Table 1
Comparison of patients with chronic hepatitis C (C-CH) and chronic hepatitis B (B-CH)

	C-CH (N = 50)	B-CH (N = 50)	P
Age (years)	57.3±9.0	55.2±11.4	0.257
Sex	30:20	30:20	–
BMI (kg/m^2)	22.4±2.9	21.9±2.9	0.434
ALT (IU/l)	50.1±40.6	57.3±70.7	0.534
Albumin (g/dl)	4.21±0.21	4.16±0.20	0.282
Prothrombin time (%)	90.2±11.9	89.8±11.6	0.859
Total cholesterol (mg/dl)	167.4±37.7	195.6±38.3	<0.0005*
HDL-cholesterol (mg/dl)	55.2±16.7	59.7±17.6	0.277
Triglyceride (mg/dl)	122.8±63.6	129.8±67.5	0.637
Apolipoprotein AI (mg/dl)	134.5±20.1	140.2±29.5	0.263
Apolipoprotein AII (mg/dl)	28.8±5.9	28.5±6.4	0.787
Apolipoprotein B (mg/dl)	78.1±21.3	105.8±24.4	<0.0001*
Apolipoprotein CII (mg/dl)	2.15±1.35	3.98±1.87	<0.0001*
Apolipoprotein CIII (mg/dl)	6.74±2.17	8.87±2.60	<0.0001*
Apolipoprotein E (mg/dl)	4.17±1.08	4.31±0.95	0.618
Serum HCV-RNA (KIU/ml)	245±200		
HCV genotype 1b	40		
2a	10		

*Statistically significant.

kit (Amplicor HCV monitor v2.0, Roche Diagnostic Systems, Branchburg, NJ). The genotype of HCV was determined by RT-PCR with primers in the core region as described previously [20].

2.3. Laboratory investigations

Levels of total and HDL cholesterol, triglycerides and other biochemical parameters including apolipoproteins measured in the serum or plasma were using an auto-analyzer (Hitachi 7600 auto-analyzer, Tokyo, Japan). All assays were performed using fresh serum samples drawn from patients after at least 12 h fasting without taking alcohol overnight.

2.4. Statistical analysis

Results are expressed as means±S.E. The significance of the difference of means was determined using Student's t-test. Differences are considered significant when $P < 0.05$.

3. Results

Serum apolipoprotein levels and other parameters were determined; serum samples were drawn from patients infected with HCV and those with HBV (control) after at least 12 h of fasting. As shown in Table 1, there was no significant difference in age, sex ratio or BMI between the patients infected with HCV and those with HBV. Moreover, there was no difference in liver function between the two groups as assessed by alanine aminotransferase (ALT) or albumin level or prothrombin time level. Total cholesterol, and apolipoproteins B, CII and CIII levels were significantly lower in patients infected with HCV than those with HBV, while there was no significant difference in the levels of apolipoprotein AI, AII or E between these two groups (Table 1). Thus, there was dyslipidemia in patients with HCV infection, although there was no significant difference in the synthesis function of the liver between the two groups. There was no significant difference in serum lipid profile between the patients with histological degrees of F1 and F2, although the number of patients with F1 was small.

By analysis of the HCV genotype, 40 of the 50 chronic hepatitis C patients were found to have genotype 1b HCV, while the remaining ten patients had genotype 2a HCV. There were no patients with genotype 2b or other genotypes. These results are compatible with the prevalence of HCV genotypes in Japan as reported previously, where the prevalence of genotype 1b is about 70%, genotype 2a about 25% and genotype 2b about 5% [21,22]. There was no significant difference in age, sex ratio, BMI, serum albumin level, prothrombin time, platelet count or serum HCV-RNA level between the patients infected with genotype 1b HCV and those with genotype 2a HCV. Total cholesterol, and apolipoproteins B,

CII and CIII levels were significantly lower in patients infected with genotype 1b HCV than those with genotype 2a HCV, while there was no significant difference in the level of apolipoprotein AI, AII or E between the two groups (Table 2). There was no significant difference in the levels of apolipoproteins between the patients infected with genotype 2a HCV and those infected with HBV. There was no significant correlation between the levels of HCV-RNA and lipid profiles in patients with genotype 1b HCV infection. However, it might be possible that analysis of a larger number of patients leads to a significant correlation.

4. Discussion

Disturbance in lipid metabolism in HCV infection has been suggested by several lines of evidence: (1) steatosis in the liver of hepatitis C patients [3–5], (2) steatosis in the liver of transgenic mice harboring the HCV core gene or the entire HCV genome [10,11], (3) a possible role of LDL receptors in HCV entry into cells [6,7] and (4) association between the HCV core protein and apolipoprotein AII in an experimental system [8,9]. In addition, hypocholesterolemia has recently been documented in HCV infection and suggested as a possible basis for steatosis [17]. Interestingly, these reports from European countries stated that only HCV of genotype 3a is associated with hepatic steatosis or hypocholesterolemia, while HCV of genotype 1, 2 or 4 is scarcely associated with lipid metabolism disturbance, particularly with dyslipidemia [15–17]. However, the association of hepatic steatosis with HCV infection has also been documented in Japan, where about 70% of HCV infection was of genotype 1b [21,22]. In addition, HCV constructs used in the experimental steatosis mouse models are of genotype 1b from Japanese patients [10,11]. Therefore, it is of a great importance to assess whether chronic hepatitis C with genotype 1b HCV in Japan has an abnormality in lipid metabolism represented by dyslipidemia.

Our current results clearly indicate that patients with HCV infection in Japan have disturbances in lipid metabolism compared with those with HBV infection, and the disorder is attributed not to genotype 2a but to genotype 1b HCV infection, although the number of patients with genotype 2a HCV may be small. In our cohort, this is not due to other common causes of dyslipidemia, such as malignancy, poor liver function, intestinal malabsorption or inherited disorders of lipids. Indeed, cirrhotic patients were excluded, and patients with

Table 2
Comparison of patients with hepatitis C virus of genotype 1b and genotype 2a

	Genotype 1b ($N = 40$)	Genotype 2a ($N = 10$)	P
Age (years)	57.6±6.1	56.1±5.9	0.493
Sex	24:16	6:4	–
BMI (kg/m^2)	22.4±3.0	22.1±2.2	0.694
Albumin (g/dl)	4.23±0.21	4.12±0.21	0.142
Platelet count (× 10^4/μl)	17.6±5.0	16.8±3.1	0.658
PT (%)	90.0±12.4	93.6±8.5	0.386
Total cholesterol (mg/dl)	159.0±27.4	211.9±44.4	<0.0001*
HDL-cholesterol (mg/dl)	53.4±13.5	60.6±24.7	0.297
Triglyceride (mg/dl)	121.5±66.0	128.4±55.5	0.788
Apolipoprotein A I (mg/dl)	133.8±20.0	137.1±21.3	0.636
Apolipoprotein AII (mg/dl)	28.6±5.9	29.8±6.0	0.541
Apolipoprotein B (mg/dl)	76.3±15.7	89.7±26.8	0.039*
Apolipoprotein CII (mg/dl)	1.86±0.92	3.18±2.04	0.003*
Apolipoprotein CIII (mg/dl)	6.35±1.40	8.09±3.62	0.018*
Apolipoprotein E (mg/dl)	4.07±0.88	4.49±1.58	0.383
Serum HCV-RNA (KIU/ml)	247±199	234±218	0.868

*Statistically significant.

or without dyslipidemia were not significantly different in terms of prothrombin time or serum albumin level. It is not clear whether or not genotype 3a HCV is more closely associated with disturbances in lipid metabolism than genotype 1b in Japan, because there are very few, if any, patients with genotype 3a HCV infection in Japan [21,22].

It is of an interest that not only apolipoprotein B but also apolipoprotein CII and CIII levels were low in patients with genotype 1b HCV infection in Japan, whereas apolipoprotein AI, AII and E levels were similar to those in control groups. Because apolipoprotein CII and CIII are present in both high-density lipoprotein (HDL) and very-low density lipoprotein) (VLDL), and apolipoprotein B is present in VLDL and LDL, impairment of synthesis or secretion of VLDL in the liver may explain these observations. In a mouse model of hepatic steatosis that is transgenic for the HCV core gene, secretion of VLDL from the liver is disrupted chiefly due to the decrease in the level of the microsomal triglyceride transfer protein [23]. Moreover, it might be interesting to know that peroxisome proliferator-activated receptor-α (PPAR-α) activates the transcription of *apolipoprotein AI* and *AII* genes and suppresses the *apolipoprotein CII* and *CIII* genes in humans [24,25]. In fact, association of the HCV core protein with PPAR-α has been observed in an experimental system (Tanaka N, Moriya K, Kamijo Y, Kiyosawa K, Koike K, Aoyama T., unpublished data). Further studies are necessary to clarify the mechanism underlying lipid disturbance in HCV infection.

It is unclear at present why there is a difference in observations for genotype 1b HCV, which may induce lipid metabolism disturbances, between Japan and European countries. It may be noteworthy that determining the amino acid sequences of nonstructural region 5A of genotype 1b HCV genome is useful for the prediction of IFN responsiveness of patients in Japan but is not effective in studies from European countries [26,27]. Thus, there may be some differences in amino acid sequences, which are responsible for the development of distinct features of hepatitis C including hepatic steatosis, in the genotype 1b HCV clones between Japan and European countries. Further studies are required to elucidate the 'steatogenic' region in the HCV genome.

References

[1] Saito I, Miyamura T, Ohbayashi A, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 1990;87:6547–9.

[2] Simonetti RG, Camma C, Fiorello F, et al. Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. Ann Intern Med 1992;116:97–102.

[3] Bach N, Thung SN, Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. Hepatology 1992;15:572–7.

[4] Lefkowitch JH, Schiff ER, Davis GL, et al. Pathological diagnosis of chronic hepatitis C: a multicenter comparative study with chronic hepatitis B. Gastroenterology 1993;104:595–603.

[5] Scheuer PJ, Ashrafzadeh P, Sherlock S, Brown D, Dusheiko GM. The pathology of chronic hepatitis C. Hepatology 1992;15:567–71.

[6] Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX. Hepatitis C virus and other flaviviridae viruses enter cells via low-density lipoprotein receptor. Proc Natl Acad Sci USA 1996;96:12766–71.

[7] Monazahian M, Bohme I, Bonk S, et al. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. J Med Virol 1999;57:223–9.

[8] Barba G, Harper F, Harada T, et al. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplet. Proc Natl Acad Sci USA 1997;94:1200–5.

[9] Sabile A, Perlemuter G, Bono F, et al. Hepatitis C virus core protein binds to apolipoprotein AII and its secretion is modulated by fibrates. Hepatology 1999;30:1064–76.

[10] Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, et al. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J Gen Virol 1997;78:1527–31.

[11] Lerat H, Honda M, Beard MR, et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterology 2002;122:352–65.

[12] Moriya K, Todoroki T, Tsutsumi T, et al. Increase of carbon 18 mono-unsaturated fatty acids in the liver of hepatitis C: Analysis in transgenic mice and humans. Biophys Biochem Res Commun 2001;281:1207–12.

[13] Moriya K, Fujie H, Shintani Y, et al. Hepatitis C virus core protein induces hepatocellular carcinoma in transgenic mice. Nature Med 1998;4:1065–8.

[14] Pawlotsky JM, Tsakiris L, Roudot-Thoraval F, et al. Relationship between hepatitis C virus genotypes and

sources of infection in patients with chronic hepatitis C. J Infect Dis 1999;171:1607–10.
[15] Rubbia-Brandt L, Quadri R, Abid K, et al. Hepatocyte steatosis is a cytopathic effect of hepatitis C virus genotype 3. J Hepatol 2000;33:106–15.
[16] Mihm S, Fayyazi A, Hartmann H, Ramadori G. Analysis of histopathological manifestations of chronic hepatitis C virus infection with respect to virus genotype. Hepatology 1997;25:735–9.
[17] Serfaty L, Andreani T, Giral P, Carbonell N, Chazouilleres O, Poupon R. Hepatitis C virus induced hypobetalipoproteinemia: a possible mechanism for steatosis in chronic hepatitis C. J Hepatol 2001;34:428–34.
[18] Fujie H, Yotsuyanagi H, Moriya K, et al. Steatosis and intrahepatic hepatitis C virus in chronic hepatitis. J Med Virol 1999;59:141–5.
[19] Haruna Y, Miyamoto T, Yasunami R, Kanda T, Fushimi H, Kotoh K. Human leukocyte antigen DRB1 1302 protects against bile duct damage and portal lymphocyte infiltration in patients with chronic hepatitis C. J Hepatol 2000;32:837–42.
[20] Yotsuyanagi H, Koike K, Yasuda K, et al. Hepatitis C virus genotypes and development of hepatocellular carcinoma. Cancer 1995;76:1352–5.
[21] Okamoto H, Mishiro S. Genetic heterogeneity of hepatitis C virus. Intervirology 1994;37:68–76.
[22] Ohno O, Mizokami M, Wu RR, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. J Clin Microbiol 1997;35:201–7.
[23] Perlemuter G, Sabile A, Letteron P, et al. Hepatitis C virus core protein inhibits triglyceride-transfer protein activity and very low-density lipoprotein secretion: a model of viral-related steatosis. FASEB J 2002;16:185–94.
[24] Vu-Dac N, Schoonjans K, Laine B, Fruchart JC, Auwerx J, Staels B. Negative regulation of the human apolipoprotein A-I promoter by fibrates can be attenuated by the interaction of the peroxisome proliferator-activated receptor with its response element. J Biol Chem 1994;269:31012–8.
[25] Vu-Dac N, Schoonjans K, Kosykh V, et al. Fibrates increase human apolipoprotein A-II expression through activation of the peroxisome proliferator-activated receptor. J Clin Invest 1995;96:741–50.
[26] Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. New Eng J Med 1996;334:77–81.
[27] Brechot C. The direct interplay between HCV NS5A protein and interferon transduction signal: from clinical to basic science. J Hepatol 1999;30:1152–4.

Despite their results, even if they seem strongly suggest an important role for TNF-α, is our opinion that the responsibility of this cytokine should be considered carefully.

In our previous study (Int J Immunopathol and Pharmacol [in press]) we assayed the TNF-α serum levels in patients with and without DM during CHC, not finding any statistical significant differences in its concentrations. In our groups, all the patients did not present obesity or hypertrigliceridemia or else hypercholesterolemia.

Taking into consideration that insulin resistance and DM type 2 are correlated to peripheral alteration of glucose metabolism TNF related[7] and on the light of the authors evidences on transgenic mice model how we could take to mean our results?

These evidences taken together seem suggest the presence of 2 different network in CHC; hepatic and peripheral.

In the liver the likely source of TNF-α production should be the Kupffer cells,[8] so have the authors evaluated these cells at a liver biopsy or TNF-α concentrations in serum?

Otherwise, how they explain the increased levels found, considering also that the transgenic mice do not present the same inflammation of an HCV infected patient?

The significance of TNF-α augmented serum levels during CHC natural history, and its possible effects on metabolism seem to be not so clear. Consequently, considering that other cytokines are been suggested in diabetes mellitus as IL-6[9]; how we can judge as guilty the TNF-α?

Moreover, making an allowance for the infective and immune adverse effects,[10] which should be the rational of an eventual anti-TNF treatment in infected patients?

In conclusion, although the interesting work of Shintani et al., on transgenic mice, focuses new attention on HCV direct role on insulin resistance, the fascinating connection with a possible cytokine environments in this metabolic disorder is really still unclear. A wider cytokine network evaluation both in liver and periphery is required to understand the intricate inflammatory network in DM pathogenesis in humans during chronic hepatitis C.

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1. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCullough AJ, Natale S, Forlani G, Melchionda N. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes 2001;50:1844–1850.
2. Aytug S, Reich D, Sapiro LE, Bernstein D, Begum N. Impaired IRS-1/PI3-kinase signaling in patients with HCV: a mechanism for increased prevalence of type 2 diabetes. Hepatology 2003;38: 1384–1392.
3. Knobler H, Zhornicky T, Sandler A, Haran N, Ashur Y, Schattner A. Tumor necrosis factor-α-induced insulin resistance may mediate the hepatitis C virus-diabetes association. Am J Gastroenterol 2003;98:2751–2756.
4. Hotamisligil GS. The role of TNF-α and TNF receptors in obesity and insulin resistance. J Intern Med 1999;245:621–625.
5. Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Tsukamoto K, Kimura S, Moriya K, Koike K. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. Gastroenterology 2004;126:840.
6. Nelson DR, Lim HL, Marousis CG, Fang JW, Davis GL, Shen L, Urdea MS, Kolberg JA, Lau JY. Activation of tumor necrosis factor-alpha system in chronic hepatitis C virus infection. Dig Dis Sci 1997;42:2487.
7. Hotamisligil GS. The role of TNF-α and TNF receptors in obesity and insulin resistance. J Intern Med 1999;245:621–625.
8. Dong W, Simeonova PP, Gallucci R, Matheson J, Fannin R, Montuschi P, Flood L, Luster MI. Cytokine expression in hepatocytes: role of oxidant stress. J Interferon Cytokine Res 1998;18:629–638.
9. Klover PJ, Zimmers TA, Koniaris LG, Mooney RA. Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice. Diabetes 2003;52:2784–2789.
10. Gomez-Reino JJ, Carmona L, Valverde VR, Mola EM, Montero MD, BIOBADASER Group. Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. Arthritis Rheum 2003;48:2122–2127.

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Reply. We appreciate the comments of Perrella et al.[1] on our recent study published in GASTROENTEROLOGY. We realized afresh that the association of diabetes and hepatitis C virus (HCV) infection is a major concern to gastroenterologists and researchers in related fields. We also appreciate their comment that our study has focused a new attention on the direct role of HCV on insulin resistance. Their key point is that it is not clear yet whether TNF-α is a central mediator for insulin resistance in chronic HCV infection according to their data and the considerations on the cytokine network.

We agree to their comment in the point that we should be careful in assessing the role of TNF-α in the genesis of insulin resistance in HCV infection, in particular, in human patients, where numerous other factors than those in an animal model would play roles in glucose homeostasis. In our article, we have stated that the increased level of TNF-α is one of the bases for insulin resistance in the mouse model but impairment in other undetermined pathways may also be responsible for the development of insulin resistance in HCV infection.[1] Insulin resistance in our mouse model is chiefly the central one, i.e., derived from the liver: suppression of insulin action on the hepatic glucose production is inhibited. Considering this specific involvement of the liver, the levels of TNF-α not in the serum but in the liver would be essential in development of insulin resistance in this mouse model. Unfortunately, we cannot access the data by Perrella et al. that there was no significant difference in serum TNF-α levels between the hepatitis C patients with and without diabetes, but we suppose the number of patients analyzed was large enough to verify the absence of difference. Nonetheless, the levels of TNF-α in the liver, where HCV replicates, may be critical in development of the central insulin resistance.

Naturally, it is possible that the core protein operates directly to inhibit insulin action of tyrosine-phosphorylation of insulin receptor sustrate-1,[1] which inhibition was, interestingly, also observed in the liver of chronic hepatitis C patients.[2] On the other hand, because the expression of the core protein was virtually limited to the liver in the mice,[3] the contribution of peripheral factor to insulin resistance,[1] which may be present in human hepatitis C patients, could not be evaluated in our study. Mitochondrial dysfunction, suggested to have

a role in insulin resistance in the elderly,[5] might also have a contribution in insulin resistance in HCV infection.

In our mouse model, we have not identified the type of cells that produce TNF-α in the liver, but determination of other cytokines including IL-6 was already done: only TNF-α and IL-1β levels were increased in the liver among numerous cytokines.[6] Serum levels of TNF-α were determined as well, resulting to be below the detection limit of sensitive Elisa assays (Moriya K, et al. unpublished data). As described previously, there was no histopathological inflammation in the liver of HCV core gene transgenic mice.[3] However, such increases in intrahepatic proinflammatory cytokine levels, combined with the overproduction of reactive oxygen species (ROS),[7] allow us to hypothesize that HCV core protein *per se* induces "biochemical inflammation" in the mouse liver in the absence of apparent inflammation.

In summary, from our data, an impairment of intracellular insulin signaling pathway in the liver is the basis for insulin resistance in HCV infection, in which an elevated intrahepatic TNF-α level would be one of the key factors. Additional factors associated with insulin resistance must be explored, in particular, associated with peripheral insulin resistance.

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2. Aytug S, Reich D, Sapiro LE, Bernstein D, Begum N. Impaired IRS-1/PI3-kinase signaling in patients with HCV: a mechanism for increased prevalence of type 2 diabetes. Hepatology 2003;38: 1384–1392.
3. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Matsuura Y, Kimura S, Miyamura T, Koike K. Hepatitis C virus core protein induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065–1068.
4. Kadowaki T. Insight into insulin resistance and type 2 diabetes from knockout mouse models. J Clin Invest 2000;206:459–465.
5. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman GI. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science 2003;300: 1140–1142.
6. Tsutsumi T, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Matsuura Y, Kimura S, Koike K, Miyamura T. Intrahepatic cytokine expression and AP-1 activation in mice transgenic for hepatitis C virus core protein. Virology 2002;304:415–424.
7. Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Miyazawa T, Ishibashi K, Horie T, Imai K, Miyamura T, Kimura S, Koike K. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocellular carcinogenesis. Cancer Res 2001;61:4365–4370.

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SPECIAL ISSUE PAPER

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Determination of serum D-lactic and L-lactic acids in normal subjects and diabetic patients by column-switching HPLC with pre-column fluorescence derivatization

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Abstract D-Lactic and L-lactic acids were simultaneously determined by means of a column-switching high-performance liquid chromatography (HPLC) with fluorescence detection. As a fluorescence reagent, 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ) was employed for the fluorescence derivatization of lactic acid. The proposed HPLC system adopted both octylsilica (Cadenza CD-C8) and amylose-based chiral columns (CHIRALPAK AD-RH), which proved to give a sufficient enantiomeric separation of the lactic acid derivatives with a separation factor (α) of 1.32 and a resolution (R_s) of 1.98. Moreover, the features of the first elution of D-lactic acid peak in the proposed HPLC were convenient for the determination of trace amount of serum D-lactic acid, which is known to increase under diabetes. Intra-day and inter-day accuracies were in the range of 90.5–101.2 and 89.0–100.7%, and the intra-day and inter-day precisions were 0.3–1.2 and 0.4–4.8%, respectively. The proposed method was applied to determine D-lactic and L-lactic acids in human serum of normal subjects and diabetic patients, showing that both D-lactic and L-lactic acid concentrations were significantly increased in the serum of diabetic patients (n=31) as compared with normal subjects (n=21). This fact was found for the first time owing to the development of the proposed HPLC method which is able to determine D-lactic and L-lactic acid simultaneously. Finally, serum D-lactic acid concentrations determined by the proposed HPLC method were compared with those from a reported enzymatic assay, and the smaller p value between normal subjects and diabetic patients was shown by the proposed HPLC method.

Keywords D-Lactic acid · L-Lactic acid · NBD-PZ · Column-switching HPLC · Amylose-based chiral column · Diabetic patients

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Introduction

Lactic acid in mammals is mainly composed of the L-form which is produced from pyruvic acid under anaerobic conditions. But its optical isomer, D-lactic acid, also exists in mammals even though the amount is approximately 1.0% relative to L-lactic acid. The endogenous D-lactic acid is produced from methyglyoxal mediated by glyoxalase I and II in the metabolic pathway of glucose [1, 2]. It has been reported that D-lactic acid concentration was significantly increased in the serum of diabetic animals [3, 4] and patients [5]. This has been considered due to the accelerated production of its precursor, methyglyoxal, which causes the patient to develop diabetic complications under the diabetic stage [6, 7, 8, 9, 10]. Also, the elevation of D-lactic acid concentration has been observed in the blood of patients suffering from short bowel syndrome, which has lead to D-lactic acidosis associated with encephalopathy [11, 12]. From these points of view, D-lactic acid in biological fluids such as serum may be a clinical marker for the diagnosis of such diseases.

For the determination of D-lactic acid in blood, serum, or plasma, the reported methods have mostly utilized D-lactic acid dehydrogenase (D-LDH), which transforms D-lactic acid to pyruvic acid [13, 14, 15, 16, 17, 18, 19]. For instance, in the enzymatic assay using D-LDH, D-lactic acid concentrations were estimated as the increase of absorption [13, 14, 17, 18], fluorescence [5], or amperometric potential [19] of NADH formed from NAD^+, which is a co-factor in the enzymatic reaction using D-LDH. Although the enzymatic assay provides a rapid determination, some disadvantages have still remained. First, D-LDH slightly cross-reacts with endogenous compounds such as L-lactic acid, pyruvic acid, 3-phosphoglyceric acid, and *S*-D-lactoylglutathione [20]; therefore the cross-reactivity may affect the accuracy of D-lactic acid determination. Secondly, L-LDH is also necessary in the case of determining L-lactic acid in the sample. Therefore, simultaneous determination of D-lactic and L-lactic acids seems to be difficult in the enzymatic assay.

For these reasons, we have developed a sensitive fluorimetric method to determine D-lactic and L-lactic acids by means of high-performance liquid chromatography (HPLC) with enantiomeric separation techniques [21, 22, 23]. Serum concentration of D-lactic acid is in the range of approximately 10–40 μM; therefore, a fluorometric detection was useful for determining D-lactic acid in small sampling volumes of serum. When 4-*N*,*N*-dimethylaminosulfonyl-7-piperazino-2,1,3-benzoxadiazole (DBD-PZ), which is a fluorescence reagent for the carboxyl group [24], was selected for the fluorescence derivatization of D-lactic and L-lactic acids, the use of a cellulose-based chiral column was effective for the enantiomeric separation of the derivative following *O*-acetylation of hydroxyl groups with acetyl chloride [22]. The HPLC method enabled us to determine D-lactic and L-lactic acids in rat plasma. In the case of using 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ) as a fluorescence reagent [24], the determination of D-lactic acid in rat plasma was possible without *O*-acetylation when the phenylcarbamoylated β-cyclodextrin-based chiral column was employed, since greater R_s of the derivative with NBD-PZ than DBD-PZ was obtained [23]. Furthermore, the introduction of the column-switching HPLC system with both non-chiral and chiral columns allowed the determination of D-lactic and L-lactic acids in rat plasma [23].

By using the HPLC method, a significant increase of D-lactic acid was observed in the plasma of streptozotocin-induced diabetic rats. In the previous HPLC method, however, a small peak of D-lactic acid eluted after the elution of a large peak of L-lactic acid. Hence, peak tailing of L-lactic acid may disturb the accurate and reproducible determination of D-lactic acid. Thus, in the present study, we tried to develop a further improved HPLC method for application to clinical human samples in which the peak of D-lactic acid is allowed to elute first. By adopting an amylose-based chiral column, the D-lactic acid derivative with NBD-PZ was found to elute first. By using a column-switching HPLC system consisting of octylsilica and the amylose-based chiral columns, D-lactic and L-lactic acid concentrations in serum of both normal subjects and diabetic patients were successfully determined. In addition, the values of D-lactic acid concentration obtained by the proposed HPLC were compared with those by the reported enzymatic assay using D-LDH [5]. D-Lactic acid concentrations in the serum of normal subjects were similar between the HPLC and enzymatic methods, whereas in the diabetic patients, a higher D-lactic acid concentration was obtained by the proposed HPLC methods. Therefore, the smaller p value (p=0.00011) between normal subjects and diabetic patients was shown by the proposed HPLC method than those by the enzymatic method (p=0.08635), suggesting that the proposed HPLC method would be useful in the clinical diagnosis of diabetes, because a more reliable concentration of D-lactic acid in serum of diabetic patients would be obtained.

Experimental

Reagents

NBD-PZ, triphenylphosphine (TPP) and 2,2′-dipyridyl disulfide (DPDS) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Lithium D-, L-, D,L-lactate, nicotinamide adenine dinucleotide (NAD^+), diethylenetriaminepentaacetic acid (DETAPAC), and D-lactic acid dehydrogenase (*Staphylococcus epidermis*, EC 1.1.1.28) were from Sigma Chemical Co. (St. Louis, MO, USA). Propionic acid, formic acid, and trifluoroacetic acid (TFA) were from Wako Pure Chemicals Industries (Tokyo, Japan). Phosphate-buffered saline (PBS) was purchased from Nissui Pharmaceutical Co (Tokyo, Japan). Empore SDB-RPS was from GL Sciences (Tokyo, Japan). Cadenza CD-C8(150×4.6-mm i.d., 3 μm) was kindly donated by Imtakt Co. (Tokyo, Japan), and CHIRALPAK AD-RH (150×4.6-mm i.d., 5 μm) was purchased from Daicel Chemical Industries (Hyogo, Japan). Methanol (MeOH), ethanol (EtOH), and acetonitrile (CH_3CN) were all HPLC grade and were purchased from Kanto Kagaku Co. (Tokyo, Japan). Hydrazine sulfate, perchloric acid, glycine, and potassium carbonate were from Kanto Kagaku Co. (Tokyo, Japan).

Procedure for fluorescent derivatization of human serum lactic acid with NBD-PZ

Fluorescent derivatization of lactic acid in human serum was carried out according to our previous method with minor modification [23]. Briefly, 40 μL of human serum was serially added with 280 μL of deproteinizing solvent, EtOH/CH_3CN (1/1), 40 μL of 0.675 mM propionic acid as an internal standard (IS), and 40 μL H_2O. The resultant solution was vigorously mixed using a vortex mixer for 1 min and then centrifuged at 3,000 rpm for 5 min. Then, 40 μL of the supernatant was transferred to a polypropylene brown eppen-tube (2 mL) and 30 μL of 200 mM TPP, 30 μL of 200 mM DPDS, and 100 μL of 2 mM NBD-PZ dissolved in CH_3CN were added. After being vortex-mixed for a few seconds, the reaction mixture was allowed to stand for 2 h at room temperature. Then, 200 μL of 5% HCOOH in H_2O was added to stop the reaction. Subsequently, in order to remove the excess of NBD-PZ, 200 μL of the resultant solution was subjected to a solid-phase extraction (cation exchange) cartridge, EmporeSDB-RPS (4 mm mL^{-1}), initialized with 200 μL of the solution composed of CH_3CN/MeOH/0.1% TFA in H_2O (9/15/76). Subsequently, the membrane filter of the EmporeSDB-RPS was flushed with 600 μL of the same solution to collect the resultant solution captured by the membrane. Finally, 25 μL of the mixed filtrate from the EmporeSDB-RPS was injected onto the HPLC.

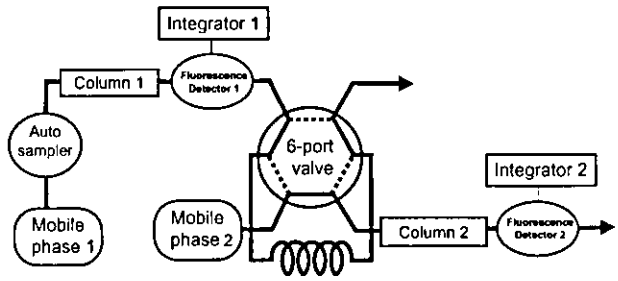

Fig. 1 Block diagram for column-switching HPLC for lactic acid enantiomers derivatized with NBD-PZ. *Column 1* Cadenza CD-C8 (Imtakt Co., Ltd. 150×4.6-mm i.d., 3 μm), *Column 2* CHIRALPAK AD-RH (Daicel Chemicals Co., Ltd. 150×4.6-mm i.d., 5 μm), *Mobile phase 1A* CH_3CN/MeOH/0.1% TFA in H_2O (7/14/79), flow rate 0.7 mL min^{-1}, *Mobile phase 1B* CH_3CN/MeOH/0.1% TFA in H_2O (10.5/17.5/72), flow rate 0.8 mL min^{-1}, *Mobile phase 2* CH_3CN/H_2O (60/40), flow rate 0.3 mL min^{-1}, *Fluorescence detectors 1* and *2* FL L-7480 (ex. 491 nm, em. 547 nm), and L-7485 (ex. 480 nm, em. 540 nm), respectively. *Integrators 1* and *2* Chromato-integrator D-7500 (Hitachi)

HPLC conditions

Figure 1 shows a block diagram for the HPLC system used in the present study. The system consists of an auto-sampler, AS-2057 (Jasco Corporation, Tokyo, Japan), two pumps, L-6200 (Hitachi, Tokyo, Japan) and PU-980 (Jasco), two fluorescence detectors, L-7480 and L-7485 (Hitachi), two integrators, both Chromato-integrator D-7500s (Hitachi), and a six-port valve, HV-992–01 (Jasco) with 100-μL sample loop. Firstly, the peak of lactic acid was separated from endogenous interfering peaks on a C_8 column. The gradient program of mobile phase 1 was as follows: CH_3CN/MeOH/0.1% TFA in H_2O (7/14/79) was isocratically eluted from 0 to 32 min at a flow rate of 0.7 mL min^{-1}, then CH_3CN/MeOH/0.1% TFA in H_2O (10.5/17.5/72) from 33 to 53 min at 0.8 mL min^{-1}, and finally, CH_3CN from 53.1 to 63 min at 0.8 mL min^{-1} for washing the C_8 column. The detector wavelength was fluorimetrically set at 547 nm with an excitation wavelength of 491 nm. Both C_8 and chiral columns were kept at 35 °C by using a column oven, 655A-52 column oven (Hitachi). The timing to change the valve position was carried out as follows: after the lactic acid derivative peak emerged in the chromatogram recorded by the integrator 1, the six-port valve was switched manually from the dotted line to the solid line pathway. Then, as soon as the peak top of the lactic acid derivative appeared in the chromatogram recorded by the integrator 1, the six-port valve was switched manually from the solid line to the dotted line pathway; thus, a portion of the peak fraction of lactic acid was introduced to the chiral column through a 100-μL sample loop. The composition of mobile phase 2 (for the chiral column) consisted of CH_3CN/H_2O (60/40), and the flow rate was constantly pumped at 0.3 mL min^{-1}. The detector wavelength was fluorimetrically set at 540 nm with an excitation wavelength of 480 nm.

Determination of D- and L-lactic acids concentrations

Each concentration of D-lactic and L-lactic acids was calculated as follows: the enantiomeric ratio of D-lactic and L-lactic acids recorded by integrator 2 was multiplied by the total (D + L)-lactic acid concentration which was determined by the peak area ratio of lactic acid derivative to IS derivative on the chromatogram recorded by integrator 1.

Calibration curve

To construct the calibration curve, 40 μL of PBS and 40 μL of the standard solution of D,L-lactic acid dissolved in H_2O (1, 2, 3, 5, 7.5, and 10 mM) were used instead of human serum and H_2O, respectively. Procedures were the same as described above, and were performed in triplicate. The calibration curve was constructed by plotting the peak area ratio of lactic acid derivative to IS derivative against the concentration of D,L-lactic acid dissolved in H_2O (1, 2, 3, 5, 7.5, and 10 mM).

Validation study

The accuracy and precision values for intra-day and inter-day determinations of D-lactic and L-lactic acids were examined as follows: 40 μL of human serum was added with 280 μL of the deproteinizing solvent (EtOH/CH_3CN, 1/1), 40 μL of 0.675 mM propionic acid (IS) and 0, 1, 2, 3, 5, and 7.5 mM lithium D,L-lactic acid dissolved in H_2O (n=3). Then, the same procedures were performed as described above. The working curve was constructed by plotting the peak area ratio against the concentration of D,L-lactic acid dissolved in H_2O (1, 2, 3, 5, and 7.5 mM). The accuracy was expressed as the ratio of the slope of the working curve to the slope of the calibration curve. The precision was expressed as coefficient variation (CV %) of the obtained values of each sample in triplicate.

Determination of D-lactic acid concentration in serum of normal subjects and diabetic patients

All of the subjects provided a written informed consent for their participation in the present study after fully explanation of the research. Blood of normal subjects and diabetic patients was collected from 21 healthy volunteers (age 22–69) and 31 diabetic patients (age 40–78). Serum was obtained from the blood by centrifugation at 3,000 g at 4 °C for 10 min. The obtained sera were treated as described above.

Glucose concentration

Glucose concentration was determined by a commercial kit, glucose C II-test Wako (Wako pure chemicals, Tokyo, Japan).

Enzymatic assay for the determination of D-lactic acid

Enzymatic assay was performed according to the method published by McLellan et al. [5] with minor modification. Briefly, 2 mL of ice-cold 0.6 M perchloric acid and 100 μL of H_2O were added to 1.0 mL of human serum. After mixing the resulting solution on a vortex mixer for 1 min, the solution was kept on ice for 10 min. Then, the solution was centrifuged at 6,000 g for 10 min at 4 °C, and 2.25 mL of the supernatant was transferred into another tube, and neutralized with 0.65 mL of 2 M potassium hydrogen carbonate. The solution was mixed and centrifuged for 5 min at 6,000 g and 4 °C to remove the precipitate. Then, the supernatant (1.0 mL) was sampled and added with 2.0 mL of enzyme reaction buffer, which was composed of 1.5 mM NAD^+, 55.5 mg mL^{-1} glycine, 39 mg mL^{-1} hydrazine sulfate, 0.6 mg mL^{-1} DETAPAC, 37.5 U mL^{-1} D-LDH, and 0.9 M potassium carbonate (pH 9.5). The resulting solution was incubated for 120 min at 25 °C. After the incubation, NADH formed from NAD^+ by the enzymatic reaction with D-LDH was measured fluorimetrically at 460 nm with an excitation wavelength of 340 nm using a fluorescence spectrophotometer, F-4500 (Hitachi, Tokyo, Japan).

Results and discussion

Enantiomeric separation of D,L-lactic acid derivative with NBD-PZ on an amylose-based chiral column

In our previous column-switching HPLC method [23], D,L-lactic acid was derivatized with a fluorescence reagent, NBD-PZ, and separated from the other endogenous compounds on an ODS column, and then, the enantiomeric separation of D,L-lactic acid was achieved on a phenylcarbamoylated β-cyclodextrin column. However, the D-lactic acid peak, which is merely approximately 1.0% of the peak area of L-lactic acid, eluted after the large L-lactic acid peak; therefore, the peak area of D-lactic acid might be affected by tailing of the large L-lactic acid peak. Therefore, it is preferable to elute D-lactic acid derivative before the L-lactic acid derivative.

As a result of the survey on the several commercially available chiral columns which could elute the D-lactic acid NBD-PZ derivative first, an amylose-based chiral column, CHIRALPAK AD-RH, was found to provide a sufficient enantiomeric separation with the first elution of the D-lactic acid peak. When the mobile phase consisted of CH_3CN/H_2O (60/40) was employed, the separation factor (α) and the resolution (R_s) values were 1.32 and 1.98, respectively. The use of $MeOH/H_2O$ as the mobile phase gave no elution of both enantiomers. Thus, in the present study, the amylose-based chiral column with the mobile phase of CH_3CN/H_2O (60 / 40) was chosen for the determination of D-lactic acid in human serum.

For the determination of D-lactic acid in human serum, the adoption of column-switching HPLC system consisting of two different types of columns, non-chiral and chiral columns (Fig. 1), was convenient [23]. In this study, an octylsilica column (Cadenza CD-C8) was selected as the first non-chiral column, because it gave good separation of lactic acid from the other endogenous compounds in human serum (Fig. 2a and c). After the separation on the octylsilica column, a portion of lactic acid fraction was introduced into the amylose-based chiral column through a six-port valve. As a result, both peaks of D-lactic and L-lactic acid were clearly observed in the chromatogram (Fig. 2b and d). The concentration detection limit for D-lactic and L-lactic acid was approximately 1.0 μM (signal to noise ratio 3).

Validation study

The calibration curve for lactic acid in the presence or absence of human serum showed an excellent linearity. The correlation coefficients of intra-day and inter-day assay were 0.999 and 0.996 for D-lactic acid, and 0.994 and 0.996 for L-lactic acid, respectively.

Intra-day and inter-day precisions for D,L-lactic acid in the absence of human serum were 0.97–4.48% and 0.76–

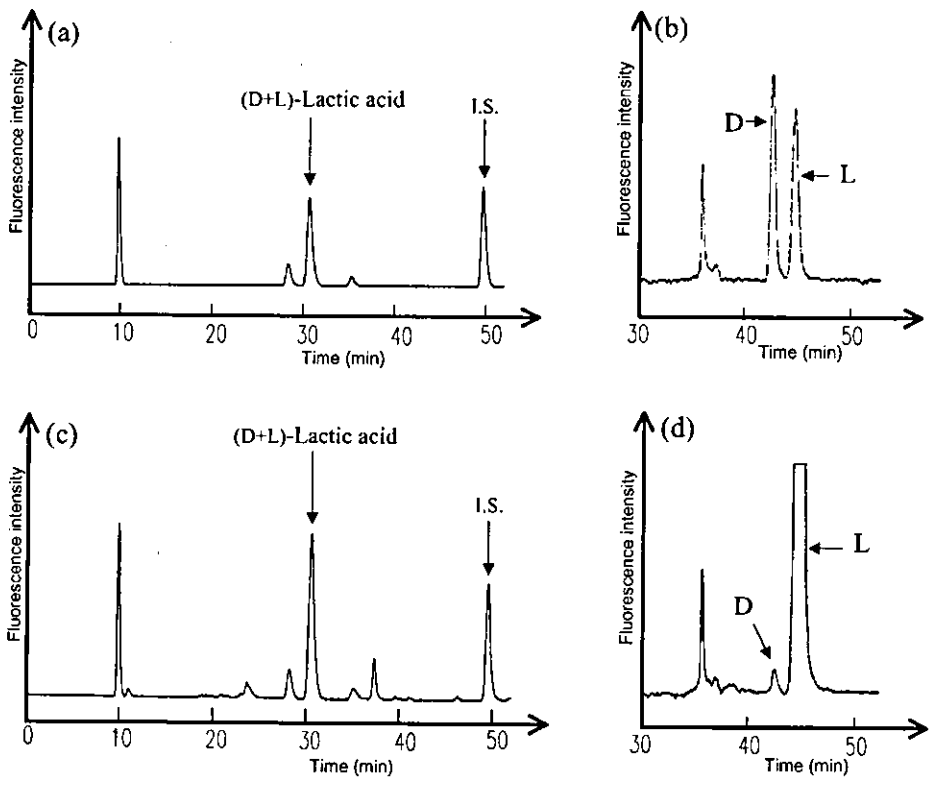

Fig. 2a–d Representative chromatograms obtained by proposed column-switching HPLC system: **a** standard of D,L- lactic acid separated on octylsilica column, **b** standard of D,L- lactic acid separated on amylose-based chiral column, **c** serum sample separated on octylsilica column, **d** serum sample separated on amylose-based chiral column

3.95%, and those for D,L-lactic acid in the presence of human serum were 0.25–1.19% and 0.66–4.79%, respectively (n=3). Intra-day and inter-day accuracies of D,L-lactic acid determination were 90.5–101.2% and 89.0–100.7%, respectively (n=3).

Based on the validation data, the proposed HPLC method was considered to be applicable to determine D-lactic and L-lactic acids in human serum for the investigation of D-lactic or L-lactic acid alterations caused by diabetes.

Determination of D-lactic and L-lactic acids in the serum of normal subjects and diabetic patients

By using the proposed column-switching HPLC method, D-lactic and L-lactic acids concentrations in the serum of normal subjects (n=21, age 22–69) and diabetic patients (n=31, age 40–78) were investigated. The results are summarized in Fig. 3a and b, respectively. In normal subjects, the concentrations of D-lactic and L-lactic acids in serum were 12.77±0.76 μM (mean±SE) and 1.14±0.04 mM, while those in serum of diabetic patients were 27.55±4.01 μM and 1.84±0.17 mM, respectively. Percentage of D-lactic acid/L-lactic acid showed no significant difference between normal subjects and diabetic patients (mean±SE= 1.15±0.08% and 1.56±0.23% for normal subjects and diabetic patients, respectively). As shown in Fig. 3a, a significant difference (p<0.001) of D-lactic acid concentration was observed between normal subjects and diabetic patients. In the diabetic stage, it is already known that the production of toxic compounds (e.g., methylglyoxal) can cause the development of diabetic complications such as cataract [25]. However, the precise and accurate determination of methylglyoxal seems to be difficult, because of its chemically unstable structure. Therefore, the final metabolite of methylglyoxal, D-lactic acid in serum may be used as a risk marker for diabetic complications.

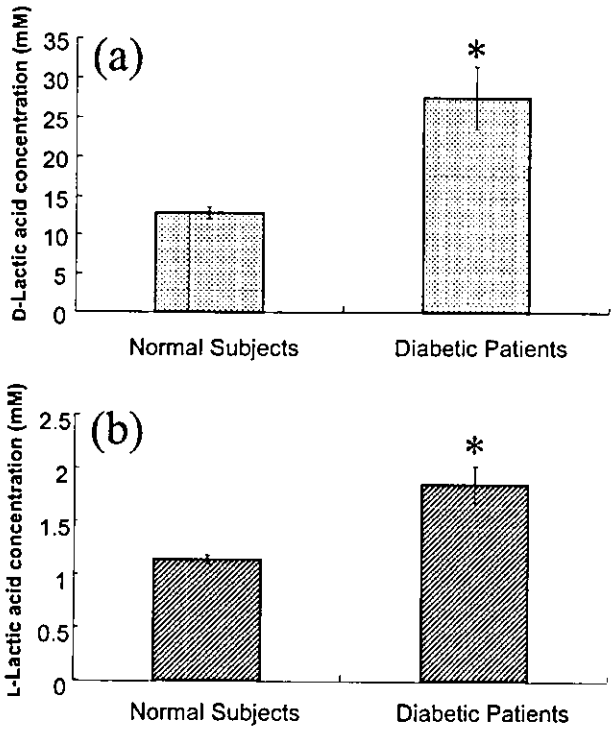

Fig. 3a,b D-Lactic acid (**a**) and L-lactic acid (**b**) concentrations in serum of normal subjects and diabetic patients (mean±SE). Normal subjects (n=21), diabetic patients (n=31), *asterisks* p<0.001 versus normal subjects

Not only D-lactic acid, but also L-lactic acid in serum, was significantly increased in the diabetic patients (p<0.001, Fig. 3b). Avogaro et al. [26, 27] have already reported that serum lactic acid, which mainly consists of L-lactic acid, was increased in diabetic patients due to the increased interconversion from pyruvate to lactate [26]. Their data support the reliability of the present results obtained by the proposed HPLC method.

Most methods for the determination of lactic acid in serum [26, 27] also employ L-lactate dehydrogenase (L-LDH); therefore, no information on the serum D-lactic acid concentration was available. Recently, Wellmer et al. have determined D-lactate and L-lactate in the cerebrospinal fluid of patients with bacterial meningitis for the diagnosis by using both D-LDH and L-LDH [14]. Therefore, the enzymatic assay has an unavoidable problem, since both D-LDH and L-LDH were needed in order to determine D-lactic and L-lactic acid simultaneously. Thus, as far as we know, the present HPLC method enabled for the first time the simultaneous determination D-lactic and L-lactic acids in human serum without using D- and L-lactate dehydrogenase. The difference between the values obtained by HPLC and enzymatic methods will be discussed later.

As both D-lactic and L-lactic acids are metabolites of glucose, the glucose concentration in the serum of patients was examined. As a result, the human serum glucose concentration was significantly higher in the diabetic patients; however, the glucose concentration was not well correlated with the concentration of both D-lactic and L-lactic acids (data not shown). Considering this result, it is suggested that serum D-lactic and L-lactic acids might be altered by metabolic disorders of not only glucose, but also the other endogenous compounds such as amino acids and lipids under the diabetic stage.

Comparison of the proposed HPLC method with the enzymatic assay

In order to evaluate the obtained values by the proposed HPLC method, D-lactic acid concentrations determined by the proposed HPLC were compared with those by the enzymatic methods with D-LDH reported by McLellan et al. [5] using the same human sera sampled from both normal subjects (n=9) and diabetic patients (n=14). As can be seen in Fig. 4, D-lactic acid concentrations in the serum of normal subjects were similar in both the HPLC and enzymatic methods, while in the diabetic patients, a higher D-lactic acid concentration was observed by the proposed

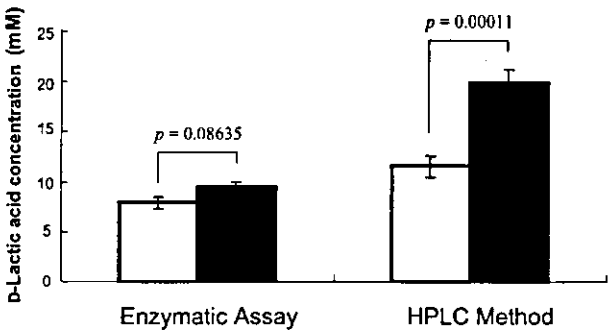

Fig. 4 D-Lactic acid concentrations determined by the enzymatic assays or the HPLC method (mean±SE). *Open column* normal subjects (n=9); *solid column* diabetic patients (n=14)

HPLC methods. In the enzymatic assay, there was a little difference between normal subjects and diabetic patients (p=0.08635), however, the difference became larger in the case of using the proposed HPLC method (p=0.00011). The correlation of D-lactic acid concentrations determined by the proposed HPLC and the enzymatic assay was not so good (r^2=0.343). It is still unclear why such discrepancy occurred. However, in the enzymatic assay of D-LDH, the cross-reactivity or disturbance of enzymatic reaction by endogenous compounds such as L-lactic acid, pyruvic acid, 3-phosphoglyceric acid, and *S*-D-lactoylglutathione should affect the values of D-lactic acid. Therefore, it seemed to be difficult to obtain accurate and precise values of D-lactic acid in the enzymatic assay, and a more reliable concentration of D-lactic acid in serum of diabetic patients would be obtained by the HPLC method, because the selectivity of the HPLC is considered to be superior as compared to the enzymatic method. In addition, the enzymatic method has another disadvantage, since the amount of serum necessary for the determination is more than 1 mL. The proposed HPLC method also overcomes this disadvantage by making it possible to determine D-lactic and L-lactic acid simultaneously with only 40 μL human serum.

Conclusions

In conclusion, a highly sensitive and simultaneous determination of both D-lactic and L-lactic acids in human serum was achieved by using a column-switching HPLC with pre-column fluorescence derivatization. The proposed HPLC method will be beneficial for clinical diagnosis of diseases such as diabetes, or to biochemical research on the metabolic pathway concerning both D-lactic and L-lactic acids.

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References

1. Racker E (1951) J Biol Chem 190:685–696
2. Thornalley PJ (1990) Biochem J 269:1-11
3. Kondoh Y, Kawase M, Kawamaki Y, Ohmori S (1992) Res Exp Med 192:407–414
4. Christopher MM, Broussard JD, Fallin CW, Drost NJ, Peterson ME (1995) Metab Clin Exp 44:287–290
5. McLellan AC, Plillips SA, Thornalley PJ (1992) Anal Biochem 206:12–16
6. Thornalley PJ, Hooper NI, Jennings PE, Florkowski CM, Jones AF, Lunec J, Barnett AH (1989) Diabetes Res Clin Prac 7: 115–120
7. McLellan AC, Plillips SA, Thornalley PJ (1992) Anal Biochem 206:17–23
8. Thornalley PJ (1994) Amino Acids 6:15–23
9. McLellan AC, Thornalley PJ, Benn J, Sonksen PH (1994) Clin Sci 87:21–29
10. Thornalley PJ, McLellan AC, Lo TWC, Benn J, Sonksen PH (1996) Clin Sci 91:575–582
11. Mayne AJ, Handy DJ, Preece MA, George RH, Booth IW (1990) Arch Dis Child 65:229–231
12. Bongaerts G, Tolboom J, Naber T, Bakkeren J, Severijnen R, Willems H (1995) Clin Chem 41:107–110
13. Haschke-Becher E, Baumgartner M, Bachmann C (2000) Clin Chim Acta 298:99–109
14. Wellmer A, Prange J, Gerber J, Zysk G, Lange P, Michel U, Eiffert H, Nau R (2001) Scand J Infect Dis 33:909–913
15. Ohmori S, Iwamoto T (1988) J Chromatogr 431:239–247
16. Kondou Y, Kawase M, Ohmori S (1992) Eur J Appl Physiol 65:88–93
17. Marti R, Varela E, Segura RM, Alegre J, Surinach JM, Pascual C (1997) Clin Chem 43:1010–1015
18. Gutheil WG (1998) Anal Biochem 259:62–67
19. Avramescu A, Noguer T, Magearu V, Marty JL (2001) Anal Chim Acta 433:81–88
20. Brandt RB, Siegel SA, Waters MG, Bloch MH (1980) Anal Biochem 102:39–46
21. Fukushima T, Adachi S, Ichihara H, Al-kindy S, Imai K (1999) Biomed Chromatogr 13:418–424
22. Ichihara H, Fukushima T, Imai K (1999) Anal Biochem 269: 379–385
23. Fukushima T, Lee JA, Korenaga T, Ichihara H, Kato M, Imai K (2000) Biomed Chromatogr 14:1–7
24. Toyo'oka T, Ishibashi M, Takeda Y (1991) J Chromatogr 588: 61–71
25. Shamsi FA, Sharkey E, Creighton D, Nagaraj RH (2000) Exp Eye Res 70:369–380
26. Avogaro A, Toffolo G, Miola M, Valerio A, Cobelli C, Del Prato S (1996) J Clin Invest 98:108–115
27. Avogaro A, Crepalgi C, Miola M, Maran A, Pengo V, Tiengo A, Del Prato S (1996) J Endocrinol Invest 19:99–105

Human Plasma Platelet-Activating Factor Acetylhydrolase Binds to All the Murine Lipoproteins, Conferring Protection Against Oxidative Stress

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Objective—Plasma platelet-activating factor (PAF) acetylhydrolase (AH) is an enzyme bound with lipoproteins that degrades not only PAF but also PAF-like oxidized phospholipids that are proposed to promote atherosclerosis. In this study, we investigated the distribution of PAF-AH protein among lipoprotein classes by using adenovirus-mediated gene transfer in mice, and we examined its effects on lipoprotein oxidation and foam cell formation of macrophages.

Methods and Results—Adenovirus-mediated overexpression of PAF-AH in mice resulted in a 76- to 140-fold increase in plasma PAF-AH activity. Contrary to the previous report, overexpressed human PAF-AH protein was bound to very low density lipoprotein, intermediate density lipoprotein, low density lipoprotein, and high density lipoprotein (HDL). All the lipoproteins with overexpressed human PAF-AH revealed more resistance against oxidative stress, which was associated with lower levels in autoantibody against oxidized low density lipoprotein in the plasma. In addition, HDL with human PAF-AH inhibited foam cell formation and facilitated cholesterol efflux in macrophages.

Conclusions—These results suggest that human plasma PAF-AH exerts an antiatherogenic effect by binding to all the lipoproteins and thereby protecting them from oxidation, producing less proatherogenic lipoproteins and preserving HDL functions. (*Arterioscler Thromb Vasc Biol.* 2003;23:829-835.)

Key Words: platelet-activating factor acetylhydrolase ■ oxidative stress ■ adenovirus ■ foam cell formation ■ cholesterol efflux

Platelet-activating factor (PAF) acetylhydrolase (AH) is a calcium-independent enzyme that degrades PAF, a bioactive phospholipid mediator for allergic and inflammatory processes, to a biologically inactive lyso-PAF. Plasma PAF-AH, 1 of the 3 PAF-AH isoforms identified so far, is produced from macrophages and exists in the plasma in the form bound with lipoproteins; the other 2 isoforms are found only in tissues. Seventy percent to 83% of the plasma PAF-AH protein exists on LDL, and 11% to 30% exists on HDL in human plasma.[1,2] An interchange between the 2 lipoproteins has been reported in plasma PAF-AH.[1] In mice, it has been recognized that PAF-AH is associated primarily with HDL and minimally with VLDL[3,4] and that neither murine PAF-AH nor human PAF-AH has been proposed to bind to murine LDL.[5]

An observational study has shown that plasma PAF-AH activity is altered in atherosclerotic diseases.[6] Oxidation of LDL, in which PAF-like oxidized phospholipids are produced on the LDL surface, is one of the key factors in the early stages of atherosclerosis.[7] Besides catalyzing PAF, plasma PAF-AH protein hydrolyzes PAF-like oxidized phospholipids, thereby most likely inactivating the biologically active mediator. However, the products of this reaction include oxidized fatty acids and lysophosphatidylcholine,[8] which are potentially inflammatory mediators that could amplify atherogenesis. Therefore, it is not fully clear whether PAF-AH is antiatherogenic or proatherogenic in humans. There was one report documenting that high PAF-AH activity is associated with an increased risk of coronary artery disease in humans.[6] However, it is not conclusive whether PAF-AH is a causative agent of coronary artery disease or just a marker. A recent animal study demonstrated that overexpression of human plasma PAF-AH protein reduced atherosclerosis in apoE-deficient mice,[9] suggesting its role in the inhibition of atherogenesis. They also clarified that antiatherogenic mechanisms of PAF-AH include reduced macrophage adhesion and homing, reduced production of oxidized LDL, and a reduced lysophosphatidylcholine/phosphatidyl-

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choline ratio.[9,10] However, the distribution of PAF-AH protein among lipoprotein particles and its effect on macrophage foaming have not been clarified.

In the present study, using gene transfer with second-generation adenoviruses, we examined the distribution of the overexpressed human PAF-AH among lipoprotein classes, its effect on lipid metabolism, and its properties for lipoprotein oxidation and foam cell formation. Our results demonstrate that human PAF-AH binds to all the lipoprotein in mice, conferring an antiatherogenic property to each of them.

Methods

Materials and Animals

Paraoxon (PON) was purchased from Sigma Chemical Co. Male wild-type mice (C57BL6/J) and male apoE-deficient mice were obtained from Jackson Laboratory (Bar Harbor, Me). All animal experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments. Total and free cholesterol concentrations were measured by a standard enzymatic method (Wako Pure Chemical Industry, Ltd). Protein concentrations were measured by Lowry's method. Goat polyclonal antibody against mouse apoA-I and rabbit polyclonal antibody against human plasma PAF-AH were purchased from Rockland Immunochemicals, Inc, and Cayman Chemical Co, respectively. Secondary peroxidase-labeled goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from Jackson Immuno Research Laboratories, Inc. Murine macrophage RAW264.7 cells were purchased from American Type Culture Collection.

Construction of Recombinant Second-Generation Adenovirus

Human plasma PAF-AH cDNA was amplified from the human cDNA library (Clontech Inc) by polymerase chain reaction. After confirming the sequence of the cDNA, the PAF-AH cDNA was subcloned into the shuttle plasmid vector pAdCMV-link,[11] which contains 0 to 1 mU adenovirus, the cytomegalovirus immediate-early gene enhancer and promoter, and 9 to 16 mU adenovirus. Recombinant second-generation adenovirus was constructed by the use of established methods.[12] Briefly, the plasmid was linearized with *Nhe*I and cotransfected into 293 cells along with LacZ second-generation adenoviral DNA digested with *Cla*I, and the cells were overlaid with agar and incubated at 32°C for 14 days. Plaques were picked and screened by polymerase chain reaction. After the confirmation of the presence of PAF-AH cDNA and the absence of LacZ and wild-type adenovirus, the new recombinant adenovirus, designated as AdPAF-AH, was expanded in 293 cells. Cell lysates were used to infect HeLa cells for confirmation of the expression of human PAF-AH by Western blotting and activity assay of the media. The recombinant adenovirus was further expanded in 293 cells and purified by cesium chloride ultracentrifugation. AdLacZ, a second-generation adenovirus carrying the β-galactosidase cDNA, was used as a control virus.[13]

Animal Studies

Male wild-type C57BL6/J mice and male apoE-deficient mice at 12 weeks of age were injected intravenously with 3×10^9 plaque-forming units of AdPAF-AH, AdLacZ, or PBS. Blood was drawn from the retro-orbital plexus after a 4-hour fast before virus injection and at 3, 7, 14, 21, and 28 days after injection, and it was collected in a heparin-coated tube.

Fractionation of Lipoproteins

Pooled plasma samples (120 μL) were fractionated by fast protein liquid chromatography (FPLC) gel filtration with the use of a Superose 6 column (Pharmacia LKB Biotechnology). Fractions were collected into 46 tubes in a volume of 375 μL per fraction. Each fraction was analyzed enzymatically for total cholesterol and PAF-AH activity.

PAF-AH Activity Assay

A spectrophotometric assay for plasma PAF-AH activity using 1-myristoyl-2-(*p*-nitrophenylsuccinyl)phosphatidylcholine, a PAF analogue with a 4-nitrophenyl group, as a substrate was performed.[14] Briefly, the rate of hydrolysis of the PAF analogue was determined by monitoring the liberation of *p*-nitrophenyl succinate at 405 nm at 37°C for 2 minutes. The assay reagents included 200 mmol/L NaCl, 15 mmol/L EDTA, 9.6 mmol/L sodium 1-nonanesulfonate, 7 mmol/L 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate, and 100 mmol/L HEPES. The activity was calculated by using the absorbance rate per minute and the extinction coefficient ($\epsilon=12.3\times10^3$ L/mol per centimeter at pH 7.6) of 4-nitrophenol.

Western Blot Assay

Plasma and FPLC fractions pooled in pairs were subjected to SDS-PAGE using 10% polyacrylamide gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane and immunoprobed with antibody against mouse apoA-I or human PAF-AH, followed by incubation with the corresponding peroxidase-labeled secondary antibody. Chemiluminescence with ECL Plus Western

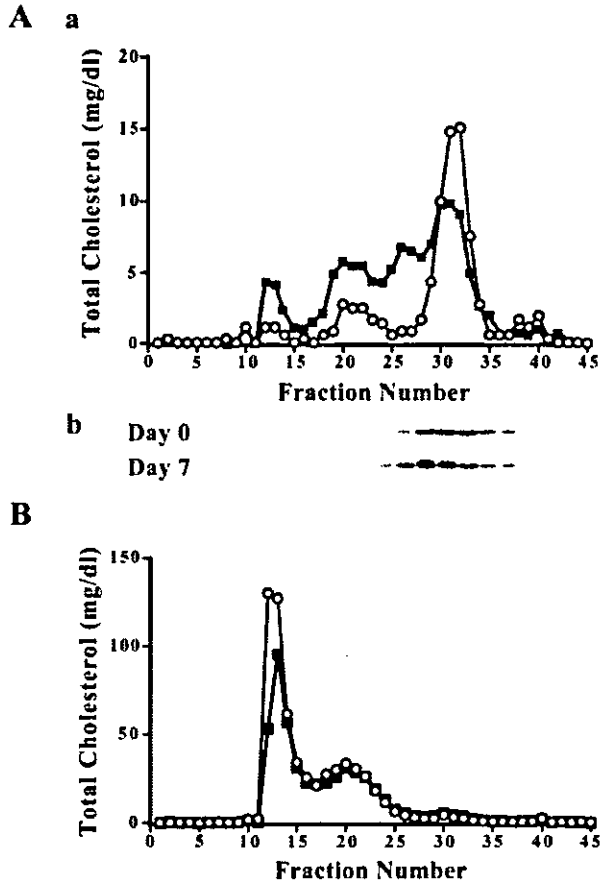

Figure 1. FPLC cholesterol profile before and after injection of AdPAF-AH. Pooled plasma samples were subjected to gel filtration by using a Superose 6 column, and the cholesterol level in each fraction was measured enzymatically. Aa, Wild-type mice. B, ApoE-deficient mice. Open circles indicate before injection; closed squares, day 7 after injection. For wild-type mice, Western blot assay of apoA-I was also performed to elucidate its distribution (Ab).

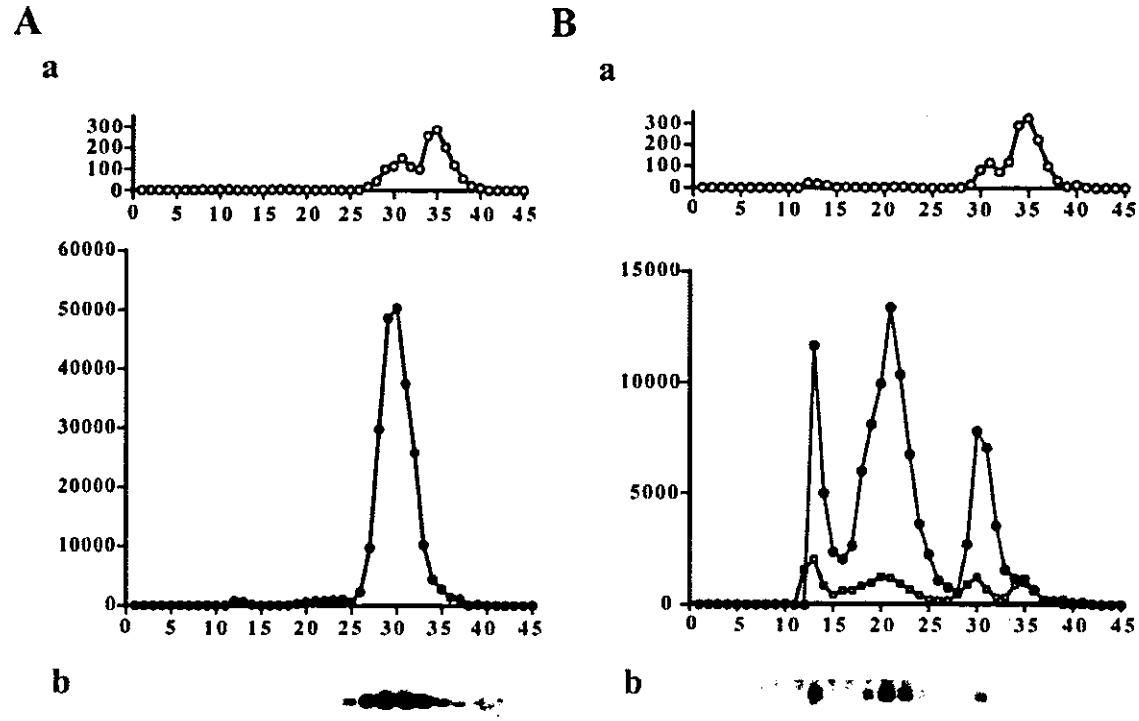

Figure 2. Distribution of human PAF-AH on lipoprotein classes. A, Wild-type mice. B, ApoE-deficient mice. a, PAF-AH activity. X-axes represent fraction numbers, and y-axes represent PAF-AH activity. Please note the difference in the scale of y-axis between the upper panels and lower panels. Open circles indicate before injection; closed circles, day 3 after injection; and open squares, day 14 after injection. b, Western blot assay on day 3.

blotting detection reagents (Amersham Pharmacia Biotech UK Ltd) was used to detect the protein bands, and they were visualized by Image Reader LAS-1000 for Macintosh (Fuji Photo Film Co, Ltd). Densitometric analysis was performed with NIH Image software.

Isolation and Modification of Lipoproteins

Human and murine serum samples were also subjected to sequential flotation ultracentrifugation at 14°C. Each lipoprotein fraction was dialyzed against PBS (pH 7.4) at 4°C. Human LDL was oxidized by 5 μmol/L $CuSO_4$.[15]

Lipid Peroxidation Assay

Each lipoprotein fraction (0.1 mg protein/mL) from apoE-deficient mice obtained through sequential flotation ultracentrifugation was exposed to $CuSO_4$ (5 μmol/L for HDL and 1.67 μmol/L for the other lipoproteins) to initiate lipid peroxidation at 37°C. Lipid peroxidation was monitored by UV absorption spectroscopy at 234 nm as formation of conjugated dienes.

Determination of Autoantibody Titers Against Oxidized LDL

Autoantibodies against oxidized LDL were measured by ELISA as described before.[16,17] Briefly, 50 μL of oxidized LDL (5 μg/mL) was plated on a microtiter plate as antigen overnight at 4°C, followed by saturation with 2% serum albumin in PBS. After incubation with 1:1000 dilutions of mouse plasma for 1 hour at room temperature, 1:500 dilutions of peroxidase-labeled secondary antibody were added to each well. One hour later, after a wash with PBS, 50 μL of freshly made substrate (0.4 mg/mL *o*-phenylenediamine [Sigma] and 0.045% H_2O_2 in 0.05 mol/L phosphate-citrate buffer, pH 5.0) was added and incubated for exactly 5 minutes at room temperature. The reaction was terminated by adding 50 μL of 2 mol/L H_2SO_4. The optical density (OD) was monitored at 492 nm by use of a microplate reader.

Foam Cell Formation Assay and Cholesterol Efflux Assay

RAW 264.7 cells were cultured in DMEM (Invitrogen Corp) supplemented with 10% FBS (Invitrogen Corp). For foam cell formation assay, cells were washed twice with PBS when confluent in 6-well dishes and incubated in medium with 10% lipoprotein-deficient serum (LPDS, Sigma) containing murine HDL (0, 10, 25, or 50 μg/mL) purified from wild-type mice and human oxidized LDL (50 μg/mL) for 48 hours in the absence of 8-bromoadenosine 3′:5′-cAMP (8-Br-cAMP, Sigma). For cholesterol efflux assay, cells were washed and incubated in medium with 10% LPDS containing human oxidized LDL (50 μg/mL) for 24 hours. Subsequently, the cells were washed again and incubated in medium with 10% LPDS containing murine HDL (0, 10, 25, or 50 μg/mL) with or without 0.3 mmol/L 8-Br-cAMP for 24 hours. After incubation, cells were washed, and the cholesterol was extracted from the cells with 600 μL hexane plus isopropyl alcohol (3:2).[18] The solvent was evaporated, and total cholesterol and free cholesterol were measured as described above. The cells were then dissolved in 500 μL NaOH (0.3N), and protein levels were measured as described above. As a marker for foaming, intracellular cholesteryl ester was calculated by subtracting free cholesterol/protein from total cholesterol/protein.

Statistical Analysis

Measurements were analyzed by the Student *t* test. A value of $P<0.05$ was considered significant.

Results

Effect of Human Plasma PAF-AH Overexpression on Cholesterol Levels in Wild-Type Mice and ApoE-Deficient Mice

Compared with baseline levels, plasma total cholesterol levels increased significantly in wild-type mice injected with

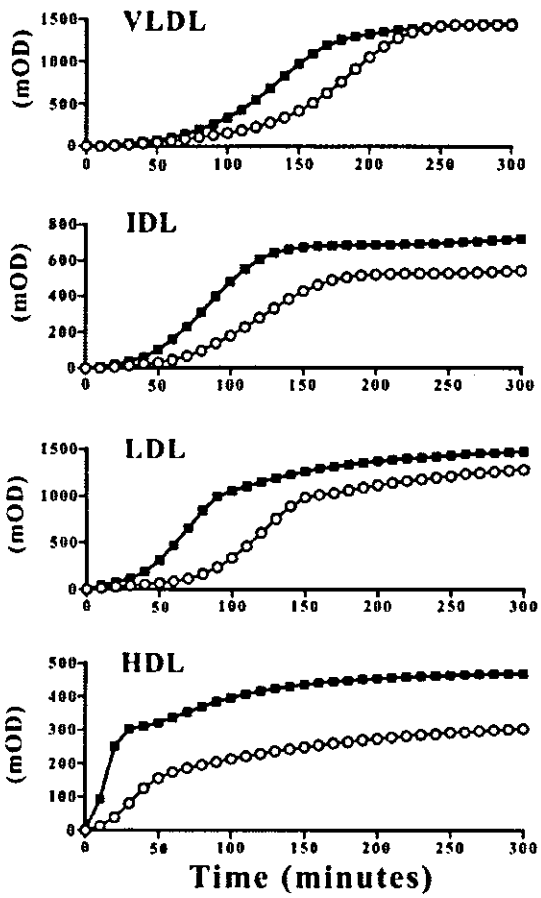

Figure 3. Oxidation assay of lipoproteins. Each lipoprotein class purified through ultracentrifugation was exposed to $CuSO_4$, and formation of conjugated dienes was monitored spectrophotometrically. Open circles indicate samples from mice injected with AdPAF-AH; closed squares, samples from control mice injected with AdLacZ. mOD indicates the measured optical density multiplied by 1000.

AdPAF-AH and AdLacZ on days 7 and 14; however, no significant differences were noted between these 2 groups at the same time points (please see online Figure IA, available at http://atvb.ahajournals.org). Injection of AdPAF-AH or AdLacZ did not alter plasma cholesterol levels in apoE-deficient mice (please see online Figure IB). The FPLC cholesterol profile revealed an appearance of lipoprotein particles (fractions 25 to 27), which are smaller than LDL and larger than normal HDL, together with an increase in LDL cholesterol and a decreased peak cholesterol level in normal HDL fractions only on day 7 in wild-type mice injected with AdPAF-AH (Figure 1Aa). In wild-type mice injected with AdLacZ, only the increase in LDL cholesterol was observed on day 7 (data not shown). Western blot analysis of apoA-I, a specific apolipoprotein of HDL, showed that the distribution of apoA-I became wider among FPLC fractions, reaching to the lipoprotein fractions appearing between LDL and HDL, with a left shift of the peak fraction after overexpression of human PAF-AH (Figure 1Ab). In apoE-deficient mice, there was no significant change in the FPLC cholesterol profile after gene transfer with AdPAF-AH (Figure 1B) and AdLacZ (data not shown).

Plasma PAF-AH Activity

Overexpression of human PAF-AH resulted in a 140-fold increase in activity in wild-type mice on day 3 (1625±133 IU/L on day 0 and $23.0\pm4.0\times10^4$ IU/L on day 3) and a 76-fold increase in apoE-deficient mice on day 3 (1515±84 IU/L on day 0 and $11.6\pm1.8\times10^4$ IU/L on day 3). The significantly increased plasma activity continued up to day 28 in wild-type mice and up to day 14 in apoE-deficient mice (please see online Figure II, available at http://atvb.ahajournals.org). The results of Western blot analysis of the plasma also confirmed the expression of human PAF-AH.

Distribution of Overexpressed Human PAF-AH on Lipoproteins

Distribution of human PAF-AH was analyzed by using FPLC fractions. Western blot analysis revealed that human PAF-AH was found to be distributed mostly in HDL in wild-type mice; however, with the activity assay, an association of PAF-AH protein was noted not only with HDL but also with VLDL, IDL, and LDL (Figure 2A).

In apoE-deficient mice, both activity assay and Western blot assay clearly showed that overexpressed human PAF-AH was associated with all the lipoproteins (Figure 2B). Densitometric analysis of the Western blot assays demonstrated that the activity per PAF-AH protein (specific activity) in the HDL fraction was 4-fold higher than that in the other fractions in apoE-deficient mice.

Effect of Human Plasma PAF-AH on Lipoprotein Oxidation

To determine whether the lipoproteins with overexpressed human PAF-AH were less susceptible to oxidative stress than were those in the control group, lipoproteins were isolated from apoE-deficient mice and incubated with $CuSO_4$. Figure 3 demonstrates a prolonged lag time for the initiation of lipoprotein oxidation, indicating that overexpressed PAF-AH provides protection against oxidation.

To examine whether the overexpressed PAF-AH affects the oxidative state of LDL in vivo, we measured the titers of autoantibody against oxidized LDL in the plasma of apoE-deficient mice. Autoantibodies against oxidized LDL were significantly lower in the AdPAF-AH group than in the AdLacZ group on day 14 (175±15 versus 220±9 OD, respectively; $P=0.03$). On day 28, no significant increase in antibodies from day 14 was seen in the AdPAF-AH group, whereas a 60% increase was noted in the AdLacZ group, although the difference between the 2 groups was nonsignificant (191±26 versus 353±78 OD, respectively; $P=0.11$).

Effects on Macrophage Foam Cell Formation and Cholesterol Efflux

When RAW 264.7 cells were incubated with oxidized LDL and HDL concomitantly, HDL with overexpressed human PAF-AH reduced intracellular cholesteryl ester accumulation (which represents foam cell formation) significantly more than HDL did (Figure 4A). On the other hand, when RAW

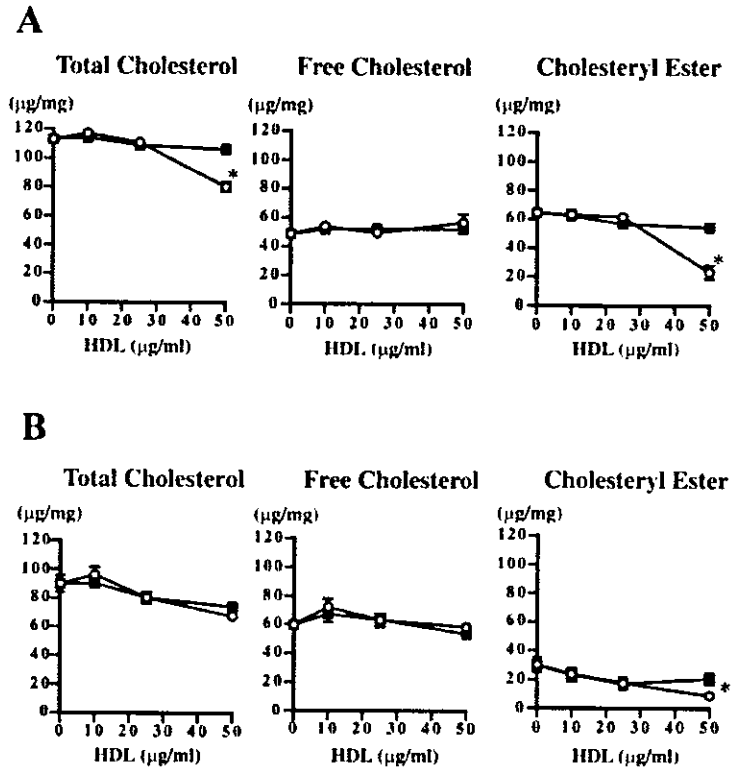

Figure 4. Assay for cellular cholesterol modulating effects by PAF-AH. A, Foam cell formation of RAW264.7 cells in the absence of 8-Br-cAMP (n=4 in each group). B, Cholesterol efflux from cholesterol-loaded RAW 264.7 cells in the presence of 8-Br-cAMP (n=6 in each group). Y-axes represent cellular cholesterol/protein. Open circles indicate AdPAF-AH group; closed squares, control group. *$P<0.05$ vs control.

264.7 cells were first made lipid-loaded with oxidized LDL and sequentially incubated with HDL, the magnitude of lipid removal was greater for HDL with human PAF-AH at a higher HDL concentration in the presence of 8-Br-cAMP (Figure 4B), whereas no cholesterol efflux by either HDL was noted in the absence of 8-Br-cAMP (data not shown).

Discussion

Plasma PAF-AH is a lipoprotein-associated enzyme that is supposed to possess an antiatherogenic effect; it hydrolyzes hyperperoxides in LDL, thereby most likely protecting against atherosclerotic vascular diseases. No clinical studies have so far clarified the antiatherogenic role of PAF-AH in humans, and the effect of PAF-AH on atherogenesis in humans remains under debate. However, a recent investigation demonstrated the inhibitory role of human PAF-AH on atherogenesis in mice.[9] This investigation clarified that antiatherogenic mechanisms of PAF-AH include reduced macrophage adhesion and homing, reduced production of oxidized LDL, and a reduced lysophosphatidylcholine/phosphatidylcholine ratio.[9,10] To clarify further the roles of PAF-AH on atherogenesis, we examined the properties of human PAF-AH by using gene delivery with second-generation adenoviruses in mice.

It has been reported that the majority of the plasma PAF-AH protein exists on LDL and the rest of it on HDL in human plasma,[1,2] and it is associated primarily with HDL and minimally with VLDL in mice.[3,4] Stafforini et al[5] reported that the carboxyl terminus of apoB plays a crucial role in the binding of human PAF-AH to LDL and human PAF-AH does not associate with murine LDL. In contrast to that report, overexpression of human PAF-AH resulted in its binding to all the murine lipoproteins in both wild-type mice and apoE-deficient mice. This association of PAF-AH on all lipoprotein classes was confirmed in apoE-deficient mice even on day 14, when the plasma activity of PAF-AH declined to approximately one sixth of that on day 3 (Figure 2), clarifying the definite preference to apoB-containing lipoproteins. The probable explanation for the association of human PAF-AH on apoB-containing lipoproteins found in the present study is that much more PAF-AH protein was introduced in the present study than in the previous study. The content of human PAF-AH used in the previous investigation was too low to be detected, although the partial contribution of murine apoB cannot be ruled out. Future studies are needed to clarify these details, including the role of apolipoproteins in the association of PAF-AH with lipoproteins.[1,3,5]

Interestingly, we observed the difference in the activity of PAF-AH among the different classes of lipoproteins: that on HDL was found to function more efficiently (ie, specific activity is higher) than that on the other lipoproteins. This phenomenon was also observed in human and PAF-AH–overexpressing LDL receptor–deficient mice (authors' unpublished data). It was possibly caused by the interaction with apoA-I and apoB, or by the different surface lipid compositions and the particle sizes; however, further research is essential to clarify this mechanism. From the viewpoint of atherogenesis, this fact may be physiologically of significance in mice because PAF-AH protein has preferential association with HDL, on which its activity is the greatest.

Rodrigo et al[19] recently proposed that PON1, another antiatherogenic enzyme on HDL that also possesses a

calcium-independent PAF-AH–like activity, is the only responsible enzyme on HDL for the hydrolysis of PAF and PAF-like phospholipids. However, as was shown by Min et al,[20] plasma PAF-AH protein possesses a broad substrate specificity, and its binding to lipoproteins including HDL does not interrupt its catalytic activity. The present study also clarified that overexpression of human plasma PAF-AH protein in mice does result in the increased hydrolyzing activity not only in LDL fractions but also in HDL fractions. Thus, we could confirm that plasma PAF-AH is absolutely functioning as a hydrolyzing enzyme on HDL for PAF and PAF-like phospholipids.

Overexpression of PAF-AH in wild-type mice resulted in an appearance of lipoprotein particles that are smaller than LDL and larger than normal HDL, a decrease in the peak cholesterol level of normal HDL, and the wider distribution of apoA-I among FPLC fractions with a left shift of the peak fraction. These observations imply that HDL particles became bigger in size with PAF-AH overexpression. Excess PAF-AH protein might have made the HDL size larger, or the abundant PAF-AH might affect the metabolism of HDL. On the other hand, elevated LDL cholesterol level, which was also observed in mice injected with AdLacZ, was probably due to a nonspecific effect of adenoviral infection; of course, it is possible that the association of PAF-AH to LDL particles modified the degradation of LDL particles. Further studies are necessary to clarify these mechanisms.

With overexpressed human PAF-AH, all the lipoproteins were found to be resistant to oxidation. Oxidation of LDL is one of the key factors in the early stages of atherosclerosis.[7] In addition, oxidized phospholipids may be the bioactive compounds in LDL and VLDL that induce growth factor expression in smooth muscle cells, thereby resulting in smooth muscle cell migration and proliferation.[21] Our result suggested that besides catalyzing oxidized lipids, PAF-AH exerts a novel antiatherogenic function through protecting all the lipoproteins from oxidation. We also demonstrated this protective effect of PAF-AH against oxidative stress in vivo, as evidenced by lower titers of autoantibodies against oxidized LDL. Concordant with this notion, adenovirus-mediated gene transfer of human PAF-AH in mice has been reported to decrease plasma malondialdehyde-modified LDL.[9]

In the present study, PAF-AH prevented HDL oxidation as well, which points to the possibility that PAF-AH on HDL may intensify the antiatherogenic functions of HDL,[22] ie, the protection of LDL from lipid peroxidation and cholesterol removal from peripheral tissues. In consequence, we further performed a macrophage foaming assay to evaluate these effects of HDL with overexpressed human PAF-AH. A previous study has shown that cholesterol efflux from RAW264.7 cells can be attained only in the presence of cAMP,[23] which induces ABCA1 expression.[24,25] In the present study, cholesterol efflux either by control HDL or HDL abundant in PAF-AH was not noted in the absence of cAMP (data not shown). HDL with overexpressed human PAF-AH exerted a more inhibitory effect on foam cell formation by oxidized LDL compared with control HDL in the absence of cAMP. It indicates that this inhibitory effect on foaming was due to the conversion of oxidized LDL to a less atherogenic lipoprotein by PAF-AH.[9,26] On the other hand, HDL abundant in PAF-AH facilitated lipid removal from foam cells when incubated with cAMP. This observation, together with the finding obtained from the oxidation assay of lipoprotein particles, supports the notion that PAF-AH prevented HDL modulation by oxidative stress from activated foam cells and maintained its function. However, we can also speculate that abundant PAF-AH contributed to facilitated ABCA1-mediated cholesterol efflux. Future studies are required to clarify the importance of PAF-AH for ABCA1-mediated cholesterol efflux. These results indicate that the enrichment of PAF-AH on HDL may be crucial in preserving the function of HDL, as was observed for PON1.[27]

In summary, we demonstrated that human PAF-AH possesses multifaceted antiatherogenic properties; it binds to all the lipoproteins and protects them from oxidation, reducing proatherogenic lipoproteins and preserving HDL functions.

Acknowledgment

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References

1. Stafforini DM, McIntyre TM, Carter ME, Prescott SM. Human plasma platelet-activating factor acetylhydrolase: association with lipoprotein particles and role in the degradation of platelet-activating factor. *J Biol Chem.* 1987;262:4215–4222.
2. Caslake MJ, Packard CJ, Suckling KE, Holmes SD, Chamberlain P, Macphee CH. Lipoprotein-associated phospholipase A(2), platelet-activating factor acetylhydrolase: a potential new risk factor for coronary artery disease. *Atherosclerosis.* 2000;150:413–419.
3. Tsaoussis V, Vakirtzi-Lemonias C. The mouse plasma PAF acetylhydrolase, II: it consists of two enzymes both associated with the HDL. *J Lipid Mediat Cell Signal.* 1994;9:317–331.
4. De Geest B, Stengel D, Landeloos M, Lox M, Le Gat L, Collen D, Holvoet P, Ninio E. Effect of overexpression of human apo A-I in C57BL/6 and C57BL/6 apo E–deficient mice on 2 lipoprotein-associated enzymes, platelet-activating factor acetylhydrolase and paraoxonase: comparison of adenovirus-mediated human apo A-I gene transfer and human apo A-I transgenesis. *Arterioscler Thromb Vasc Biol.* 2000;20:E68–E75.
5. Stafforini DM, Tjoelker LW, McCormick SP, Vaitkus D, McIntyre TM, Gray PW, Young SG, Prescott SM. Molecular basis of the interaction between plasma platelet-activating factor acetylhydrolase and low density lipoprotein. *J Biol Chem.* 1999;274:7018–7024.
6. Packard CJ, O'Reilly DS, Caslake MJ, McMahon AD, Ford I, Cooney J, Macphee CH, Suckling KE, Krishna M, Wilkinson FE, Rumley A, Lowe GD. Lipoprotein-associated phospholipase A2 as an independent predictor of coronary heart disease: West of Scotland Coronary Prevention Study Group. *N Engl J Med.* 2000;343:1148–1155.
7. Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: an overview. *Free Radic Biol Med.* 2000;28:1815–1826.
8. MacPhee CH, Moores KE, Boyd HF, Dhanak D, Ife RJ, Leach CA, Leake DS, Milliner KJ, Patterson RA, Suckling KE, Tew DG, Hickey DM. Lipoprotein-associated phospholipase A2, platelet-activating factor acetylhydrolase, generates two bioactive products during the oxidation of low-density lipoprotein: use of a novel inhibitor. *Biochem J.* 1999;338:479–487.
9. Quarck R, De Geest B, Stengel D, Mertens A, Lox M, Theilmeier G, Michiels C, Raes M, Bult H, Collen D, Van Veldhoven P, Ninio E, Holvoet P. Adenovirus-mediated gene transfer of human platelet-activating factor-acetylhydrolase prevents injury-induced neointima formation and reduces spontaneous atherosclerosis in apolipoprotein E–deficient mice. *Circulation.* 2001;103:2495–2500.
10. Theilmeier G, De Geest B, Van Veldhoven PP, Stengel D, Michiels C, Lox M, Landeloos M, Chapman MJ, Ninio E, Collen D, Himpens B, Holvoet P. HDL-associated PAF-AH reduces endothelial adhesiveness in apoE-/- mice. *FASEB J.* 2000;14:2032–2039.

11. Ye X, Robinson MB, Batshaw ML, Furth EE, Smith I, Wilson JM. Prolonged metabolic correction in adult ornithine transcarbamylase-deficient mice with adenoviral vectors. *J Biol Chem.* 1996;271: 3639–3646.
12. Kozarsky KF, Wilson JM. Gene therapy: adenovirus vectors. *Curr Opin Genet Dev.* 1993;3:499–503.
13. Tsukamoto K, Smith P, Glick JM, Rader DJ. Liver-directed gene transfer and prolonged expression of three major human apoE isoforms in apoE-deficient mice. *J Clin Invest.* 1997;100:107–114.
14. Kosaka T, Yamaguchi M, Soda Y, Kishimoto T, Tago A, Toyosato M, Mizuno K. Spectrophotometric assay for serum platelet-activating factor acetylhydrolase activity. *Clin Chim Acta.* 2000;296:151–161.
15. Hakamata H, Miyazaki A, Sakai M, Suginohara Y, Sakamoto Y, Horiuchi S. Species difference in cholesteryl ester cycle and HDL-induced cholesterol efflux from macrophage foam cells. *Arterioscler Thromb.* 1994;14: 1860–1865.
16. Lehtimaki T, Lehtinen S, Solakivi T, Nikkila M, Jaakkola O, Jokela H, Yla-Herttuala S, Luoma JS, Koivula T, Nikkari T. Autoantibodies against oxidized low density lipoprotein in patients with angiographically verified coronary artery disease. *Arterioscler Thromb Vasc Biol.* 1999; 19:23–27.
17. Shih DM, Xia YR, Wang XP, Miller E, Castellani LW, Subbanagounder G, Cheroutre H, Faull KF, Berliner JA, Witztum JL, Lusis AJ. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J Biol Chem.* 2000; 275:17527–17535.
18. Hara A, Radin NS. Lipid extraction of tissues with a low-toxicity solvent. *Anal Biochem.* 1978;90:420–426.
19. Rodrigo L, Mackness B, Durrington PN, Hernandez A, Mackness MI. Hydrolysis of platelet-activating factor by human serum paraoxonase. *Biochem J.* 2001;354:1–7.
20. Min JH, Wilder C, Aoki J, Arai H, Inoue K, Paul L, Gelb MH. Platelet-activating factor acetylhydrolases: broad substrate specificity and lipoprotein binding does not modulate the catalytic properties of the plasma enzyme. *Biochemistry.* 2001;40:4539–4549.
21. Subbanagounder G, Watson AD, Berliner JA. Bioactive products of phospholipid oxidation: isolation, identification, measurement and activities. *Free Radic Biol Med.* 2000;28:1751–1761.
22. Thomas MJ, Chen Q, Zabalawi M, Anderson R, Wilson M, Weinberg R, Sorci-Thomas MG, Rudel LL. Is the oxidation of high-density lipoprotein lipids different than the oxidation of low-density lipoprotein lipids? *Biochemistry.* 2001;40:1719–1724.
23. Smith JD, Miyata M, Ginsberg M, Grigaux C, Shmookler E, Plump AS. Cyclic AMP induces apolipoprotein E binding activity and promotes cholesterol efflux from a macrophage cell line to apolipoprotein acceptors. *J Biol Chem.* 1996;271:30647–30655.
24. Marcil M, Brooks-Wilson A, Clee SM, Roomp K, Zhang LH, Yu L, Collins JA, van Dam M, Molhuizen HO, Loubster O, Ouellette BF, Sensen CW, Fichter K, Mott S, Denis M, Boucher B, Pimstone S, Genest J Jr, Kastelein JJ, Hayden MR. Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet.* 1999;354: 1341–1346.
25. Zheng P, Horwitz A, Waelde CA, Smith JD. Stably transfected ABCA1 antisense cell line has decreased ABCA1 mRNA and cAMP-induced cholesterol efflux to apolipoprotein AI and HDL. *Biochim Biophys Acta.* 2001;1534:121–128.
26. Watson AD, Navab M, Hama SY, Sevanian A, Prescott SM, Stafforini DM, McIntyre TM, Du BN, Fogelman AM, Berliner JA. Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. *J Clin Invest.* 1995; 95:774–782.
27. Aviram M, Rosenblat M, Bisgaier CL, Newton RS, Primo-Parmo SL, La Du BN. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J Clin Invest.* 1998;101:1581–1590.

Isoform-Dependent Cholesterol Efflux From Macrophages by Apolipoprotein E Is Modulated by Cell Surface Proteoglycans

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Objective—Apolipoprotein E (apoE) mediates cellular cholesterol efflux and plays a crucial role in the inhibition of atherogenesis. We investigated whether there is an isoform-specific difference in its function for cholesterol efflux from cholesterol-loaded RAW264.7 cells, a murine macrophage cell line that lacks endogenous apoE expression.

Methods and Results—When human apoE was expressed in RAW264.7 cells, apoE2 reduced cellular total cholesterol (TC) and esterified cholesterol (EC) levels significantly, whereas apoE3 and apoE4 had no effect. However, treatment of cells with 4-methylumbelliferyl-7-β-D-xyloside (β-DX) resulted in all 3 isoforms' reducing cellular TC and EC contents significantly. We also investigated the effect of exogenously derived apoE on cholesterol efflux by utilizing the medium harvested from HeLa cells expressing apoE. ApoE2 and E3 reduced both cellular TC and EC contents significantly, whereas apoE4 did not. However, treatment of the cells with β-DX resulted in all 3 exogenously derived apoE isoforms' reducing TC and EC contents significantly. The binding ability of apoE to heparan sulfate proteoglycans examined by heparinase I treatment revealed less binding ability of apoE2 compared with that of apoE3 or apoE4.

Conclusions—The present study clarified the differential cellular cholesterol–modulating effect of apoE isoforms in macrophages, which would be due to the difference in their binding to proteoglycans. (***Arterioscler Thromb Vasc Biol.* 2003;23:269-274.**)

Key Words: apolipoprotein E ■ isoforms ■ cholesterol efflux ■ RAW264.7 cells ■ heparan-sulfate proteoglycans

Macrophages in the arterial wall play a crucial role in the progression of atherosclerosis.[1] Macrophages are transformed to foam cells by their uptake cholesterol, form fatty streak lesions, and secrete cytokines that trigger the development of advanced complex atherosclerotic lesions.[1] Cholesterol efflux is a pivotal mechanism that reduces the accumulation of cholesterol in macrophages and in which HDL has been proposed to play an important role.[2]

Apolipoprotein E (apoE) is a glycoprotein produced not only by the liver but also from macrophages.[3,4] Besides its classic role in lipoprotein metabolism as a ligand for lipoprotein receptors,[3,5] several studies have suggested additional roles for apoE in the protection against atherosclerosis. Overexpression of apoE in the liver of atherosclerosis-prone mice reduced atherosclerosis, accompanied by the deposition of hepatic-derived apoE in the atherosclerotic lesions.[6,7] Macrophage-specific expression of apoE in apoE-deficient mice resulted in reduced atherosclerosis,[8] and bone marrow transplantation from apoE-deficient mice to normal mice increased atherosclerotic lesions[9] without affecting plasma lipid profiles. Mice overexpressing apoE in the arterial wall show reduced atherosclerotic lesions.[10] These studies suggest that both macrophage-derived and liver-derived apoE possess direct antiatherogenic properties.

There are 3 major isoforms in human apoE: apoE2, E3, and E4. Many studies have clarified differences among these isoforms in terms of their effects on lipoprotein metabolism and atherosclerosis.[7,11–16] However, few studies have been performed to prove a differential effect of these isoforms on cholesterol-loaded macrophages. Cullen et al[17] clarified a differential effect on cholesterol homeostasis in macrophages by apoE isoforms by using monocytes from humans homozygous for these isoforms. On the other hand, Smith et al[18] did not find any difference in the efficiency for cholesterol efflux among these isoforms in RAW264.7 cells engineered to stably express the apoE isoform. Furthermore, no study has been performed to examine the isoform-specific cholesterol efflux by exogenously derived apoE under condition of nonendogenous apoE production in macrophages.

To address apoE isoform–specific effects on cholesterol metabolism in macrophages, we used adenovirus-mediated gene transfer to attain the same level of expression among the apoE isoforms. We also used 4-methylumbelliferyl 7-β-D-xyloside (β-DX)[19,20] to inhibit the cellular production of

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