi-factorial disease caused by the interaction of environmental and genetic factors. In Caucasian populations,

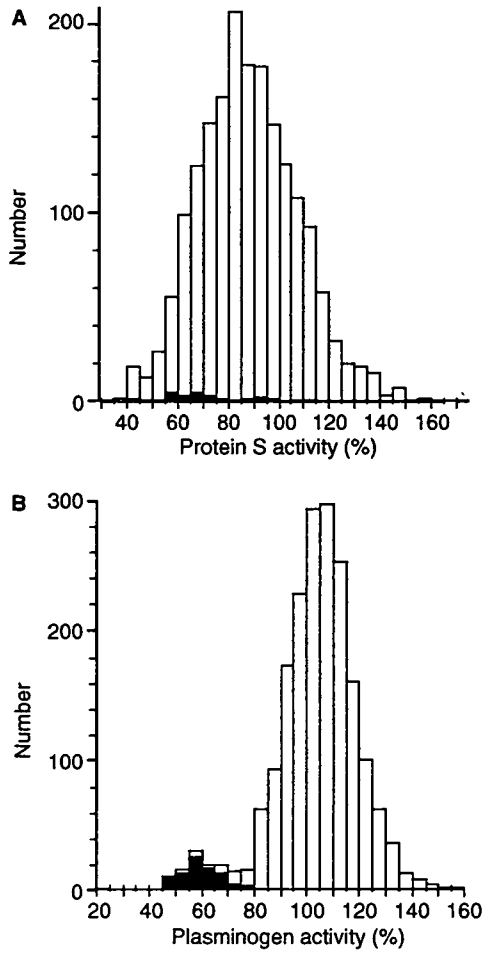

Fig. 2. Histogram representation of protein S (PS) (A) and plasminogen (B) activity in wildtype and heterozygous individuals. PS activity was measured in 1862 individuals, and plasminogen activity was measured in 3295 individuals. Activity was divided into groups by 5% increments, and mutation carriers are shown in closed bars.

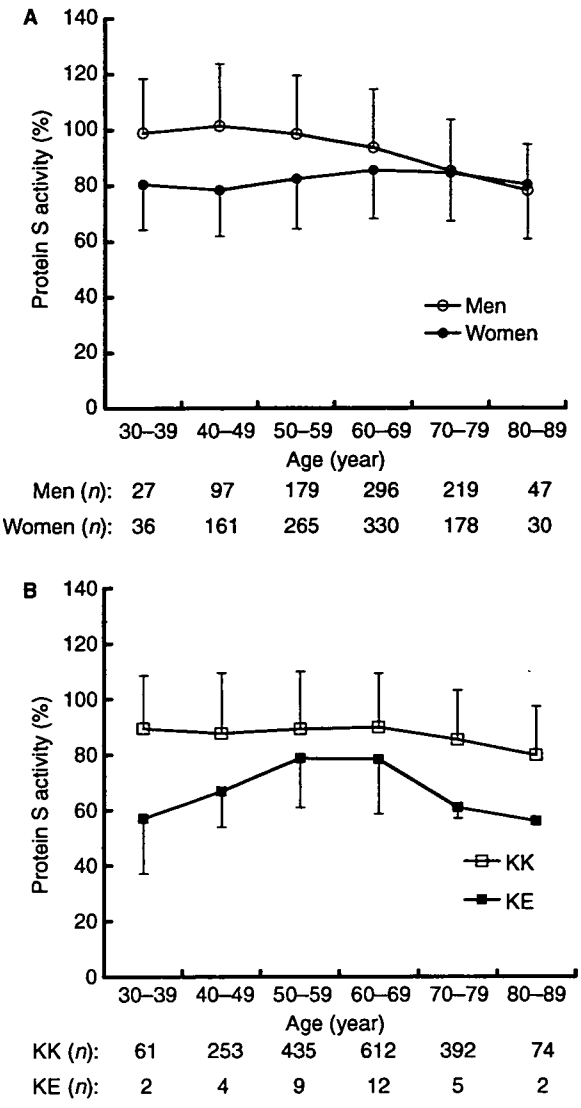

Fig. 3. Protein S (PS) activity divided in sex, age, and genotype. Open circles and closed circles in (A) show the mean PS activity in men and women, respectively. Open squares and closed squares in (B) show the mean PS activity in wild-type (KK-genotype) and heterozygote (KE-genotype). Error bars represent SD.

the FV Leiden (FVL) mutation, R506Q mutation in FV, is an important risk factor for the development of DVT. FVL carriers can be readily identified using the APC resistance test [15]. A FVL carrier will exhibit a prolonged clotting time in an activated thromboplastin time assay following the addition of APC. The incidence of this particular mutation varies in different ethnic populations [16,17] and is not observed in the Japanese [18]. In contrast, the PS K196E mutation present in the Japanese population is a genetic risk factor for DVT [4,5]. Therefore, a plasma assay for detecting PS 196E carriers should be developed. To understand the relation of the PS activity with the K196E mutation, we examined the PS activity and the K196E genotype in the Japanese population enrolled in the Suita Study.

The plasma PS activity in individuals with the PS K196E mutation remained controversial [6,9,10]. In one report, four members in a family who carried this mutation showed the PS activity with 37%, 72%, 101%, and 77%, respectively [10]. In a second family in this report, two members carried this mutation with the PS activity with 87% and 92%. On the basis of these results, the authors suggested this mutation as a phenotypically neutral polymorphism. In contrast, another study identified the same mutation correlated with low PS activity [6,9]. In this study, the authors identified this mutation in three patients with DVT. In addition, four individuals who did not show history of thrombosis were carriers of this mutation. All of these carriers showed low PS activity (mean ± SD, 43.1% ± 9.1%). Thus, so far, the relationship between the plasma PS activity and K196E mutation has not been settled. To address this issue, we have measured the PS activity and determined the genotype in the general Japanese population. As the results, we found that individuals heterozygous for the PS K196E mutation had reduced plasma PS activity compared to wildtype subjects, but this difference was relatively small and did not sufficiently differentiate between the two genotypes. In contrast, plasma

plasminogen activity was an effective test for segregating wildtype individuals and those heterozygous for the plasminogen A620T mutation. Thus, plasma PS activity is influenced by environmental factors to a greater extent than plasminogen activity.

The environmental factors such as age, sex hormone, and inflammation, are known to influence the PS activity [19]. As shown in Fig. 3, gender- and age-related differences in PS activity were observed in the general Japanese population. In addition, plasma PS activity might be influenced by other genetic factors. Genome scan for plasma free PS levels indicated a quantitative trait locus on human chromosome 1q [20]. This region contains *C4BPA* and *C4BPB* genes that are differentially regulated by acute phase cytokines [21]. PS can bind to the β-chain of C4 binding protein and not to the α-chain. The resulting alterations in the synthesis of C4 binding protein isoforms may affect the equilibrium between bound and free PS. Alternative means must be developed for the identification of PS K196E carriers to reduce the risk of DVT in affected individuals.

Acknowledgements

This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan, a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We would like to express our highest gratitude to Mr Y. Sakaguchi, the mayor of Suita city, and Dr S. Kitamura, President of the National Cardiovascular Center, for their support of the genome project. We would like to express our gratitude to Dr O. Hishikawa, Dr K. Kawanishi, Dr Y. Kotani, Mr T. Fujii, and Dr T. Mannami for their continuous support of our population survey in Suita city. We thank the members of the Satsuki-Junyukai.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Dahlback B. Blood coagulation. *Lancet* 2000; **355**: 1627–32.
2 Lane DA, Mannucci PM, Bauer KA, Bertina RM, Bochkov NP, Boulyjenkov V, Chandy M, Dahlback B, Ginter EK, Miletich JP, Rosendaal FR, Seligsohn U. Inherited thrombophilia: Part 1. *Thromb Haemost* 1996; **76**: 651–62.
3 Bucciarelli P, Rosendaal FR, Tripodi A, Mannucci PM, De Stefano V, Palareti G, Finazzi G, Baudo F, Quintavalla R. Risk of venous thromboembolism and clinical manifestations in carriers of antithrombin, protein C, protein S deficiency, or activated protein C resistance: a multicenter collaborative family study. *Arterioscler Thromb Vasc Biol* 1999; **19**: 1026–33.
4 Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, Tsuda H, Kang D, Hamasaki N. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem* 2005; **38**: 908–15.
5 Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, Kojima T, Murata M, Nishigami K, Chiku M, Hayashi T, Kokubo Y, Okayama A, Tomoike H, Ikeda Y, Miyata T. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. *Blood* 2006; **107**: 1737–8.
6 Hayashi T, Nishioka J, Shigekiyo T, Saito S, Suzuki K. Protein S Tokushima: abnormal molecule with a substitution of Glu for Lys-155 in the second epidermal growth factor-like domain of protein S. *Blood* 1994; **83**: 683–90.
7 Hayashi T, Nishioka J, Suzuki K. Molecular mechanism of the dysfunction of protein S (Tokushima) (Lys155–> Glu) for the regulation of the blood coagulation system. *Biochim Biophys Acta* 1995; **1272**: 159–67.
8 Hayashi T, Nishioka J, Suzuki K. Characterization of dysfunctional protein S-Tokushima (K155–> E) in relation to the molecular interactions required for the regulation of blood coagulation. *Pol J Pharmacol* 1996; **48**: 221–3.
9 Shigekiyo T, Uno Y, Kawauchi S, Saito S, Hondo H, Nishioka J, Hayashi T, Suzuki K. Protein S Tokushima: an abnormal protein S found in a Japanese family with thrombosis. *Thromb Haemost* 1993; **70**: 244–6.
10 Yamazaki T, Sugiura I, Matsushita T, Kojima T, Kagami K, Takamatsu J, Saito H. A phenotypically neutral dimorphism of protein S: the substitution of Lys155 by Glu in the second EGF domain predicted by an A to G base exchange in the gene. *Thromb Res* 1993; **70**: 395–403.
11 Miyata T, Kimura R, Kokubo Y, Sakata T. Genetic risk factors for deep vein thrombosis in Japanese, importance of protein S K196E mutation. *Int J Hematol* 2006; **83**: 217–23.
12 Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T. Prevalence of protein S deficiency in the Japanese general population: the Suita Study. *J Thromb Haemost* 2004; **2**: 1012–3.
13 Okamoto A, Sakata T, Mannami T, Baba S, Katayama Y, Matsuo H, Yasaka M, Minematsu K, Tomoike H, Miyata T. Population-based distribution of plasminogen activity and estimated prevalence and relevance to thrombotic diseases of plasminogen deficiency in the Japanese: the Suita Study. *J Thromb Haemost* 2003; **1**: 2397–403.
14 Henkens CM, Bom VJ, Van der Schaaf W, Pelsma PM, Sibinga CT, de Kam PJ, van der Meer J. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. *Thromb Haemost* 1995; **74**: 1271–5.
15 Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993; **90**: 1004–8.
16 Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet* 1995; **346**: 1133–4.
17 Zivelin A, Griffin JH, Xu X, Pabinger I, Samama M, Conard J, Brenner B, Eldor A, Seligsohn U. A single genetic origin for a common Caucasian risk factor for venous thrombosis. *Blood* 1997; **89**: 397–402.
18 Fujimura H, Kambayashi J, Monden M, Kato H, Miyata T. Coagulation factor V Leiden mutation may have a racial background. *Thromb Haemost* 1995; **74**: 1381–2.
19 Rezende SM, Simmonds RE, Lane DA. Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. *Blood* 2004; **103**: 1192–201.
20 Almasy L, Soria JM, Souto JC, Coll I, Bacq D, Faure A, Mateo J, Borrell M, Munoz X, Sala N, Stone WH, Lathrop M, Fontcuberta J, Blangero J. A quantitative trait locus influencing free plasma protein S levels on human chromosome 1q: results from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project. *Arterioscler Thromb Vasc Biol* 2003; **23**: 508–11.
21 Garcia de Frutos P, Alim RI, Hardig Y, Zoller B, Dahlback B. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. *Blood* 1994; **84**: 815–22.

plasminogen activity was an effective test for segregating wildtype individuals and those heterozygous for the plasminogen A620T mutation. Thus, plasma PS activity is influenced by environmental factors to a greater extent than plasminogen activity.

The environmental factors such as age, sex hormone, and inflammation, are known to influence the PS activity [19]. As shown in Fig. 3, gender- and age-related differences in PS activity were observed in the general Japanese population. In addition, plasma PS activity might be influenced by other genetic factors. Genome scan for plasma free PS levels indicated a quantitative trait locus on human chromosome 1q [20]. This region contains *C4BPA* and *C4BPB* genes that are differentially regulated by acute phase cytokines [21]. PS can bind to the β-chain of C4 binding protein and not to the α-chain. The resulting alterations in the synthesis of C4 binding protein isoforms may affect the equilibrium between bound and free PS. Alternative means must be developed for the identification of PS K196E carriers to reduce the risk of DVT in affected individuals.

Acknowledgements

This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan, a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We would like to express our highest gratitude to Mr Y. Sakaguchi, the mayor of Suita city, and Dr S. Kitamura, President of the National Cardiovascular Center, for their support of the genome project. We would like to express our gratitude to Dr O. Hishikawa, Dr K. Kawanishi, Dr Y. Kotani, Mr T. Fujii, and Dr T. Mannami for their continuous support of our population survey in Suita city. We thank the members of the Satsuki-Junyukai.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Dahlback B. Blood coagulation. *Lancet* 2000; **355**: 1627–32.
2 Lane DA, Mannucci PM, Bauer KA, Bertina RM, Bochkov NP, Boulyjenkov V, Chandy M, Dahlback B, Ginter EK, Miletich JP, Rosendaal FR, Seligsohn U. Inherited thrombophilia: Part 1. *Thromb Haemost* 1996; **76**: 651–62.
3 Bucciarelli P, Rosendaal FR, Tripodi A, Mannucci PM, De Stefano V, Palareti G, Finazzi G, Baudo F, Quintavalla R. Risk of venous thromboembolism and clinical manifestations in carriers of antithrombin, protein C, protein S deficiency, or activated protein C resistance: a multicenter collaborative family study. *Arterioscler Thromb Vasc Biol* 1999; **19**: 1026–33.
4 Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, Tsuda H, Kang D, Hamasaki N. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem* 2005; **38**: 908–15.
5 Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, Kojima T, Murata M, Nishigami K, Chiku M, Hayashi T, Kokubo Y, Okayama A, Tomoike H, Ikeda Y, Miyata T. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. *Blood* 2006; **107**: 1737–8.
6 Hayashi T, Nishioka J, Shigekiyo T, Saito S, Suzuki K. Protein S Tokushima: abnormal molecule with a substitution of Glu for Lys-155 in the second epidermal growth factor-like domain of protein S. *Blood* 1994; **83**: 683–90.
7 Hayashi T, Nishioka J, Suzuki K. Molecular mechanism of the dysfunction of protein S (Tokushima) (Lys155–> Glu) for the regulation of the blood coagulation system. *Biochim Biophys Acta* 1995; **1272**: 159–67.
8 Hayashi T, Nishioka J, Suzuki K. Characterization of dysfunctional protein S-Tokushima (K155–> E) in relation to the molecular interactions required for the regulation of blood coagulation. *Pol J Pharmacol* 1996; **48**: 221–3.
9 Shigekiyo T, Uno Y, Kawauchi S, Saito S, Hondo H, Nishioka J, Hayashi T, Suzuki K. Protein S Tokushima: an abnormal protein S found in a Japanese family with thrombosis. *Thromb Haemost* 1993; **70**: 244–6.
10 Yamazaki T, Sugiura I, Matsushita T, Kojima T, Kagami K, Takamatsu J, Saito H. A phenotypically neutral dimorphism of protein S: the substitution of Lys155 by Glu in the second EGF domain predicted by an A to G base exchange in the gene. *Thromb Res* 1993; **70**: 395–403.
11 Miyata T, Kimura R, Kokubo Y, Sakata T. Genetic risk factors for deep vein thrombosis in Japanese, importance of protein S K196E mutation. *Int J Hematol* 2006; **83**: 217–23.
12 Sakata T, Okamoto A, Mannami T, Tomoike H, Miyata T. Prevalence of protein S deficiency in the Japanese general population: the Suita Study. *J Thromb Haemost* 2004; **2**: 1012–3.
13 Okamoto A, Sakata T, Mannami T, Baba S, Katayama Y, Matsuo H, Yasaka M, Minematsu K, Tomoike H, Miyata T. Population-based distribution of plasminogen activity and estimated prevalence and relevance to thrombotic diseases of plasminogen deficiency in the Japanese: the Suita Study. *J Thromb Haemost* 2003; **1**: 2397–403.
14 Henkens CM, Bom VJ, Van der Schaaf W, Pelsma PM, Sibinga CT, de Kam PJ, van der Meer J. Plasma levels of protein S, protein C, and factor X: effects of sex, hormonal state and age. *Thromb Haemost* 1995; **74**: 1271–5.
15 Dahlback B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993; **90**: 1004–8.
16 Rees DC, Cox M, Clegg JB. World distribution of factor V Leiden. *Lancet* 1995; **346**: 1133–4.
17 Zivelin A, Griffin JH, Xu X, Pabinger I, Samama M, Conard J, Brenner B, Eldor A, Seligsohn U. A single genetic origin for a common Caucasian risk factor for venous thrombosis. *Blood* 1997; **89**: 397–402.
18 Fujimura H, Kambayashi J, Monden M, Kato H, Miyata T. Coagulation factor V Leiden mutation may have a racial background. *Thromb Haemost* 1995; **74**: 1381–2.
19 Rezendė SM, Simmonds RE, Lane DA. Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. *Blood* 2004; **103**: 1192–201.
20 Almasy L, Soria JM, Souto JC, Coll I, Bacq D, Faure A, Mateo J, Borrell M, Munoz X, Sala N, Stone WH, Lathrop M, Fontcuberta J, Blangero J. A quantitative trait locus influencing free plasma protein S levels on human chromosome 1q: results from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project. *Arterioscler Thromb Vasc Biol* 2003; **23**: 508–11.
21 Garcia de Frutos P, Alim RI, Hardig Y, Zoller B, Dahlback B. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. *Blood* 1994; **84**: 815–22.

Journal of the American College of Cardiology
© 2006 by the American College of Cardiology Foundation
Published by Elsevier Inc.

Vol. 48, No. 7, 2006
ISSN 0735-1097/06/$32.00
doi:10.1016/j.jacc.2006.05.069

Impact of Blockade of Histamine H_2 Receptors on Chronic Heart Failure Revealed by Retrospective and Prospective Randomized Studies

Jiyoong Kim, MD, Akiko Ogai, MS, Satoshi Nakatani, MD, PhD, Kazuhiko Hashimura, MD, Hideaki Kanzaki, MD, Kazuo Komamura, MD, PhD, Masanori Asakura, MD, PhD, Hiroshi Asanuma, MD, PhD, Soichiro Kitamura, MD, PhD, Hitonobu Tomoike, MD, PhD, Masafumi Kitakaze, MD, PhD

Suita, Japan

OBJECTIVES The goal of this work was to determine whether the blockade of histamine H_2 receptors is beneficial for the pathophysiology of chronic heart failure (CHF).

BACKGROUND Because CHF is one of the major life-threatening diseases, we need to find a novel effective therapy. Intriguingly, our previous study, which predicts the involvement of histamine in CHF, suggests that we should test this hypothesis in patients with CHF.

METHODS We selected 159 patients who received famotidine among symptomatic CHF patients for the retrospective study. We blindly selected age- and gender-matched CHF patients receiving drugs for gastritis other than histamine H_2 receptor blockers as a control group. For the prospective study, 50 symptomatic CHF patients were randomly divided into 2 groups. One group received famotidine of 30 mg/day for 6 months, and the other group received teprenone.

RESULTS In the retrospective study, famotidine of 20 to 40 mg decreased both left ventricular end-diastolic and end-systolic lengths (LVDd and LVDs, respectively) and the plasma B-type natriuretic peptide (BNP) levels (182 ± 21 vs. 259 ± 25 pg/ml, $p < 0.05$) with unaltered fractional shortening (FS). In a randomized, open-label study, compared with teprenone, famotidine of 30 mg prospectively decreased both New York Heart Association functional class ($p < 0.05$) and plasma BNP levels (183 ± 26 pg/ml vs. 285 ± 41 pg/ml, $p < 0.05$); this corresponded to decreasing both LVDd (57 ± 2 mm vs. 64 ± 2 mm, $p < 0.05$) and LVDs (47 ± 2 mm vs. 55 ± 2 mm, $p < 0.05$) with unaltered FS (15 ± 1% vs. 17 ± 1%). The frequency of readmission because of worsening of CHF was lower in the famotidine group (4% and 24%, $p < 0.05$). On the other hand, teprenone had no effects on CHF.

CONCLUSIONS Famotidine improved both cardiac symptoms and ventricular remodeling associated with CHF. Histamine H_2 receptor blockers may have therapeutic benefits for CHF. (J Am Coll Cardiol 2006;48:1378–84) © 2006 by the American College of Cardiology Foundation

Despite current medical therapy for patients with chronic heart failure (CHF) such as angiotensin-converting enzyme (ACE) inhibitors or beta-adrenergic receptor blockers (1), CHF remains one of the major causes of high morbidity and mortality worldwide. Chronic heart failure is characterized by cardiac symptoms, impaired cardiac performance, cardiac

See page 1385

mechanical stress, and neurohormonal imbalance (2). Indeed, increased levels of catecholamines, cytokines, and angiotensin II are thought to play important roles in the pathophysiology and development of CHF (3,4). Histamine is one of the neurohormonal factors that provoke various cellular functions via stimulation of histamine H_1-H_3 receptors (5,6). Specifically, because histamine H_2 receptors are known to be located in gastric cells and enhance the production of acids that cause gastric ulcers, the blocker of histamine H_2 receptors is developed as the drug for the treatment of gastric ulcers (7). Interestingly, we have previously predicted that histamine H_2 receptor blockers may be cardioprotective in patients with CHF using the data mining technique (8). The histamine H_2 receptor is also located in the cardiomyocytes, and this receptor is coupled to Gs protein as well as is the beta receptor (9–13). Indeed, it is reported that: 1) histamine provokes positive inotropic effects (11,14); and 2) the blocker of histamine H_2 receptors decreases cardiac output (14). The important roles of mast cells and released histamine are also accepted in the cardiovascular system (15).

We tested the hypothesis that the blockade of histamine H_2 receptors by famotidine is beneficial for the pathophys-

From the Cardiovascular Division, National Cardiovascular Center, Suita, Japan.
This study was supported by Grants-in-Aid for Human Genome, Tissue Engineering and Food Biotechnology (H13-Genome-011), Health and Labour Sciences Research Grants, Comprehensive Research on Aging and Health (H13-21seiki(seikatsu)-23), Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare, Japan, and a grant from the Japan Cardiovascular Research Foundation. The funding sources had no involvement in the current study.

Manuscript received April 11, 2006; revised manuscript received May 18, 2006, accepted May 23, 2006.

Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
AMP = adenosine monophosphate
BNP = brain natriuretic peptide
CHF = chronic heart failure
FS = fractional shortening
GERD = gastroesophageal reflux disease
LVDd = left ventricular end-diastolic volume
LVDs = left ventricular end-systolic volume
NYHA = New York Heart Association

iology of CHF in retrospective and prospective randomized studies.

METHODS

This study was approved by the ethical committee of National Cardiovascular Center. Informed consent was obtained from all patients before participation in this study in accordance with institutional approved protocols.

Study population and protocols. THE RETROSPECTIVE STUDY. A total of 1,104 consecutive subjects who were admitted to our hospital for treatment of CHF between January 2002 and April 2004 were candidates for this study. The criteria for enrollment in this study were: 1) clinical evidence of heart failure despite the conventional therapy; and 2) left ventricular fractional shortening (FS) below 30%, as assessed by 2-dimensional echocardiography. All the patients had New York Heart Association (NYHA) functional classifications of II to III, but were stable for 2 months after their discharge. Among these patients, we selected the patients who received famotidine of 20 to 40 mg (n = 159, the famotidine group). In the control group, the patients were selected so as to be matched for age, gender, and the cause of CHF (n = 159). We randomly selected age-, gender-, and cause-matched patients for the other drug of non-histamine H_2 blocker for gastritis (n = 159). Among the 159 patients in each group, the number of patients who suffered from dilated cardiomyopathy, hypertensive heart disease, ischemic cardiomyopathy, and valvular heart disease were 71, 11, 39, and 38, respectively. Clinical parameters of the plasma brain natriuretic peptide (BNP) levels and echocardiography were obtained. We estimated the NYHA functional classification of each patient by 3 independent cardiologists who were blinded to the medical treatment of CHF. If the estimations of all 3 doctors did not agree, we decided to take the median among 3 values of NYHA functional classification.

The prospective studies: the effects of famotidine. We studied 50 patients with symptomatic CHF and gastroesophageal reflux disease (GERD) in our institute. Gastroesophageal reflux disease was diagnosed by questionnaires reported previously (16). The criteria for enrollment in this study were clinical evidence of heart failure despite the conventional therapy and a left ventricular FS below 30%, as assessed by 2-dimensional echocardiography, and existence of GERD. All the patients had NYHA functional classifications of II to III. There were 32 men and 18 women with a mean age of 65 years. The number of patients diagnosed as CHF because of dilated cardiomyopathy, hypertensive heart disease, ischemic cardiomyopathy, and valvular heart disease were 17, 2, 4, and 2 in each group, respectively. Exclusion criteria included chronic obstructive pulmonary disease, pregnancy, and severe liver disease as defined by having hepatic enzymes >2 times the upper limit of normal values. All patients were treated by optimal and stable doses of beta-blockers and ACE inhibitors for at least 3 months before screening echocardiography and randomization. We did not change the doses of these drugs after the enrollment. Patients were randomly divided into 2 treatment groups: famotidine (n = 25, the famotidine group) and teprenone (n = 25, the control group). The doses of famotidine and teprenone were 30 and 150 mg per day, respectively, and there were no patients who discontinued the intake of either famotidine or teprenone, and drugs for CHF.

In the current study, we tested the hypothesis that famotidine, the histamine H_2 receptor blocker, may have therapeutic benefits for CHF in the clinical settings. The primary end point is to assess the changes in NYHA functional class and the plasma BNP levels from the baseline to 24 weeks. We estimated the NYHA functional classification of each patient by 3 independent cardiologists who were blinded to the treatment assignment of famotidine. If the estimations of all 3 doctors did not agree, we decided to take the median among 3 values of NYHA functional classification. Additional analyses were done using the echocardiogram to obtain the changes in left ventricular or atrial volume, and the pressure differences across the tricuspid valve from baseline to 24 weeks. Furthermore, the frequency of readmission because of worsening of CHF within 24 weeks was investigated.

Estimating from retrospective study results showing that the reduction of the plasma BNP levels was about 30%, 25 patients were required for each study group. A randomization was performed according to a computer generated randomization list by central telephone call or fax to Clinical Study Support Center Japan (Suita Osaka, Japan).

Effects of teprenone. There is a possibility that teprenone has deleterious effects on the pathophysiology of CHF, and if this were the case, famotidine would appear to be beneficial, when famotidine has no cardioprotective effects. To examine this possibility, we administered teprenone to 10 patients with CHF for 24 weeks, and compared 10 CHF patients without the teprenone treatment. The criteria for the enrollment, evaluated parameters, and the evaluation procedure were the same as in the study of famotidine described earlier in the text.

Analysis of parameters for CHF. Blood samples were collected in test tubes containing ethylenediaminetetraacetic acid at baseline and after 24 weeks of the treatment. The plasma was separated from blood cells by centrifugation and frozen at −80°C. Plasma concentrations of BNP were

JACC Vol. 48, No. 7, 2006
October 3, 2006:1378–84

measured using a specific immunoradiometric assay (17). The personnel performing these assays were blinded to the patients' treatment assignments.

M-mode echocardiography was performed with 2-dimensional monitoring using a Sono layer phased-array sector scanner (SONOS 5500, Hewlett Packard, Palo Alto, California) before and after 24 weeks of the treatment with famotidine or teprenone (18). All echocardiograms were read by the same physician, at baseline and after 24 weeks of the treatment, who was blinded to patients' treatment, assignment, and time point.

Statistical analysis. Data are presented as mean ± SEM. Statistical analysis was performed using paired or unpaired *t* test for numerical values, and either chi-square tests or Wilcoxon signed rank test for categorical values. B-type natriuretic peptide levels were logarithmically transformed to perform the statistical analysis. Furthermore, we used two-way repeated-measures analysis of variance when we compared the changes of each parameter in 2 groups. The chi-square tests were also performed to test the differences of the incidence of the readmission. All statistical analyses were performed using Stat View version 5.0 for Windows (SAS Institute, Cary, North Carolina) and SPSS 10.0.5J software (SPSS Inc., Chicago, Illinois).

Table 1. Clinical Parameters of CHF With or Without Famotidine

	Control Group (n = 159)	Famotidine Group (n = 159)
Age (yrs)	66 ± 1	66 ± 1
M/F gender (%)	97/62 (61/39)	97/62 (61/39)
Hypertension (%)	11 (7)	11 (7)
Duration of CHF (yrs)	8.7 ± 0.7	8.5 ± 0.8
Systolic blood pressure (mm Hg)	112 ± 9	105 ± 8*
Diastolic blood pressure (mm Hg)	67 ± 4	62 ± 5*
Heart rate (beats/min)	73 ± 5	66 ± 5*
Fractional shortening (%)	24 ± 1	23 ± 1
LV diastolic diameter (mm)	58 ± 2	54 ± 1*
LV systolic diameter (mm)	44 ± 1	41 ± 1*
LA diameter (mm)	40 ± 3	39 ± 3
Pressure across tricuspid valve (mm Hg)	30 ± 2	28 ± 2
Plasma BNP levels (pg/ml)	259 ± 25	182 ± 21*
NYHA functional class: II/III (%)	75/84 (47/53)	97/62 (61/39)*
Concomitant drugs, n (%)		
Digoxin	126 (80)	134 (84)
Diuretics except spironolactone	140 (88)	137 (86)
Nitrates	80 (25)	32 (20)
Beta-blockers	143 (90)	137 (86)
ACE inhibitors	127 (80)	121 (76)
ARB	118 (20)	38 (24)
Spironolactone	25 (20)	25 (20)

Values are either numbers of each group, range, or mean ± SEM. *$p < 0.05$ vs. the control group.

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; BNP = brain natriuretic peptide; CHF = chronic heart failure; LA = left atrium; LV = left ventricular; NYHA = New York Heart Association; Fractional shortening (%) = (left ventricle end-diastolic diameter − left ventricle end-systolic diameter)/left ventricle end-diastolic diameter.

Table 2. Baseline Characteristics of the Study Population

	Teprenone Group (n = 25)	Famotidine Group (n = 25)
Age (yrs)	65 ± 2	65 ± 2
M/F gender (%)	16/9 (64/36)	16/9 (64/36)
NYHA functional class: II/III (%)	8/17 (32/68)	10/15 (40/60)
Hypertension (%)	4 (16)	6 (24)
Duration of CHF (yrs)	11.2 ± 1.7	13.2 ± 2.3
Systolic blood pressure (mm Hg)	113 ± 3	112 ± 3
Diastolic blood pressure (mm Hg)	68 ± 3	67 ± 2
Heart rate (beats/min)	83 ± 3	83 ± 2
Fractional shortening (%)	15 ± 1	15 ± 1
LV diastolic diameter (mm)	65 ± 2	64 ± 2
LV systolic diameter (mm)	55 ± 2	55 ± 2
LA diameter (mm)	43 ± 2	42 ± 2
Pressure across tricuspid valve (mm Hg)	33 ± 3	36 ± 3
Plasma BNP levels (pg/ml)	268 ± 28	286 ± 41
Concomitant drugs, n (%)		
Digoxin	24 (96)	22 (84)
Diuretics except spironolactone	25 (100)	25 (100)
Nitrates	7 (28)	4 (16)
Beta-blockers	25 (100)	25 (100)
ACE inhibitors	23 (92)	21 (84)
ARB	2 (8)	4 (16)
Spironolactone	10 (40)	8 (32)

Values are either numbers of each group, range, or mean ± SEM. Abbreviations as in Table 1.

RESULTS

After age and gender matching, as shown in Table 1, the gender ratio and the average age of the 2 groups were similar. There were no significant differences of the variety of medical treatment drugs between the 2 groups. Blood pressure, heart rate, NYHA functional class, the plasma BNP levels, and left ventricular dimensions were smaller in the famotidine group compared with the control group. There were no differences between FS in the 2 groups. This result suggests that famotidine may be beneficial for pathophysiology of CHF.

As for the prospective randomized famotidine treatment protocol, medications were well tolerated over the 24-week period. The participants were recruited from September 2004 to October 2004. Participants attended clinic visits at the time of randomization (baseline) and at 4- to 8-week intervals for 24 weeks. All patients completed the protocol (50 of 50). No patients died during the 24-week study. In addition, the doses of beta-blockers, ACE inhibitors, and diuretics were not altered during the course of the entire study.

There were no differences in age, gender, or concurrent medications between the control and famotidine groups (Table 2). Blood pressure and heart rate were not different between the groups with and without famotidine before the treatment. Famotidine administration slightly decreased blood pressure (systolic and diastolic blood pressure 107 ± 3 mm Hg vs. 112 ± 3 mm Hg, $p < 0.01$ and 60 ± 3 mm Hg vs. 67 ± 2 mm Hg, $p < 0.05$) and heart rate (79 ± 2

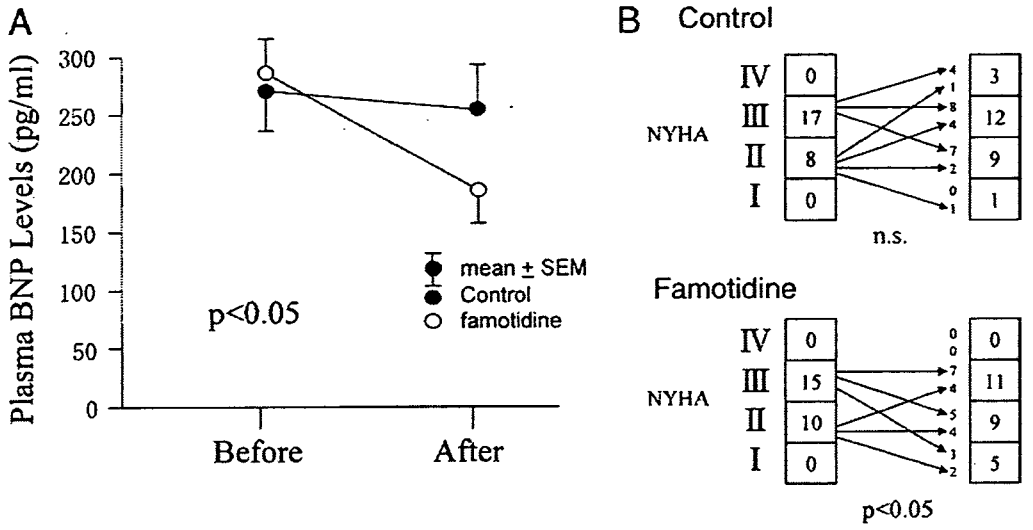

Figure 1. Changes in the plasma B-type natriuretic peptide (BNP) levels **(A)** and New York Heart Association (NYHA) functional classification **(B)** before and after the treatment with (the famotidine group) or without famotidine (the control group). The plasma BNP levels are statistically analyzed after the log transformation. The p values are obtained using 2-way repeated-measures analysis of variance **(A)** or Wilcoxon signed rank test **(B)**.

min^{-1} vs. 83 ± 2 min^{-1}, $p < 0.05$), whereas the control group did not exhibit changes in either blood pressure (systolic and diastolic blood pressure 113 ± 3 mm Hg vs. 113 ± 3 mm Hg and 68 ± 3 mm Hg vs. 68 ± 3 mm Hg) or heart rate (81 ± 3 min^{-1} vs. 83 ± 3 min^{-1}) before and 24 weeks after the treatment. The patients who received famotidine demonstrated improved functional capacity assessed by the plasma BNP levels and NYHA functional class (Fig. 1). Plasma BNP level and NYHA functional class were unchanged after 24 weeks in the group without famotidine. The functional improvement in the famotidine group was associated with improved cardiac performance. Compared with the group without famotidine, the patients treated with famotidine had lower left ventricular end-diastolic volume (LVDd) and left ventricular end-systolic volume (LVDs) while keeping FS unchanged (Fig. 2). The

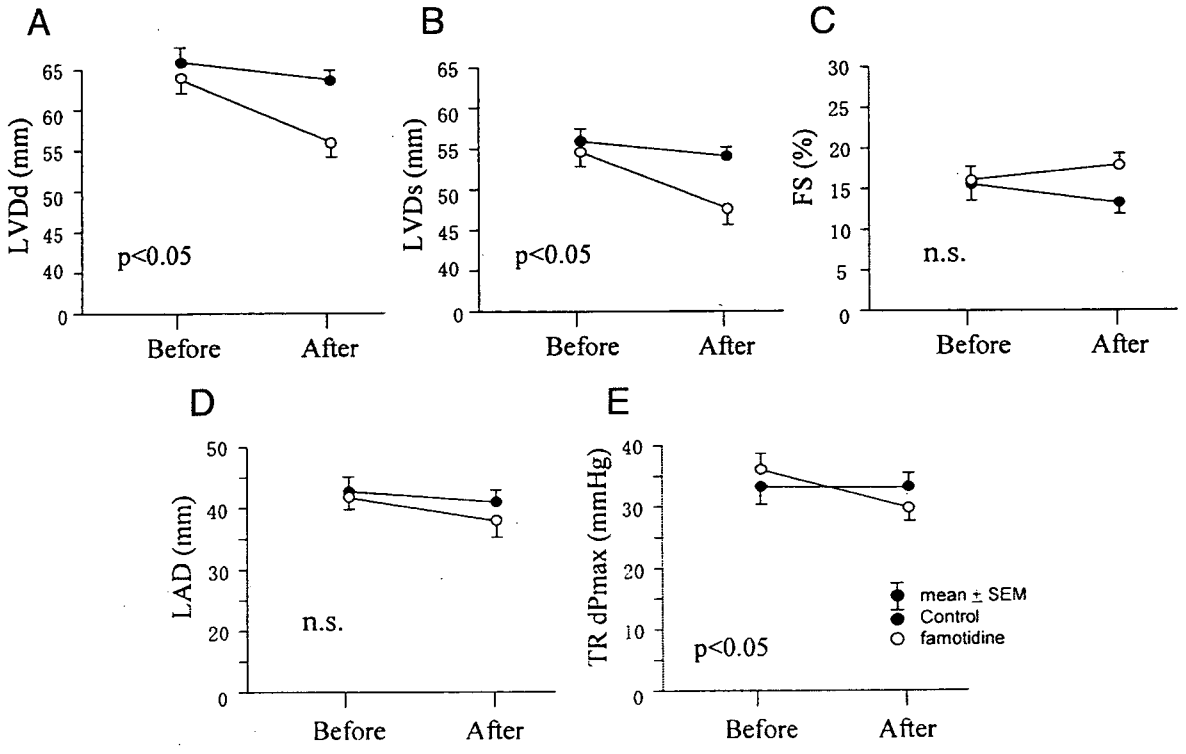

Figure 2. Changes in left ventricular (LV) end-diastolic volume (LVDd) **(A)** or end-systolic volume (LVDs) **(B)**, LV fractional shortening (FS) **(C)**, left atrial diameter (LAD) **(D)**, and the pressure differences across the tricuspid valve (TR dPmax) **(E)** before and after 24 weeks of treatment in the control and famotidine groups. The p values are tested using 2-way repeated-measures analysis of variance.

Table 3. Baseline Characteristics of the Study Population

	Teprenone Group (n = 10)	No Treatment Group (n = 10)
Age (yrs)	67 ± 4	68 ± 3
M/F gender (%)	7/3 (70/30)	7/3 (70/30)
NYHA functional class: II/III (%)	2/8 (20/80)	2/8 (20/80)
Hypertension (%)	2 (20)	2 (20)
Duration of CHF (yrs)	12.1 ± 2.8	12.0 ± 1.3
Systolic blood pressure (mm Hg)	109 ± 5	114 ± 4
Diastolic blood pressure (mm Hg)	64 ± 4	65 ± 32
Heart rate (beats/min)	75 ± 4	75 ± 2
Fractional shortening (%)	18 ± 3	18 ± 1
LV diastolic diameter (mm)	66 ± 4	65 ± 1
LV systolic diameter (mm)	53 ± 3	54 ± 1
LA diameter (mm)	45 ± 2	45 ± 2
Pressure across tricuspid valve (mm Hg)	36 ± 3	34 ± 2
Plasma BNP levels (pg/ml)	246 ± 48	248 ± 26
Concomitant drugs, n (%)		
Digoxin	10 (100)	10 (100)
Diuretics except spironolactone	10 (100)	10 (100)
Nitrates	2 (20)	1 (10)
Beta-blockers	10 (100)	10 (100)
ACE inhibitors	9 (90)	7 (70)
ARB	0 (0)	3 (30)
Spironolactone	5 (50)	5 (50)

Values are either numbers of each group, range, or mean ± SEM.
Abbreviations as in Table 1.

frequency of readmission because of worsening of CHF was lower in the famotidine group compared with the control group (1 [4%] and 6 [24%], difference [95% confidence interval] 20% [2 to 38], $p < 0.05$).

As for the prospective randomized teprenone treatment protocol, medications were well tolerated over the 24-week period. The participants were recruited from September 2004 to October 2004. Participants attended clinic visits at the time of randomization (baseline) and at 4- to 8-week intervals for 24 weeks. All patients completed the protocol (20 of 20). No patients died during the 24-week study. In addition, the doses of beta-blockers, ACE inhibitors, and diuretics were not altered during the course of entire study. There were no differences in age, gender, or concurrent medications between the control and famotidine groups (Table 3). We found that teprenone does not affect the severity of CHF (the plasma BNP levels: 246 ± 48 pg/ml vs. 234 ± 49 pg/ml, LVDd: 65.8 ± 3.6 mm vs. 65.6 ± 3.7 mm, LVDs: 53.2 ± 2.9 mm vs. 53.8 ± 3.6 mm, FS: 18.7 ± 2.8% vs. 18.3 ± 1.3% before and 24 weeks after an administration of teprenone) in comparison with the patients without the teprenone treatment (the plasma BNP levels: 248 ± 26 pg/ml vs. 238 ± 18 pg/ml, LVDd: 65.1 ± 1.3 mm vs. 66.7 ± 1.5 mm, LVDs: 53.6 ± 1.4 mm vs. 55.1 ± 1.2 mm, FS: 17.7 ± 1.3% vs. 17.2 ± 1.0% at observation time of 0 and 24 weeks). The frequency of readmission because of worsening of CHF was identical between the teprenone and control groups (2 [20%] and 2 [20%]).

DISCUSSION

In the present study, we demonstrate that the blockade of histamine H_2 receptors favors the improvements of the pathophysiology of CHF via retrospective and prospective clinical trials. These conclusions propose the novel findings that histamine that stimulates histamine H_2 receptors is one of the neurohumoral factors for the worsening of CHF, and that the blockade of histamine H_2 receptors becomes the novel strategy for the treatment of CHF.

Histamine in failing hearts. We have shown that histamine release is augmented in the ischemic myocardium compared with the non-ischemic myocardium in dogs (unpublished data). When the mast cells that store histamine are stimulated by ischemia or mechanical stress, mast cells actively release histamine. There are reports that mast cells are found in the human heart (19) and have been implicated in cardiovascular diseases (15,20,21). Indeed, the increase of mast cells have been observed in the hearts of patients with hypertrophy (22), dilated cardiomyopathy, ischemic cardiomyopathy (23), and ischemia/reperfusion (24), and the infarction-related coronary arteries (25). Furthermore, histamine is present in high concentrations in cardiac tissues in most animal species, including humans (10,26,27), and its release from cardiac stores and the subsequent actions on the heart may be of importance in the pathophysiology of heart disease. These lines of evidence agree with the present observation that the blockade of histamine H_2 receptors in failing hearts has an impact on the pathophysiology of CHF.

The role of histamine receptors in failing hearts. The histamine receptors (H_1, H_2, H_3, and H_4) are all G protein-coupled molecules, and they transduce extracellular signals via Gq, Gs, and Gi/o, respectively (5,6,28). Specifically, histamine H_2 receptors are linked to Gs proteins that facilitate the production of cyclic adenosine monophosphate (AMP) as beta-adrenoreceptors are (29). Histamine H_2-receptor-stimulated cAMP accumulation or adenylyl cyclase activator has been demonstrated in a variety of tissues including gastric cells (10,30), vascular smooth muscle cells (31), brain (10,32), and cardiac tissue (10,33). Beta-adrenoreceptor blockers are known to be cardioprotective in failing hearts because the accumulation of cyclic AMP after the activation of beta-adrenoreceptors enhances both myocardial contractility and oxygen consumption, which deteriorates heart function in patients with CHF (34,35). In addition, it has been reported that histamine is a powerful vasoconstrictor in atherosclerotic coronary arteries (36), which may locally provoke coronary spasm and thus contribute to the onset of myocardial infarction (23). The importance of beta-adrenoreceptor blockers depends on the presence of both catecholamine and beta-adrenoreceptors in the heart. Therefore, because histamine H_2 receptors and histamine are located in failing human hearts, it is likely that blockers of histamine H_2 receptors are as cardioprotective against failing hearts as beta-adrenoreceptor blockers are.

Because famotidine decreases both blood pressure and heart rate, this may improve the pathophysiology of CHF. Indeed, the reduction of afterload or preload and heart rate seems to be an important factor in the treatment of CHF. This is also the case in either beta-adrenoreceptor blockers or ACE inhibitors in patients with heart failure. Either beta-adrenoreceptor blockers or ACE inhibitors are still effective independent of the reduction of loading condition to the heart, because they inhibit the signal transduction for deterioration of cardiac function. Because histamine increases cyclic AMP levels in the cardiomyocytes via histamine H_2 receptors, famotidine may be beneficial through both load-reduction-dependent and -independent mechanisms.

Clinical importance. Beta-adrenoreceptor blockers have been shown to be effective for treating ischemic heart diseases and heart failure (37), and histamine receptor blockers are similar to beta-adrenoreceptor blockers. Histamine plays an important role in the regulation and malregulation of cardiac and coronary function. Furthermore, the histamine receptor blockers such as famotidine that are used for peptic ulcers or GERD all over the world could be used for ischemic heart diseases. Furthermore, beta-adrenoreceptor blockers ameliorate the severity of heart failure, and histamine receptor blockers may be beneficial for patients with CHF. However, we should note that the 3 H_2 receptor blockers administered for 7 days at clinical dosages had no significant effect on left ventricular systolic function, aerobic metabolic performance, or exercise capacity in men with class II or III stable CHF (38). This suggests that a relatively long-term administration of histamine receptor blockers is necessary to mediate the cardioprotective effects of histamine receptor blockers in patients with CHF as a relatively long-term administration of beta-adrenoreceptor blockers is necessary for the treatment of CHF (37).

Moreover, because famotidine was administered in addition to the aggressive treatments with beta-adrenoreceptors blockers, ACE inhibitors, and diuretics, and we proved that famotidine further improves the pathophysiology of CHF, it is possible to develop famotidine for the drug of CHF, although we need to plan and perform a large-scale clinical trial for the investigation of the effects of famotidine on CHF. We also need to clarify the best dose of famotidine for the treatment of CHF.

Study limitations. The present study has several limitations that we need to pay attention to. First of all, the first part of the data was obtained from the retrospective analysis, and seemed to be influenced by many factors, although Table 1 showed low BNP levels and low ventricular volumes in the famotidine group suggested the preventive effects of famotidine on cardiac remodeling. To strengthen the hypothesis obtained by the retrospective study, we performed the prospective analysis using either famotidine or teprenone.

The second limitation is that the second part of the study was an open-labeled, randomized trial using small sampling size. However, to decrease these weaknesses, we used the objective end points such as the plasma BNP levels and left ventricular dimensions, and we also tried to exclude the subjective scope of the assessment of NYHA functional classification.

Third, the severities of pathophysiology of CHF in the retrospective and prospective studies were different. The severity of CHF in enrolled patients in prospective study is higher than that in the retrospective study. This is because we enrolled the patients from the different protocols. Nevertheless, because both studies suggest that famotidine is effective for patients with CHF, we may be able to suggest the beneficial effects of famotidine to treat patients with CHF.

Fourth, if teprenone could be deleterious to the pathophysiology of CHF, famotidine seemed to be beneficial compared with teprenone even if famotidine has no beneficial effects on CHF. Before planning the present study, we tested the effects of pathophysiology of CHF, and we found that teprenone has no beneficial or deleterious effects on CHF in the present study.

Fifth, either famotidine or teprenone may directly affect the plasma half-life or excretion of BNP. If this is the case, the plasma BNP levels may be altered independent of the improvements of CHF. We cannot deny this possibility, however, because left ventricular dimension becomes smaller in the famotidine group, suggesting that famotidine is beneficial for the heart of CHF patients.

Sixth, we should notice that an interaction of gastritis with heart failure could confound their conclusion regarding the effect of histamine blockade. Indeed, it may be still possible to consider that famotidine improves cardiac function via an improvement of GERD if GERD worsens CHF, because we have no positive or negative data to link GERD and CHF. We should investigate this possibility to explain the effects of H_2 receptor blockers on CHF in further study.

In summary, despite these limitations, we proposed the hypothesis that H_2 receptor blockers are effective for the treatment of CHF, and we need to verify the beneficial effects of H_2 receptor blockers such as ranitidine or cimetidine as well as famotidine in CHF patients with a large-scale trial.

Reprint requests and correspondence: Dr. Masafumi Kitakaze, Cardiovascular Division, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita City, Osaka Pref. 565-8565, Japan. E-mail: kitakaze@zf6.so-net.ne.jp.

REFERENCES

1. Braunwald E, Bristow MR. Congestive heart failure: fifty years of progress. Circulation 2000;102:14–23.

2. Torre-Amione G. The syndrome of heart failure: emerging concepts in the understanding of its pathogenesis and treatment. Curr Opin Cardiol 1999;14:193–5.
3. Packer M. Neurohormonal interactions and adaptations in congestive heart failure. Circulation 1988;77:721–30.
4. Drexler H, Hornig B. Importance of endothelial function in chronic heart failure. J Cardiovasc Pharmacol 1996;27:S9–12.
5. Hough LB. Genomics meets histamine receptors: new subtypes, new receptors. Mol Pharmacol 2001;59:415–9.
6. Leurs R, Bakker RA, Timmerman H, et al. The histamine H3 receptor: from gene cloning to H3 receptor drugs. Nat Rev Drug Discov 2005;4:107–20.
7. Gantz I, Schaffer M, DelValle J, et al. Molecular cloning of a gene encoding the histamine H2 receptor. Proc Natl Acad Sci U S A 1991;88:429–33.
8. Kim J, Washio T, Yamagishi M, et al. A novel data mining approach to the identification of effective drugs or combinations for targeted endpoints—application to chronic heart failure as a new form of evidence-based medicine. Cardiovasc Drugs Ther 2004;18:483–9.
9. Matsuda N, Jesmin S, Takahashi Y, et al. Histamine H1 and H2 receptor gene and protein levels are differentially expressed in the hearts of rodents and humans. J Pharmacol Exp Ther 2004;309: 786–95.
10. Hill SJ, Ganellin CR, Timmerman H, et al. International Union of Pharmacology. XIII. Classification of histamine receptors. Pharmacol Rev 1997;49:253–78.
11. Eckel L, Gristwood RW, Nawrath H, et al. Inotropic and electrophysiological effects of histamine on human ventricular heart muscle. J Physiol 1982;330:111–23.
12. Du XY, Schoemaker RG, Bos E, et al. Effects of histamine on porcine isolated myocardium: differentiation from effects on human tissue. J Cardiovasc Pharmacol 1993;22:468–73.
13. Hattori Y. Cardiac histamine receptors: their pharmacological consequences and signal transduction pathways. Methods Find Exp Clin Pharmacol 1999;21:123–31.
14. Kirch W, Halabi A, Hinrichsen H. Hemodynamic effects of quinidine and famotidine in patients with congestive heart failure. Clin Pharmacol Ther 1992;51:325–33.
15. Maronle G. The role of mast cells in cardiovascular disease. In: Matsumori A, editor. Cardiomyopathies in Heart Failure: Biomolecular, Infectious and Immune Mechanisms. Norwell, MA: Kluwer Academic Publishers, 2003:185–228.
16. Carlsson R, Dent J, Bolling-Sternevald E, et al. The usefulness of a structured questionnaire in the assessment of symptomatic gastroesophageal reflux disease. Scand J Gastroenterol 1998;33:1023–9.
17. Pollock ML, Lowenthal DT, Phillips MI, Staples ED. Abnormal neuroendocrine responses during exercise in heart transplant recipients. Circulation 1992;86:1453–63.
18. Goldstein M, Vincent JL, Kahn RJ. Evaluation of cardiac function by echo-Doppler studies in critically ill patients. Intensive Care Med 1988;14:406–10.
19. Dvorak AM. Mast-cell degranulation in human hearts. N Engl J Med 1986;315:969–70.
20. Marone G, de Crescenzo G, Adt M, Patella V, Arbustini E, Genovese A. Immunological characterization and functional importance of human heart mast cells. Immunopharmacology 1995;31:1–18.
21. Hara M, Ono K, Hwang MW, et al. Evidence for a role of mast cells in the evolution to congestive heart failure. J Exp Med 2002;195: 375–81.
22. Panizo A, Mindan FJ, Galindo MF, Cenarruzabeitia E, Hernandez M, Diez J. Are mast cells involved in hypertensive heart disease? J Hypertens 1995;13:1201–8.
23. Patella V, Marino I, Arbustini E, et al. Stem cell factor in mast cells and increased mast cell density in idiopathic and ischemic cardiomyopathy. Circulation 1998;97:971–8.
24. Frangogiannis NG, Perrard JL, Mendoza LH, et al. Stem cell factor induction is associated with mast cell accumulation after canine myocardial ischemia and reperfusion. Circulation 1998;98:687–98.
25. Laine P, Kaartinen M, Penttila A, et al. Association between myocardial infarction and the mast cells in the adventitia of the infarct-related coronary artery. Circulation 1999;99:361–9.
26. Wolff AA, Levi R. Histamine and cardiac arrhythmias. Circ Res 1986;58:1–16.
27. Bristow MR, Ginsburg R, Harrison DC. Histamine and the human heart: the other receptor system. Am J Cardiol 1982;49:249–51.
28. Hofstra CL, Desai PJ, Thurmond RL, et al. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. J Pharmacol Exp Ther 2003;305:1212–21.
29. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? Circ Res 2003;93:896–906.
30. Gespach C, Bouhours D, Bouhours JF, et al. Histamine interaction on surface recognition sites of H2-type in parietal and non-parietal cells isolated from the guinea pig stomach. FEBS Lett 1982;149:85–90.
31. Ottosson A, Jansen I, Edvinsson L. Pharmacological characterization of histamine receptors in the human temporal artery. Br J Clin Pharmacol 1989;27:139–45.
32. Al-Gadi M, Hill SJ. The role of calcium in the cyclic AMP response to histamine in rabbit cerebral cortical slices. Br J Pharmacol 1987;91: 213–22.
33. Johnson CL, Weinstein H, Green JP. Studies on histamine H2 receptors coupled to cardiac adenylate cyclase. Blockade by H2 and H1 receptor antagonists. Mol Pharmacol 1979;16:417–28.
34. Packer M, Coats AJ, Fowler MB, et al. Effect of carvedilol on survival in severe chronic heart failure. N Engl J Med 2001;344:1651–8.
35. Dargie HJ. Effect of carvedilol on outcome after myocardial infarction in patients with left-ventricular dysfunction: the CAPRICORN randomised trial. Lancet 2001;357:1385–90.
36. Ginsburg R, Bristow MR, Davis K, et al. Quantitative pharmacologic responses of normal and atherosclerotic isolated human epicardial coronary arteries. Circulation 1984;69:430–40.
37. Metra M, Nardi M, Giubbini R, et al. Effects of short- and long-term carvedilol administration on rest and exercise hemodynamic variables, exercise capacity and clinical conditions in patients with idiopathic dilated cardiomyopathy. J Am Coll Cardiol 1994;24:1678–87.
38. Lucas BD Jr., Williams MA, Mohiuddin SM, LaMadrid LJ, Schroeder LJ, Hilleman DE. Effect of oral H2-receptor antagonists on left ventricular systolic function and exercise capacity in patients with chronic stable heart failure. Pharmacotherapy 1998;18:824–30.

Clinical Chemistry 52:11
2049–2053 (2006)

Lipids, Lipoproteins and Cardiovascular Risk Factors

Component Analysis of HPLC Profiles of Unique Lipoprotein Subclass Cholesterols for Detection of Coronary Artery Disease

MITSUYO OKAZAKI,[1*] SHINICHI USUI,[2] AKIO FUKUI,[3] ISAO KUBOTA,[3] and HITONOBU TOMOIKE[4]

Background: **Patients with coronary artery disease (CAD) are known to have several lipoprotein abnormalities. We examined plasma cholesterol concentrations of major lipoproteins and their subclasses, using a gel permeation HPLC, to establish an association between a lipoprotein subclass pattern and the presence of CAD.**
Methods: **We performed a simple and fully automated HPLC, followed by mathematical treatment on chromatograms, for measuring cholesterol concentrations of major lipoproteins and their subclasses in 62 male patients (45 with CAD and 17 controls without CAD) who underwent cardiac catheterization.**
Results: **For major lipoprotein classes, the patient group had a significantly (P <0.05) higher LDL-cholesterol (LDL-C) and lower HDL-cholesterol (HDL-C), but no difference in VLDL-cholesterol (VLDL-C) concentrations. For lipoprotein subclasses, the patient group had a significantly higher small VLDL-C (mean particle diameter of 31.3 nm, P <0.001), small LDL-C (23.0 nm, P <0.05), and very small LDL-C (16.7–20.7 nm, P <0.001), but a significantly lower large HDL-C (12.1 nm, P <0.001) concentrations. Combined variables of "small VLDL-C + small LDL-C + very small LDL-C – large HDL-C" differentiated the patient from the control group more clearly than single-subclass measurements or calculated traditional lipid markers.**
Conclusions: **These results suggest the usefulness of multiple and simultaneous subclass analysis of proatherogenic and antiatherogenic lipoproteins and indicate that HPLC and its component analysis can be used for easy detection and evaluation of abnormal distribution of lipoprotein subclasses associated with CAD.**

Patients with coronary artery disease (CAD)[5] have several lipoprotein abnormalities, such as increased triglyceride (TG) and LDL-cholesterol (LDL-C), and decreased HDL-cholesterol (HDL-C) *(1, 2)*. In epidemiological studies, calculated non–HDL-C concentration [the difference of total cholesterol (TC) and HDL-C] was demonstrated to be a stronger predictor of cardiovascular events than plasma TC alone *(3, 4)*. Partially catabolized TG-rich lipoproteins such as intermediate-density lipoproteins and remnant-like particles (RLP) are considered to be highly atherogenic *(5)*. The atherogenic lipoprotein phenotype has been defined as the presence of a predominance of small, dense LDL particles and high TG and low HDL-C concentrations *(6, 7)*. Prospective studies have reported the small LDL phenotype to be an important predictor of subsequent CAD *(8, 9)*. Therefore, detailed analysis of both major lipoproteins and their subclasses, including remnant lipoproteins, might be required for more effective assessment of CAD risk status. Many techniques have been used for lipoprotein subclass analysis; analytical ultracentrifugation *(10)*, sequential separation at various densities *(11)*, rate zonal ultracentrifugation *(12)*, and density-gradient ultracentrifugation *(13)*. All these techniques are laborious and not available in routine clinical laboratories.

[1] Laboratory of Chemistry, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Japan.
[2] Faculty of Health Sciences, Okayama University Medical School, Okayama, Japan.
[3] Department of Cardiology, Pulmonology and Nephrology, Course of Internal Medicine and Therapeutics, Yamagata University School of Medicine, Yamagata, Japan.
[4] National Cardiovascular Center, Osaka, Japan.
* Address correspondence to this author at: Laboratory of Chemistry, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, 2-8-30, Kohnodai, Ichikawa-shi, Chiba 272-0827, Japan. e-mail okazaki.las@tmd.ac.jp.
Received March 12, 2006; accepted August 24, 2006.
Previously published online at DOI: 10.1373/clinchem.2006.070094

[5] Nonstandard abbreviations: CAD, coronary artery disease; TG, triglyceride; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; TC, total cholesterol; RLP, remnant-like particle; MI, myocardial infarction; AUC, area under the curve.

Lipoprotein particle size analysis is usually performed with nondenaturing gradient gel electrophoresis *(8, 9)*. Alternative techniques include a Lipoprint LDL system with a nongradient (3%) polyacrylamide gel electrophoretic method *(14)* and nuclear magnetic resonance *(15–17)*. In addition, Hirano et al. *(18)* reported a new technique for small LDL-C measurement that combines selective precipitation and a homogeneous LDL-C assay. Techniques for remnant lipoprotein measurement include a homogeneous assay developed by Miyauchi et al. *(19)* and an immunoseparation method by Nakajima et al. *(20)*. Detailed HDL subclass analysis has been performed with 2-dimentional gel electrophoresis *(21, 22)*.

HPLC with gel permeation columns is an alternative method for classifying and quantifying lipoproteins on the basis of differences of particle size *(23–25)*. Component analysis of cholesterol profiles after HPLC can provide useful information about almost all of the above-mentioned atherogenic lipoprotein subclasses *(26, 27)*.

In the current study, we applied simple and fully automated HPLC, followed by mathematical treatment analysis of chromatograms, to identify lipoprotein subclass patterns associated with the presence of CAD in men who underwent cardiac catheterization.

Materials and Methods

STUDY PARTICIPANTS

The 62 study patients, [mean (SD) age 62 (9) years, range 41–76 years], were selected from 609 consecutive male patients who underwent cardiac catheterization in Yamagata University Hospital. We excluded patients who had diabetes mellitus, renal or liver disease, or were receiving any lipid-lowering medication. All participants gave their informed consent before entering the study. The study was carried out according to guidelines of the institutional review board.

The presence of CAD was assessed by coronary angiography. Narrowing of the luminal diameter of the coronary artery by ≥75% was considered significant. Coronary vasospasm was induced by a provocation test with ergonovine (2–20 μg), and only total or subtotal occlusion was considered positive. According to these assessments, the CAD patient group (n = 45) comprised 21 patients who were myocardial infarction (MI) survivors (acute MI, 3; MI within 1 month, 11; previous MI, 7), 22 patients with angina (effort angina, 7; unstable angina, 4; vasospastic angina, 11), and 2 patients with silent myocardial ischemia with considerable coronary stenosis. The non-CAD group (n = 17) comprised patients without substantial coronary stenosis (atypical chest pain, 4; cardiomyopathy, 6; aortic valve disease, 4; electrocardiogram abnormality, 3).

After patients had fasted overnight, venous blood was drawn and placed in tubes containing disodium EDTA (1 g/L). Plasma samples were kept in a refrigerator and analyzed within 7 days after blood collection.

HPLC METHOD

Plasma lipoproteins were analyzed by HPLC, as previously described *(24, 27, 28)*. We defined 3 VLDL subclasses, 4 LDL subclasses, and 5 HDL subclasses according to lipoprotein particle size (diameter) from 20 component peaks [see Table 1. in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue11] determined on the basis of mean profiles of healthy and hyperlipidemic persons *(27)*.

OTHER CLINICAL AND LIPID PARAMETER ANALYSIS

We used commercial enzymatic reagent sets (Kyowa Medex) to measure plasma TG. Fasting blood sugar was measured by the enzymatic method (Arkray Inc.). We used medical records and questionnaires to obtain data on age, weight, height, smoking history, family history, hypertension, previous MI, angina pectoris, diabetes mellitus, and medication use. Body mass index was calculated from weight and height as weight (kg) per [height (m)]^2.

STATISTICAL ANALYSIS

Data were analyzed with SPSS (Version 10.0, SPSS Inc.). Continuous measures are expressed as mean (SD), and judged by Student *t*-test. Dichotomous variables were tested by means of χ^2 analysis. Area under the curve (AUC) of ROC curve was calculated to describe the power of variables to distinguish the CAD patients from the control individuals. A value of $P < 0.05$ was considered to be statistically different.

Results

Clinical characteristics, lipid and major lipoprotein concentrations in 62 men in this study are shown in Table 1. The patient group had significantly ($P < 0.05$) higher LDL-C and lower HDL-C, but there was no difference in VLDL-C concentrations.

Cholesterol concentrations in each lipoprotein subclass

Table 1. Clinical characteristics, lipid and major lipoprotein concentrations in studied participants.[a]

Variables	Patients with CAD, n = 45	Control participants without CAD, n = 17
Age, years	63.7 (8.7)	58.8 (9.1)
BMI, kg/m^2	23.6 (3.1)	22.4 (2.9)
FBS, mg/dL[b]	98.6 (16.2)	93.8 (13.1)
Current smoker, %	23 (51.1%)	5 (29.4%)
Hypertension, %	21 (46.7%)	10 (58.8%)
Family history, %	14 (31.1%)	7 (41.1%)
Plasma TG, mg/dL	103.8 (51.4)	84.9 (48.9)
Plasma TC, mg/dL	193.1 (36.6)	175.8 (33.1)
VLDL-C, mg/dL	27.9 (11.2)	22.4 (12.7)
LDL-C, mg/dL	121.7 (29.6)[c]	103.2 (24.9)
HDL-C, mg/dL	43.4 (10.0)[c]	50.1 (10.0)

[a] Data represent mean (SD) or the number (%) of participants.
[b] BMI, body mass index; FBS, fasting blood sugar.
[c] $P < 0.05$ (vs control participants).

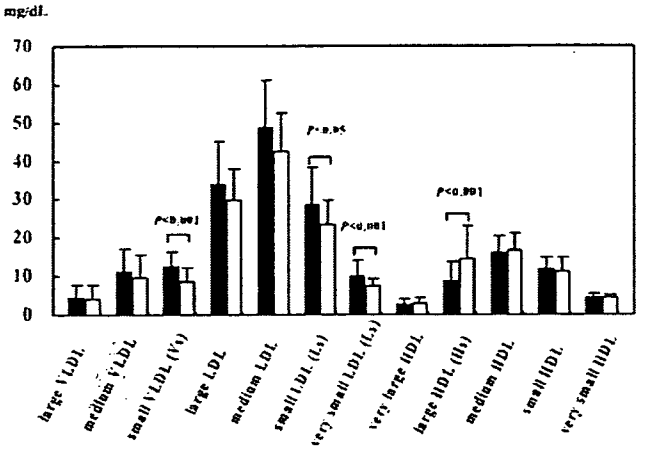

Fig. 1. Comparison of cholesterol concentrations in lipoprotein subclasses between the CAD patients (n = 45) and the control participants (n = 17).

The values are shown as mean (SD). *Closed* and *open columns* represent the CAD patient and the control groups, respectively.

are summarized in Fig. 1. The patient group had significant increases in small VLDL-C (P <0.001), small LDL-C (P <0.05), and very small LDL-C (P <0.001), but a significant decrease in large HDL-C (P <0.001).

To more clearly distinguish between the 2 groups, several derived variables were calculated from each subclass. Three subclasses (small VLDL, small LDL, and very small LDL), which were significantly increased in the patient group, were combined and expressed as Vs + Ls. Ls represented the sum of small LDL and very small LDL. The difference or the ratio of increased subclasses (Vs + Ls) and a decreased subclass (large HDL) was expressed as Vs + Ls − Hs, and (Vs + Ls)/Hs or Ls/Hs, respectively.

In Table 2, these derived variables by HPLC and the calculated traditional risk markers, non-HDL-C, TC/HDL-C, and LDL-C/HDL-C ratios, were compared between the patient and the control groups. All derived HPLC variables were significantly higher in the patient group, and Vs + Ls − Hs showed the highest significant differences (P <0.001) in the 2 groups. All the traditional risk markers, non-HDL-C, TC/HDL-C, and LDL-C/HDL-C ratios, were significantly higher in the patient group.

On ROC analysis, AUCs for the derived HPLC variables and the calculated traditional risk markers were estimated to describe the power of the variables to distinguish between the 2 groups. AUCs and the range of 95% confidence intervals are presented in Table 2. From ROC analysis, the derived HPLC variable Vs + Ls − Hs produced the greatest AUC, indicating that this variable was a more powerful discriminator than any other derived variable, including traditional risk markers.

Discussion

In this study, we compared for the first time lipoprotein cholesterol profiles between CAD patients and controls and propose a unique lipoprotein subclass pattern for identifying the patients at increased risk for CAD. HPLC has been used for decades in lipoprotein research applications and as a routine method *(24, 25, 27)*, but the present study is a first report for clinical and diagnostic testing of lipoprotein subclass analysis by HPLC in CAD patients. Analytical precision of HPLC was previously demonstrated to be acceptable in the determination of 3 VLDL, 4 LDL, and 5 HDL subclasses, with CV values of 1%–4% (n = 5), results that were comparable to those obtained with major lipoprotein quantification *(27, 28)*. Our HPLC and the traditional methods [Friedewald equation for LDL-C *(29)* and the precipitation method for

Table 2. Comparison of derived HPLC and traditional variables between the patients with CAD and the control participants without CAD.

Derived variables[a]	Patients with CAD, n = 45	Control particpants without CAD, n = 17	AUC[b] n = 62	95% CI[c]
HPLC				
Vs+Ls−Hs, mg/dL	42.9 (16.7)[d]	25.1 (16.8)	0.773[d]	0.645–0.900
Vs+Ls, mg/dL	51.3 (14.5)[e]	39.6 (10.2)	0.749[e]	0.620–0.878
Ls, mg/dL	38.8 (13.4)[e]	30.9 (7.9)	0.677[f]	0.543–0.811
(Vs+Ls)/Hs	9.5 (6.9)[e]	4.2 (3.4)	0.759[e]	0.631–0.888
Ls/Hs	7.3 (5.7)[e]	3.2 (2.6)	0.749[e]	0.618–0.880
Traditional				
Non HDL-C (mg/dL)	149.6 (34.1)[f]	125.7 (34.7)	0.710[f]	0.555–0.865
TC/HDL-C	4.6 (1.1)[e]	3.7 (1.1)	0.745[e]	0.596–0.894
LDL-C/HDL-C	2.9 (0.8)[e]	2.2 (0.8)	0.748[e]	0.592–0.903

[a] Vs+Ls−Hs, small VLDL-C + small LDL-C + very small LDL-C − large HDL-C; Vs + Ls, small VLDL-C + small LDL-C + very small LDL-C; Ls, small LDL-C + very small LDL-C; (Vs+Ls)/Hs, (small VLDL-C + small LDL-C + very small LDL-C)/large HDL-C ratio; Ls/Hs, (small LDL-C + very small LDL-C)/large HDL-C ratio; non-HDL-C, the difference of TC and HDL-C.

[b] The area under the ROC curve (AUC) discriminating the patients with CAD from the control participants without CAD.

[c] CI, confidence interval.

[d] P <0.001. [e] P <0.01. [f] P <0.05.

HDL-C *(30)*] were in agreement for LDL-C and HDL-C values ($r > 0.97$), as described previously *(27)*.

As presented in Tables 1 and 2, significantly higher LDL-C and lower HDL-C concentrations ($P < 0.05$) were observed in the patient group, and these significant differences were more clearly differentiated by computed values of TC/HDL-C or LDL-C/HDL-C ($P < 0.01$). Non-HDL-C has been recently recognized as one of the calculated risk markers for CAD *(3, 4)* but was less significant ($P < 0.05$) in this study (Table 2.).

In the subclass analysis by HPLC (Fig. 1), small VLDL-C, small LDL-C, very small LDL-C, and large HDL-C were found to be significantly different between the 2 groups. Increased small VLDL-C in the CAD group might represent an increase in remnant lipoproteins, although neither intermediate-density lipoprotein-C nor RLP-C was measured in this study. Our previous study showed that RLP fractions isolated by an immunoaffinity separation were very heterogeneous *(31)*, but the particle size of RLP from Type III hyperlipidemia corresponded mainly to small VLDL (peak 7). In another previous study, all VLDL subclasses were positively correlated with visceral fat area, and small VLDL remained considerable after adjustment for serum TG concentration *(27)*. In conjunction with these previous studies, the increase of small VLDL-C in CAD patients also supports the concept that smaller, partially catabolized triglyceride-rich lipoprotein (VLDL remnants) and/or a part of intermediate-density lipoprotein are atherogenic.

Although HDL subclasses are also heterogeneous and their atherogenic properties differ between subclasses *(32)*, many investigators suggest that measuring HDL subclasses may provide additional information about risk for the development of CAD. In this study, AUC for large HDL was larger than total HDL-C (results not shown), indicating the potential usefulness of HDL subclass analysis.

Increased small LDL-C and very small LDL-C in the CAD patients were consistent with atherogenic profiles of increasing remnant lipoproteins as well as small, dense LDL reported by Cohn et al. *(33, 34)*, but their ability to differentiate between the CAD patients and the controls was not as strong when compared with small VLDL-C, judged by AUC (results not shown).

To more clearly differentiate between the 2 groups, several derived variables were calculated from each subclass, as shown in Table 2. AUC for all derived HPLC variables except for Ls (small LDL + very small LDL) was larger than that for traditional risk marker, and Vs + Ls − Hs produced the largest AUC. These observations indicate that the new parameter, Vs + Ls − Hs, might be useful for total interpretation of both proatherogenic and antiatherogenic lipoproteins and provide additional clinical information to evaluate the risk status for CAD.

In conclusion, component analysis after HPLC provided the cholesterol concentrations of major lipoproteins and their subclasses within 16 min with a small volume of plasma or serum (<10 μL). Our results support the general concept of the usefulness of lipoprotein subclass analysis for diagnostic testing. Larger clinical trials are needed to establish the diagnostic significance of our proposed parameter, Vs + Ls − Hs, for identifying the patients at increased risk for CAD.

We gratefully acknowledge Kyowa Medex, Japan for providing enzyme reagents for the cholesterol measurement by HPLC. We greatly thank Skylight Biotech Inc. (Akita, Japan) for technical assistances in this study.

References

1. Kannel WB, Castelli WP, Gordon T, McNamara PM. Serum cholesterol, lipoproteins and risk of coronary heart diseases. The Framingham study. Ann Intern Med 1971;74:1–12.
2. Wilson PW, Abbott RD, Castelli WP. High density lipoprotein cholesterol and mortality. The Framingham Heart Study. Arteriosclerosis 1988;8:737–41.
3. Grundy SM. Low-density lipoprotein, non-high-density lipoprotein, and apolipoprotein B as targets of lipid-lowering therapy. Circulation 2002;106:2526–9.
4. Frost PH, Havel RJ. Rationale for use of non-high-density lipoprotein cholesterol rather than low-density lipoprotein cholesterol as a tool for lipoprotein cholesterol screening and assessment of risk and therapy. Am J Cardiol 1998;81:26B–31B.
5. Zilversmit DB. Atherogenic nature of triglycerides, postprandial lipidemia, and triglyceride-rich remnant lipoproteins. Clin Chem 1995;41:153–8.
6. Austin MA, King MC, Vranizan KM, Krauss RM. Atherogenic lipoprotein phenotype: a proposed genetic marker for coronary heart disease risk. Circulation 1990;82:495–506.
7. Campos H, Genest JJ Jr, Blijlevens E, McNamara JR, Jenner JL, Ordovas JM, et al. Low density lipoprotein particle size and coronary artery disease. Arterioscler Thromb 1992;12:187–95.
8. Lamarche B, Tchernof A, Moorjani S, Cantin B, Dagenais GR, Lupien PJ, et al. Small, dense low-density lipoprotein particles as a predictor of the risk of ischemic heart disease in men. Circulation 1997;95:69–75.
9. St-Pierre AC, Cantin B, Dagenais GR, Mauriege P, Bernard PM, Despres JP, et al. Low-density lipoprotein subfractions and the long-term risk of ischemic heart disease in men: 13-year follow-up data from the Quebec Cardiovascular Study. Arterioscler Thromb Vasc Biol 2005;25:553–9.
10. Krauss RM, Lindgren FT, Ray RM. Interrelationship among subgroups of serum lipoproteins in normal human subjects. Clin Chim Acta 1980;104:275–90.
11. Havel RJ, Eder HA, Bragdon J. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955;34:1345–53.
12. Patsch JR, Patsch W. Zonal ultracentrifugation. Method Enzymol 1986;129:3–26.
13. Shen MM, Krauss RM, Lindgren FT, Forte TM. Heterogeneity of serum low density lipoproteins in normal human subjects. J Lipid Res 1981;22:236–44.
14. Hoefner DM, Hodel SD, O'Brien JF, Branum EL, Sun D, Meissner I, et al. Development of a rapid, quantitative method for LDL subfractionation with use of the Quantimetrix Lipoprint LDL System. Clin Chem 2001;47:266–74.
15. Otvos JD, Jeyarajah EJ, Bennett DW, Krauss RM. Development of a proton nuclear magnetic resonance spectroscopic method for

determining plasma lipoprotein concentrations and subspecies distributions from a single, rapid measurement. Clin Chem 1992; 38:1632–8.
16. Kuller L, Arnold A, Tracy R, Otvos J, Burke G, Psaty B, et al. Nuclear magnetic resonance spectroscopy of lipoproteins and risk of CHD in the Cardiovascular Health Study. Arterioscler Thromb Vasc Biol 2002;22:1175–80.
17. Freedman DS, Otvos JD, Jeyarajah EJ, Shalaurova I, Cupples LA, Parise H, et al. Sex and age differences in lipoprotein subclasses measured by nuclear magnetic resonance spectroscopy: The Framingham Study. Clin Chem 2004;50:1189–200.
18. Hirano T, Ito Y, Saegusa H, Yoshino G. A novel and simple method for quantification of small, dense LDL. J Lipid Res 2003;44: 2193–201.
19. Miyauchi K, Kayahara N, Mori H, Sugiuchi H, Irie T. Development of homogeneous assay for measuring remnant-like particle cholesterol in serum [abstract]. Clin Chem 2005;51:A129.
20. Nakajima K, Saito T, Tamura A, Suzuki M, Nakano T, Adachi M, et al. Cholesterol in remnant-like lipoproteins in human serum using monoclonal anti apolipoprotein B-100 and anti apolipoprotein A-I immunoaffinity mixed gels. Clin Chim Acta 1993;223:53–71.
21. Ishida BY, Frolich J, Fielding CJ. Prebeta-migrating high density lipoprotein: quantitation in normal and hyperlipidemic plasma by solid phase radioimmunoassay following electrophoretic transfer. J Lipid Res 1987;28:778–86.
22. Asztalos BF, Lefevre M, Foster TA, Tulley R, Windhauser M, Wong L, et al. Arterioscler Thromb Vasc Biol 1997;17:1885–93.
23. Hara I, Okazaki M. High-performance liquid chromatography of serum lipoproteins. Methods Enzymol 1986;129:57–78.
24. Okazaki M, Usui S, Hosaki S. Analysis of plasma lipoproteins by gel permeation chromatography. In: Rifai N, Warnick GR, Dominiczak MH, eds. Handbook of Lipoprotein Testing. Washington DC: AACC Press, 2000:647–69.
25. Usui S, Hara Y, Hosaki S, Okazaki M. A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. J Lipid Res 2002;43:805–14.
26. Kazama H, Usui S, Okazaki M, Hosoi T, Ito H, Orimo H. Effects of bezafibrate and pravastatin on remnant-like lipoprotein particles and lipoprotein subclasses in type 2 diabetes. Diabetes Res Clin Pract 2003;59:181–9.
27. Okazaki M, Usui S, Ishigami M, Sakai N, Nakamura T, Matsuzawa Y, Yamashita S. Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in high-performance liquid chromatography. Arterioscler Thromb Vasc Biol 2005;25:578–84.
28. Usui S, Nakamura M, Jitsukata K, Nara M, Hosaki S, Okazaki M. Assessment of between-instrument variations in a HPLC method for serum lipoproteins and its traceability to reference methods for total cholesterol and HDL-cholesterol. Clin Chem 2000;46:63–72.
29. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499–502.
30. Okazaki M, Sasamoto K, Muramatsu T, Hosaki S. Evaluation of precipitation and direct methods for HDL-cholesterol assay by HPLC. Clin Chem 1997;43:1885–90.
31. Okazaki M, Usui S, Tada N, Nakano T, Nakajima K. The relation between RLP-triglyceride to RLP-cholesterol ratio in RLP-cholesterol profiles by HPLC. Clin Chim Acta 2000;296:135–49.
32. Silverman DI, Ginsburg GS, Pasternak RC. High-density lipoprotein subfractions. Am J Med 1993;94:636–45.
33. Cohn JS, Marcoux C, Davignon J. Detection, quantification, and characterization of potentially atherogenic triglyceride-rich remnant lipoproteins. Arterioscler Thromb Vasc Biol 1999;19:2474–86.
34. Twickler TB, Dallinga-Thie GM, Cohn JS, Chapman MJ. Elevated remnant-like particle cholesterol concentration: a characteristic feature of the atherogenic lipoprotein phenotype. Circulation 2004;109:1918–25.

Assessment of Genetic Effects of Polymorphisms in the MCP-1 Gene on Serum MCP-1 Levels and Myocardial Infarction in Japanese

Naoharu Iwai, MD; Kazuaki Kajimoto, PhD; Yoshihiro Kokubo, MD; Akira Okayama, MD; Shunichi Miyazaki, MD; Hiroshi Nonogi, MD; Yoichi Goto, MD; Hitonobu Tomoike, MD

Circulation Journal
Vol.70 No.7 July 2006
(Pages 805–809)