Table 2. Baseline characteristics of patients in secondary prevention group

Characteristics	Secondary prevention No. (%) of patients			*p*
	All *n*=88	Exposed *n*=74 (84.1)	Unexposed *n*=14 (15.9)	
Age, mean (range)	52 (23-71)	51 (29-70)	53 (23-71)	0.62
Men, No. (%)	53 (60.2%)	46 (62.2%)	7 (50.0%)	0.55
BMI ≥25	21 (25.3%)	17 (24.3%)	4 (30.8%)	0.73
Smoker	42 (50.0%)	38 (53.5%)	4 (30.8%)	0.23
Drinker	39 (46.4%)	33 (46.5%)	6 (46.2%)	1.00
Xanthoma	75 (85.2%)	63 (85.1%)	12 (85.7%)	1.00
Tendon xanthoma	71 (80.7%)	61 (82.4%)	10 (71.4%)	0.46
Nodular xanthoma	7 (8.0%)	6 (8.1%)	1 (7.1%)	1.00
Palpebral xanthoma	8 (9.1%)	5 (6.8%)	3 (21.4%)	0.11
PAD	2 (2.3%)	2 (2.7%)	0 (0.0%)	1.00
Hypertension	36 (40.9%)	30 (40.5%)	6 (42.9%)	1.00
Diabetes	14 (15.9%)	9 (12.2%)	5 (35.7%)	0.04
Lipid profile, (mg/dL)				
TC†	332 (191-469)	334 (191-469)	322 (229-444)	0.41
TG†	128 (37-636)	128 (37-636)	136 (63-318)	0.85
HDL-C†	42 (20-90)	42 (20-90)	39 (26-73)	0.91
LDL-C†	249 (117-381)	256 (117-381)	245 (138-354)	0.57
Blood Pressure, mmHg				
SBP†	129 (90-180)	128 (96-180)	136 (90-166)	0.97
DBP (mmHg)†	80 (52-114)	80 (52-114)	78 (60-104)	0.33
FBS (mg/dL)†	96 (72-252)	97 (72-197)	94 (79-252)	0.96
HbA1c (%)†	5.8 (4.1-10.6)	5.5 (4.1-8.1)	6.4 (5.3-10.6)	0.06
Tendon xanthoma thickness (mm)†	14.5 (5.8-25.0)	15.0 (5.8-25.0)	10.0 (8.5-18.8)	0.09
Prior CV events				
Angina Pectoris	45 (51.1%)	36 (48.6%)	9 (64.3%)	0.39
Myocardial Infarction	34 (38.6%)	33 (44.6%)	1 (7.1%)	<0.01
Stroke	7 (8.0%)	4 (5.4%)	3 (21.4%)	0.08
Heart failure	2 (2.3%)	2 (2.7%)	0 (0.0)	1.00
TIA	2 (2.3%)	1 (1.4%)	1 (7.1%)	0.29
Treatment				0.08
Cholesterol-lowering drugs (non-probucol)	81 (92.0%)	70 (94.6%)	11 (78.6%)	
LDL-apheresis	13 (14.8%)	11 (14.9%)	2 (14.3%)	1.00
Anti-platelet drugs	50 (56.8%)	44 (59.5%)	6 (42.9%)	0.38
Anti-hypertensive drugs	47 (53.4%)	42 (56.8%)	5 (35.7%)	0.24
Diabetic drugs	6 (6.8%)	3 (4.1%)	3 (21.4%)	0.05

†Data are the median (range). All data are numbers (%) unless otherwise indicated. Each percentage is related to the total number with measurement data. TIA indicates transient ischemic attack.

147 (124-197) and 33 (17-70) mg/dL. Sub-analysis of changes in the lipid profile after probucol treatment detected significant three predictors of CV event risk: higher baseline TC (HR 2.74, 95% CI 1.05-7.16; p=0.04) in the primary prevention group; reduction in TG (HR 0.22, 95% CI 0.06-0.86; p=0.03); and reduction in LDL-C (HR 0.17,95% CI 0.03-0.90; p=0.04) after treatment in the subset of the secondary prevention group on stable doses of probucol. Neither TC nor HDL-C after treatment was associated with CV event risk in the probucol-exposed group, which indicates that reduction of the HDL-C level after probucol treatment is not related to CV event risk for probucol-exposed patients.

We evaluated the safety of probucol for all collected data from 541 patients, and found 56 adverse

Table 3. Incidence of cardiovascular events

		Cardiovascular Event		No event	Total	*p*
Primary prevention ($n=322$)	Exposed ($n=233$)		27 (11.6%)	206	233	
		MI	4			
		AP	18			
		Str.	3			
		TIA	1			
		PAD	1			0.058
	Unexposed ($n=89$)		4 (4.5%)	85	89	
		AP	1			
		Str.	2			
		TIA	1			
Secondary prevention ($n=88$)	Exposed ($n=74$)		20 (27.0%)	54	74	
		MI	6			
		AP	12			
		HF	1			
		Str.	1			0.012
	Unexposed ($n=14$)		9 (64.3%)	5	14	
		MI	2			
		AP	6			
		Str.	1			

MI, myocardial infarction; AP, angina pectoris; HF, heart failure; Str., stroke; TIA, transient ischemic attack; PAD, peripheral artery disease.
†One of the 4 patients died after 12 months of probucol termination.

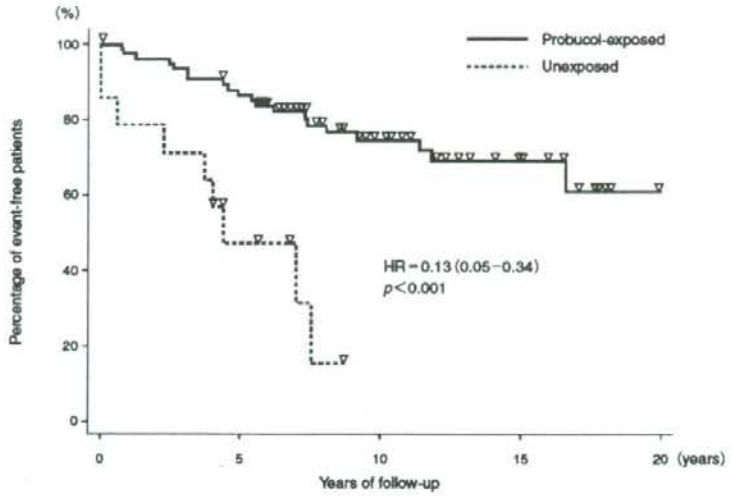
events in 18 patients. Malaise, pruritus, macrocytic anemia and pain in the extremities were recorded as adverse drug reactions associated with probucol. We noted and reported gastric cancer stage III immediately to the Ministry of Health and Welfare as an unexpected serious event, because of an unknown drug relation due to many concomitant drugs, although probucol was found to be non-carcinogenic alone[21]. Six deaths were observed in the population not taking probucol or stopping probucol. There was no other difference in the incidence of adverse events, including serious events, between probucol exposure and non-exposure.

Discussion

Many data from large-scale randomized controlled trials have overwhelmingly demonstrated the clinical benefits of lowering cholesterol with statins[22, 23], yet the rapid and extensive prophylactic use of cholesterol-lowering drugs remains controversial. Few studies have addressed the clinical risks and benefits of long-term treatment of hyperlipidemia among women[24] or elderly patients[25]. The safety of long-term cholesterol-lowering therapy, including the issue of associated cancer risk or benefit, remains inconclusive because of conflicting clinical evidence[26]. More importantly, conclusions from the results of randomized controlled trials are limited by their relatively short follow-up periods (generally less than 5 years) in the analyzed studies.

In long-term treatment for FH, probucol was used with other cholesterol lowering drugs in over 80% of the secondary prevention group–those with a more severe clinical outlook than the primary prevention group: a higher prevalence of hypertension and diabetes, significant thicker tendon xanthoma, more combined therapy with LDL-apheresis, anti-platelet drugs, and anti-hypertensive drugs. The high rate of probucol use in FH was surprising, different from expected. This might partly reflect the prescription behavior of experts with the result that intractable patients responded to the regimen.

In the secondary prevention, the higher-risk group, probucol exposure was associated with a reduction in the risk of cardiovascular events (HR 0.13; 95% CI 0.05–0.34) with high significance ($p<0.001$), while it was not significant in the primary prevention group. This result was also contrary to our expectation that probucol exposure would likely be associated with increased event risk due to a confounding indication–that patients considered more severe at diagnosis would receive more treatment, including probucol. We did not collect the details of non-probucol drugs



Number at risk

Years	0				5					10					15					20
Exposed	74	71	70	68	66	62	54	50	42	38	34	30	25	19	17	13	12	8	3	1	0
Unexposed	14	11	11	10	9	5	4	2	1	0	0	0	0	0	0	0	0	0	0	0	0

Estimates of event-free rates are according to whether patients received probucol. The cumulative probability of remaining without events was higher in patients treated with probucol ($p<0.001$; log-rank test).

Fig. 2. Kaplan-Meier Estimates of Event-free Rate.

For secondary prevention, the incidence of cardiovascular events was 27.0% in the exposed group and 64.3% in the unexposed group. An event-free survival curve for the secondary prevention group is given.

Table 4. The results of multivariate analysis using Cox regression procedure

Factor	Primary prevention			Secondary prevention		
	HR	95% CI	*p*	HR	95% CI	*p*
Baseline variables						
Total cholesterol	1.58	1.06–2.33	0.02	–	–	–
Drinking	2.43	1.09–5.44	0.03	–	–	–
Peripheral artery disease	5.27	0.51–54.63	0.16	–	–	–
Palpebral xanthoma	–	–	–	2.94	1.02–8.47	0.05
Diabetes	–	–	–	2.58	0.76–8.76	0.13
Treatment in follow-up						
Probucol use	1.50	0.48–4.67	0.49	0.13	0.05–0.34	<0.001
Anti-platelet drug use	–	–	–	2.48	1.00–6.17	0.05

to simplify the study procedure. However, we would likely exclude underused statins because of the reduced use of non-probucol drugs from the possible factors of the higher event rate in the unexposed group, because statins were available when all of the 9 recurrent patients (**Table 3**) started and the patients continued on cholesterol-lowering drugs. We suppose, therefore, that the reasons for this unanticipated great risk reduction include some antioxidant and anti-atherogenic actions[3, 4, 27] of probucol. The finding in second prevention may be suggested by the report[27] that probucol significantly decreased *in vitro* LDL oxidizability measured under typically strong oxidative conditions, and that long-term treatment with probucol had an

anti-atherogenic effect in Watanabe Heritable Hyperlipidemic rabbits. From the observation that the baseline lipid profile was not different between the two groups of exposure and non-exposure in secondary prevention, the drug might exhibit greates effectiveness in post-cardiovascular disease patients, in possibly advanced lipid accumulation and inflammation, which are associated with the circulation of oxidized LDL[28].

In primary prevention, we observed an almost significant increase of events in the exposed group (**Table 3**), and an apparently increased risk (HR 1.5), although not statistically significant after adjustment (**Table 4**). We suppose, however, that the ideal effects of probucol might be concealed by the following factors noted in primary prevention. The exposed group had a worse lipid profile (TC, LDL-C and HDL-C levels), higher HbA_{1c}, and thus definitely a higher risk than the unexposed group. Furthermore, 8 (nearly 30%) of the 27 patients experiencing cardiovascular events in the exposed group discontinued probucol when they had events. This was consistent with the different finding between primary and secondary preventions in the exposed group: less than half of the patients (113 of 233) in primary prevention continued on probucol, while 53 (72%) of 74 patients continued in secondary prevention. This estimation might be conservative.

The controversial and paradoxical action of probucol– lowering HDL-C– level was not associated with the risk of CV events in the cohort, therefore, the association between low levels of HDL-C and an increased risk for CV events or death indicated by the early Framingham Heart Study[29] may not be extrapolated to probucol-treated patients. This proposition is consistent with recent findings that a lowered HDL-C level is not always atherogenic, but that the quality or function of HDL-C is more important than the HDL-C levels[30]. In fact, increased levels of HDL-C with torcetrapib, a CETP inhibitor, were not associated with a significant clinical benefit in patients with coronary disease[31], FH[32] or mixed dyslipidemia[33].

We speculate that enhanced reverse cholesterol transport by CETP activation as a result of probucol treatment also contributed to the detected risk reduction in the cohort. The observed positive outcome of probucol, a CETP activator, might be a mirror image of the negative clinical trial results for the CETP inhibitor[34]. Reports[35, 36] of increased coronary heart disease in CETP deficiency despite increased HDL-C levels, and the molecular approach to review CETP deficiency[37] support our hypothesis, at least in Japanese genealogy. Interestingly, a recent basic research reports that human CETP expression enhances the mouse survival rate in an experimental systemic inflammation model[38], indicating for the first time a role for CETP in the defense against the exacerbated production of proinflammatory mediators.

For the safety evaluation, we found no cardiotoxic adverse drug reaction including QT/QTc prolongation or torsade de pointes, in this study, although probucol can cause them[16, 39, 40].

We obtained these results from an observational study with no control for inaccuracy, unexpected bias or confounding factors. We could not assure the precision of the baseline measurements due to unrecorded data. The participant centers were major hospitals for FH, but not all hospitals in Japan, because the study was conducted as part of a post-marketing study by a pharmaceutical manufacturer within the framework of the Japanese government regulations. Some restrictions on collecting data might have resulted in unexpected small numbers in the unexposed group in secondary prevention, although we think that the study cohort represents nearly a nationwide population of heterozygous FH in Japan. The results derived from patient data in Japan can not necessarily be generalized to patients in western countries.

Despite these limitations of the study, however, we could evaluate the outcome of long-term probucol treatment in the medical practice setting for FH, a high-risk population, for as long as 20 years in Japan. The significant risk reduction of CV events observed in the secondary prevention group holds clinical significance and suggests some beneficial therapeutic actions of this drug in arteriosclerotic diseases. The hypothesis from the findings warrants a randomized controlled trial for verification of the secondary prevention, and needs further research into the molecular mechanisms or roles of CETP in pathogenesis.

Author Contributions

Dr. Yamashita had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Matsuzawa, Kita, Saito, Fukushima, Matsui. Acquisition of data: Yamashita, Bujo, Arai, Harada-Shiba, Saito, Kita, Matsuzawa. Analysis and interpretation of data: Yamashita, Bujo, Arai, Harada-Shiba, Matsui, Saito, Fukushima, Kita, Matsuzawa.

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and Matsuzawa. Statistical analysis: Matsui and Fukushima. Administrative, technical, or material support: Fukushima, Matsui, Kita, Saito, and Matsuzawa. Study supervision: Yamashita, Fukushima, Matsui, Kita, Saito, and Matsuzawa.

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Disclosures

From the formerly Daiichi and Otsuka, Dr. Matsui, Dr. Fukushima, Dr. Matsuzawa, and Dr. Kita received fees and expenses for meetings related to protocol design, statistical and clinical interpretation of the data; Dr. Bujo, Dr. Arai, Dr. Harada-Shiba received honoraria and travel expenses for lectures, Dr.Yamashita, Dr. Bujo, Dr. Arai received fees and travel expenses for a meeting related to clinical interpretation of the data. Dr. Yamashita received consultancy fees from Otsuka. Dr. Matsuzawa is contracted as a short-term adviser to Otsuka in medical science. Dr. Saito received travel expenses only.

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References

1) Barnhart JW, Sefranka JA, McIntosh DD: Hypocholesterolemic effect of 4,4'-(isopropylidenedithio)-bis (2,6-di-t-butylphenol) (probucol). Am J Clin Nutr, 1970; 23:1229-1233
2) Tawara K, Tomikawa M, Abiko Y: Mode of action of probucol in reducing serum cholesterol in mice. Jpn J Pharmacol, 1986; 40:123-133
3) Kita T, Nagano Y, Yokode M, Ishii K, Kume N, Ooshima A, Yoshida H, Kawai C: Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. Proc Natl Acad Sci USA, 1987; 84:5928-5931
4) Carew TE, Schwenke DC, Steinberg D: Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci USA, 1987; 84:7725-7729
5) Siveski-Iliskovic N, Kaul N, Singal PK: Probucol promotes endogenous antioxidants and provides protection against adriamycin-induced cardiomyopathy in rats. Circulation, 1994; 89:2829-2835
6) McPherson R, Hogue M, Milne RW, Tall AR, Marcel YL: Increase in plasma cholesteryl ester transfer protein during probucol treatment. Relation to changes in high density lipoprotein composition. Arterioscler Thromb, 1991; 11:476-481
7) Chiesa G, Michelagnoli S, Cassinotti M, Gianfranceschi G, Werba JP, Pazzucconi F, Sirtori CR, Franceschini G: Mechanisms of high-density lipoprotein reduction after probucol treatment: changes in plasma cholesterol esterification/transfer and lipase activities. Metabolism, 1993; 42:229-235
8) Ishigami M, Yamashita S, Sakai N, Hirano K, Arai T, Maruyama T, Takami S, Koyama M, Kameda-Takemura K, Matsuzawa Y: High-density lipoproteins from probucol-treated patients have increased capacity to promote cholesterol efflux from mouse peritoneal macrophages loaded with acetylated low-density lipoproteins. Eur J Clin Invest, 1997; 27:285-292
9) Rinninger F, Wang N, Ramakrishnan R, Jiang XC, Tall AR: Probucol enhances selective uptake of HDL-associated cholesteryl esters in vitro by a scavenger receptor B-I-dependent mechanism. Arterioscler Thromb Vasc Biol, 1999; 19:1325-1332
10) Hirano K, Ikegami C, Tsujii K, Zhang Z, Matsuura F, Nakagawa-Toyama Y, Koseki M, Masuda D, Maruyama T, Shimomura I, Ueda Y, Yamashita S: Probucol enhances the expression of human hepatic scavenger receptor class B type 1, possibly through a species-specific mechanism. Arterioscler Thromb Vasc Biol, 2005; 25:2422-2427
11) Matsuzawa Y, Yamashita S, Funahashi T, Yamamoto A, Tarui S: Selective reduction of cholesterol in HDL2 frac-

tion by probucol in familial hypercholesterolemia and hyper HDL2 cholesterolemia with abnormal cholesteryl ester transfer. Am J Cardiol, 1988; 62:66B-72B
12) Yamamoto A, Matsuzawa Y, Yokoyama S, Funahashi T, Yamamura T, Kishino B: Effects of probucol on xanthomata regression in familial hypercholesterolemia. Am J Cardiol, 1986; 57:29H-35H
13) Shinomiya M, Nishide T, Tashiro J, Shirai K, Saito Y, Yoshida S: Effect of 5-year administration of probucol on development of myocardial infarction in heterozygous familial hypercholesterolemia. Current Ther Res, 1993; 54:142-151
14) Daida H, Kuwabara Y, Yokoi H, Nishikawa H, Takatsu F, Nakata Y, Kutsumi Y, Oshima S, Nishiyama S, Ishiwata S, Kato K, Nishimura S, Miyauchi K, Kanoh T, Yamaguchi H: Effect of probucol on repeat revascularization rate after percutaneous transluminal coronary angioplasty (from the Probucol Angioplasty Restenosis Trial [PART]). Am J Cardiol, 2000; 86:550-552, A9
15) Tardif JC, Côté G, Lespérance J, Bourassa M, Lambert J, Doucet S, Bilodeau L, Nattel S, de Guise P: Probucol and multivitamins in the prevention of restenosis after coronary angioplasty. Multivitamins and Probucol Study Group. N Engl J Med, 1997; 337:365-372
16) Tardif JC, Grégoire J, Schwartz L, Title L, Laramée L, Reeves F, Lespérance J, Bourassa MG, L'Allier PL, Glass M, Lambert J, Guertin MC; Canadian Antioxidant Restenosis Trial (CART-1) Investigators: Effects of AGI-1067 and probucol after percutaneous coronary interventions. Circulation, 2003; 107:552-558
17) Baldassarre D, Franceschini G, Peruzzotti G, Brusoni B, Sirtori CR: Clinical evaluation of probucol in hypercholesterolemia: individual lipoprotein responses and inhibitory effect on carotid atherosclerosis progression. J Cardiovasc Pharmacol, 1997; 30:784-789
18) Sawayama Y, Shimizu C, Maeda N, Tatsukawa M, Kinukawa N, Koyanagi S, Kashiwagi S, Hayashi J: Effects of probucol and pravastatin on common carotid atherosclerosis in patients with asymptomatic hypercholesterolemia. Fukuoka Atherosclerosis Trial (FAST). J Am Coll Cardiol, 2002; 39:610-616
19) Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, Daida H, Biro S, Hirobe K, Funahashi T, Yokote K, Yokode M: Committee for Epidemiology and Clinical Management of Atherosclerosis: Primary hyperlipidemia. J Atheroscler Thromb, 2008; 15:49-51
20) DeLong DM, DeLong ER, Wood PD, Lippel K, Rifkind BM: A comparison of methods for the estimation of plasma low- and very low-density lipoprotein cholesterol. The Lipid Research Clinics Prevalence Study. JAMA, 1986; 256:2372-2377
21) Newman TB, Hulley SB: Carcinogeniciy of lipid-lowering drugs. JAMA, 1996; 275:55-60
22) Cholesterol Treatment Trialists' (CTT) Collaborators: Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90056 participants in 14 randomised trilas of statins. Lancet, 2005; 366:1267-1278
23) Nakamura H, Arakawa K, Itakura H, Kitabatake A, Goto Y, Toyota T, Nakaya N, Nishimoto S, Muranaka M, Yamamoto A, Mizuno K, Ohashi Y; MEGA Study Group: Primary prevention of cardiovascular disease with pravastatin in Japan (MEGA Study): a prospective randomised controlled trial. Lancet, 2006; 368:1155-1163
24) Walsh JM, Pignone M: Drug treatment of hyperlipidemia in women. JAMA, 2004; 291:2243-2252
25) Ali R, Alexander KP: Statins for the primary prevention of cardiovascular events in older adults: a review of the evidence. Am J Geriatr Pharmacother, 2007; 5:52-63
26) Bonovas S, Filioussi K, Tsavaris N, Sitaras NM: Statins and cancer risk: a literature-based meta-analysis and meta-regression analysis of 35 randomized controlled trials. J Clin Oncol, 2006; 24:4808-4817
27) Bräsen JH, Koenig K, Bach H, Kontush A, Heinle H, Witting PK, Ylä-Herttuala S, Stocker R, Beisiegel U: Comparison of the effects of alpha-tocopherol, ubiquinone-10 and probucol at therapeutic doses on atherosclerosis in WHHL rabbits. Atherosclerosis, 2002; 163:249-259
28) Tsimikas S, Brilakis ES, Miller ER, McConnell JP, Lennon RJ, Kornman KS, Witztum JL, Berger PB: Oxidized phospholipids, Lp(a) lipoprotein, and coronary artery disease. N Engl J Med, 2005; 353:46-57
29) Wilson PW, Abbott RD, Castelli WP: High density lipoprotein cholesterol and mortality. The Framingham Heart Study. Arteriosclerosis, 1988; 8:737-741
30) Navab M, Anantharamaiah GM, Reddy ST, Van Lenten BJ, Ansell BJ, Fogelman AM: Mechanisms of disease: proatherogenic HDL--an evolving field. Nat Clin Pract Endocrinol Metab, 2006; 2:504-511
31) Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Tuzcu EM; ILLUSTRATE Investigators. Effect of torcetrapib on the progression of coronary atherosclerosis. N Engl J Med, 2007; 356:1304-1316
32) Kastelein JJ, van Leuven SI, Burgess L, Evans GW, Kuivenhoven JA, Barter PJ, Revkin JH, Grobbee DE, Riley WA, Shear CL, Duggan WT, Bots ML; RADIANCE 1 Investigators. Effect of torcetrapib on carotid atherosclerosis in familial hypercholesterolemia. N Engl J Med, 2007; 356:1620-1630
33) Bots ML, Visseren FL, Evans GW, Riley WA, Revkin JH, Tegeler CH, Shear CL, Duggan WT, Vicari RM, Grobbee DE, Kastelein JJ; RADIANCE 2 investigators. Torcetrapib and carotid intima-media thickness in mixed dyslipidaemia (RADIANCE 2 study): a randomized, double-blind trial. Lancet, 2007; 370:153-160
34) Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B; ILLUMINATE Investigators: Effects of torcetrapib in patients at high risk for coronary events. N Engl J Med, 2007; 357:2109-2122
35) Zhong S, Sharp DS, Grove JS, Bruce C, Yano K, Curb JD, Tall AR: Increased coronary heart disease in Japanese-American men with mutation in the cholesteryl ester transfer protein gene despite increased HDL levels. J Clin Invest 1996; 97:2917-2923
36) Hirano K, Yamashita S, Nakajima N, Arai T, Maruyama T, Yoshida Y, Ishigami M, Sakai N, Kameda-Takemura K,

Matsuzawa Y: Genetic cholesteryl ester transfer protein deficiency is extremely frequent in the Omagari area of Japan. Marked hyperalphalipoproteinemia caused by CETP gene mutation is not associated with longevity. Arterioscler Thromb Vasc Biol, 1997; 17:1053-1059
37) Nagano M, Yamashita S, Hirano K, Takano M, Maruyama T, Ishihara M, Sagehashi Y, Kujiraoka T, Tanaka K, Hattori H, Sakai N, Nakajima N, Egashira T, Matsuzawa Y: Molecular mechanisms of cholesteryl ester transfer protein deficiency in Japanese. J Atheroscler Thromb, 2004; 11:110-121
38) Cazita PM, Barbeiro DF, Moretti AI, Quintão EC, Soriano FG: Human cholesteryl ester transfer protein expression enhances the mouse survival rate in an experimental systemic inflammation model: a novel role for CETP. Shock, 2008; 30:590-595
39) Viskin S, Justo D, Halkin A, Zeltser D: Long QT syndrome caused by noncardiac drugs. Prog Cardiovasc Dis, 2003; 45:415-427
40) Guo J, Massaeli H, Li W, Xu J, Luo T, Shaw J, Kirshenbaum LA, Zhang S. Identification of IKr and its trafficking disruption induced by probucol in cultured neonatal rat cardiomyocytes. J Pharmacol Exp Ther, 2007; 321:911-920

Opinion

Proposed Guidelines for Hypertriglyceridemia in Japan with Non-HDL Cholesterol as the Second Target

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The Japan Atherosclerosis Society (JAS) guidelines for the prevention of atherosclerotic diseases, proposing management for LDL cholesterol as the primary target, have successfully contributed to the prevention of cardiovascular events; however, recently, the impact of hypertriglyceridemia as an additional cardiovascular risk has become understood, especially in light of the rise in obesity, metabolic syndrome, and diabetes in the Japanese population. Rather than waiting to obtain conclusive domestic data confirming that hypertriglyceridemia is a cardiovascular risk factor and that its management is efficacious, we propose guidelines for hypertriglyceridemia using non-HDL cholesterol as a second target.

J Atheroscler Thromb, 2008; 15:116-121.

Key words; Hyperlipidemia, Dyslipidemia, Triglycerides, HDL cholesterol, LDL cholesterol

Introduction

Many prospective epidemiological studies have indicated a positive relationship between serum triglyceride (TG) levels and the incidence of coronary heart disease (CHD)[1, 2]. TG-rich lipoproteins such as remnant lipoproteins and small dense LDL particles are increased in hypertriglyceridemia and have been established to be atherogenic by numerous clinical and experimental studies[3-6]; however, classification of the plasma TG level as an independent risk factor for atherosclerosis has been controversial. This is partly because plasma TG levels are inversely intercorrelated by other well-established risk factors, such as low HDL cholesterol. To date, large scale trials for intervention targeting plasma TGs with TG reducing agents such as fibrates have not reached definitive conclusions about their effectiveness on primary endpoints, although fibrates have some impact on both primary and secondary prevention in small scale studies[7-9].

The precise estimation of plasma TGs as a cardiovascular risk is confounded by other risk factors, such as obesity, diabetes, hypertension and smoking. In addition, a cluster of metabolic risk factors, such as visceral obesity and insulin resistance with hypertriglyceridemia, referred to as metabolic syndrome, indicates that plasma TG concentrations are tightly linked to other strong risk factors for CHD. Thus, patients with elevated TGs are at increased risk for CHD, although greater risk cannot be independently explained by TGs. Meanwhile, recent meta-analyses suggested that plasma TGs could be an independent factor for CHD[1, 2]. Supportively, many experimental studies indicated that triglyceride-rich lipoproteins as well as LDL are atherogenic. Taken together, these data suggest that hypertriglyceridemia should be regarded as a semi-independent risk factor and should be included as a clinical target for the prevention of CHD. Considering the increasing prevalence of obesity, metabolic syndrome, and diabetes in this country, guidelines specialized for patients with hypertriglyceridemia need to be immediately established. In this study, we propose new guidelines for Japanese patients with hypertriglyceridemia

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Table 1. Plasma lipid profile of severe and mild type IIb hyperlipidemic patients sub-grouped by non-HDL cholesterol level

Male	severe type IIb non-HDLc >190 mg/dL	mild type IIb non-HDLc <190 mg/dL	*p*
n	51	54	
Total Cholesterol	270±41.8	234±40.3	0.001
Triglycerides	347±286	236±110	0.031
HDL Cholesterol	42.4±8.0	54.9±15.2	0.000
LDL Cholesterol	159±51.6	135±38.1	0.029
non-HDL Cholesterol	228±41.6	182±39.1	0.000

Female	severe type IIb non-HDLc >180 mg/dL	mild type IIb non-HDLc <180 mg/dL	*p*
n	52	48	
Total Cholesterol	265±29.6	231±20.2	0.000
Triglycerides	242±120	218±56	0.1
HDL Cholesterol	47.3±14.1	63.2±19.5	0.000
LDL Cholesterol	175±40.4	125±17.9	0.000
non-HDL Cholesterol	224±30.2	168±14.9	0.000

Subjects were patients who visited the outpatient clinic of the Endocrinology and Metabolism Unit of Tsukuba University Hospital on a regular basis (monthly or bimonthly) as described in Materials and Methods. Data are the means±SD (mg/dL).

using non-HDL as a secondary target after the goal for LDL cholesterol as the primary target is achieved.

Materials and Methods

A total of 1,124 patients in Tsukuba University hospital in 2006 were consecutively included in the study (**Table 1**). Patients with severe illness were excluded. Plasma total cholesterol (TC), LDL-C, TG, HDL-C, glucose and HbA1c in either the fasted or fed state were determined enzymatically with the Hitachi 7070. Plasma HDL-C concentration was measured by a direct method using polyethylene-glycoso-pretreated enzymes. We calculated LDL-C concentration with Friedewald's formula (TC-TG/5-HDL-C) when TG was less than 400 mg/dL. Plasma non-HDL-C concentration was calculated as TC−HDL-C. One hundred and five male and 100 female patients were diagnosed with Type IIb hyperlipidemia (TC>220 mg/dL and TG>150 mg/dL). They were subcategorized into two groups according to their non-HDL cholesterol level (**Table 1**).

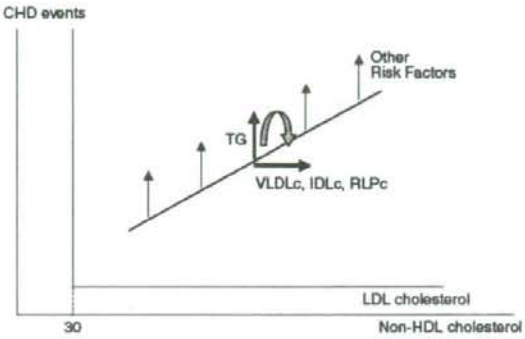

Fig. 1. Rationale for usage of non-HDL cholesterol: impact of TG and other risk factors on correlation between LDL-cholesterol CHD event.

nonHDL cholesterol = Total cholesterol − HDL cholesterol = VLDL cholesterol + IDL cholesterol (remnant lipoprotein cholesterol) + LDL cholesterol (Friedewald formula).
VLDL cholesterol + IDL cholesterol (RLP cholesterol) = TG/5
The risk of hypertriglyceridemia is approximated to VLDL, IDL, and RLP cholesterol estimated as TG/5, and incorporated into non-HDLC. The difference between non-HDL cholesterol and LDL cholesterol on X-axis was set up at 30 mg/dL based upon the data from Fig. 2.

Results and Discussion

Advantage of Non-HDL Cholesterol as a Marker for Hypertriglyceridemia

LDL cholesterol has been established as the most potent predictor of CHD and is currently the primary target for treatment and prevention. Other risk factors, including TG, diabetes, obesity, and metabolic syndrome, do not directly elevate plasma LDL cholesterol, but could enhance the risk of LDL cholesterol by shifting up the curve, as depicted in **Fig. 1**. To evaluate and manage the risk of hypertriglyceridemia, the TG level must be interpolated into the risk of plasma cholesterol. In patients with high TGs, most VLDL cholesterol resides in the smaller (remnant) VLDL fraction. Cholesterol of remnant lipoproteins (VLDL and IDL), which is concomitantly increased by elevation of plasma TG is an appropriate surrogate marker of hypertriglyceridemia. TG-rich remnant lipoproteins have been established as atherogenic lipoproteins[4, 5]. Thus, RLPc, a commercially available laboratory test for remnant lipoprotein cholesterol, could be a suitable marker for the atherogenicity of hypertriglyceridemia; however, this test is expensive and is not practical for use as a routine parameter. In contrast, non-HDL cholesterol, defined as total cholesterol − HDL cholesterol, is easily calculated, and represents the sum-

mation of VLDL/IDL (remnant) cholesterol and LDL cholesterol. It reflects the risks for all apoB-containing lipoproteins and could be an excellent marker for atherogenic lipoproteins. Plasma TG itself is not an appropriate marker for CHD risk due to its internal and dietary variability. In contrast, non-HDL cholesterol is not affected by dietary states and has much less daily variability than TG.

Predictive Power of Non-HDL Cholesterol

Non-HDL cholesterol reflects the risks of both hypertrigyceridemia and LDL-cholesterol[10, 11]. Several studies have indicated that non-HDL cholesterol is better than LDL cholesterol in its predictive power of cardiovascular diseases, indicating that VLDL cholesterol could contribute to CVD[12]. Non-HDL cholesterol is also a useful marker in a variety of subpopulations: men, the elderly, and patients with high-risk diseases such as diabetes and end-stage renal disease[13-16]. Our current clinical data from patients with type IIb hyperlipidemia also support the usefulness of non-HDL cholesterol (**Table 1**). In our outpatient clinic, 70% of patients had diabetes and roughly 10% were type IIb hyperlipidemia (cholesterol > 220 mg/dL and TG > 150 mg/dL). These type IIb hyperlipidemic patients were equally divided into two sub-groups: severe (non-HDL cholesterol levels ≥ 190 mg/dL for male patients and 180 mg/dL for female patients) and mild < 190 mg/dL for male patients and 180 mg/dL for female patients. When the severe and mild IIb groups were compared, total, LDL, HDL cholesterol, and TG levels were significantly different among these two groups for both genders, except for serum triglyceride in females (**Table 1**). These data indicate that non-HDL cholesterol is an excellent marker representing all the components of dyslipidemia. The usefulness of non-HDL cholesterol rather than low-density lipoprotein cholesterol as a tool for lipoprotein cholesterol screening and assessment of risk and therapy has been already recognized in the USA[17, 18]. Another candidate marker for both remnant and LDL cholesterol is plasma apoB level[19]. ApoB is a direct marker for the particle number of apoB-containing lipoproteins and reflects risks of both remnants and LDL. Non-HDL cholesterol is highly correlated with apoB, and should replace this specialized and expensive laboratory test despite some reports indicating that apoB is better than non-HDL cholesterol for the predictive power of CHD[13, 20].

However, according to the Friedewald formula, the TG risk in non-HDL cholesterol represents only one fifth of TG levels as remnant cholesterol, and thus, the contribution of the risk is relatively weak compared to that of LDL cholesterol. Our previous data indicated that the correlation of non-HDL cholesterol to LDL cholesterol was much stronger than that to the TG level (**Fig. 2**)[21]. It should be noted that non-HDL cholesterol is not a specific marker for hypertriglyceridemia. Rather, non-HDL cholesterol should be regarded as a general single marker for both hypercholesterolemia and/or hypertriglyceridemia.

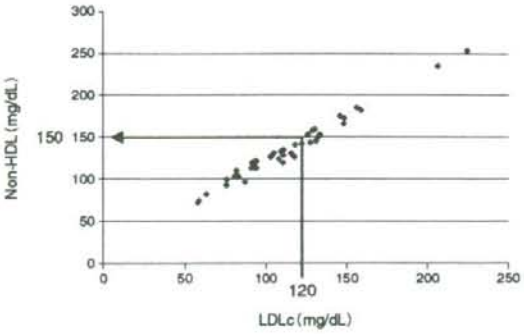

Fig. 2. Distribution of non-HDL cholesterol vs. calculated LDL cholesterol in normolipidemic patients.

Non-HDL cholesterol and LDL cholesterol calculated from Friedewald formula were highly correlated. Subjects were from the outpatient clinic of Tsukuba University Hospital[21].

Proposed Guidelines for Hypertriglyceridemia

Based upon these considerations, we propose guidelines for hypertriglyceridemia in Japanese patients using non-HDL cholesterol as a secondary target, as shown in **Table 2**. This is an extended version of the 2007 edition of the Japan Atherosclerosis Society (JAS) guidelines for the prevention of atherosclerotic diseases in which LDL cholesterol is the primary marker and target. It is essentially similar to the AHA-ATP III guidelines for hyperTG in USA[22]. ATP III recommends using non-HDL cholesterol as a secondary target when plasma TG is greater than 200 mg/dL because VLDL cholesterol is not significantly accumulated if TG is less than 200 mg/dL[23]. We do not have enough clinical data for Japanese on the relationship between TG and VLDL cholesterol to provide the appropriate TG level where the use of a non-HDL marker should be considered. Currently, we recommend using non-HDL for patients with hypertriglyceridemia (TG > than 150 mg/dL). Even for patients with hypertriglyceridemia, the primary target is still LDL cholesterol. In the 2007 JAS guidelines, goals of LDL for the secondary prevention group and the primary prevention group with category I, II, and III are 100, 120, 140, and 160 mg/

Table 2. Proposed Japanese Guidelines for Hypertriglyceridemia

Treatment	Categories		Goal for plasma lipids (mg/dL)		
		Coronary Risk Factors other than LCL-C	Primary LDL-C	Secondary nonHDL-C	HDL-C
Primary Prevention Improving lifestyle as the first line, followed by medication	I (Low Risk Group)	0	<160	<190	≥ 40
	II (Intermediate)	1～2	<140	<170	
	III (High)	≥ 3	<120	<150	
Secondary Prevention Improving lifestyle & medication	Past History of CHD		<100	<130	

Goals for control depend upon categories of LDL cholesterol and non-HDL cholesterol. The primary target in hypertriglyceridemia is LDL-cholesterol. If the goal for LDL-cholesterol in the Japanese Guidelines for Atherosclerosis 2007 is already achieved, nonHDL-C is the secondary target. For the patients with TG > 500 mg/dL, potential genetic disorders and the prevention of acute pancreatitis should be considered. Coronary risk factors other than LDL-cholesterol include low HDL cholesterol, aging, diabetes, hypertenstion, smoking, past history of CHD, and obesity (visceral obesity).

dL, respectively. Goals for non-HDL cholesterol in each group are those for LDL cholesterol plus 30 mg/dL. This is based upon our outpatient clinic data that non-HDL cholesterol was 30 mg/dL higher than LDL cholesterol (**Fig. 2**)[21]. ATPIII also recommends using LDL cholesterol goal + 30 mg/dL[24]. This also corresponds to the calculated VLDL cholesterol of the cut-off point of normal TGs (150/5 mg/dL). This goal is arbitrarily set and could be modified in the future, especially when the relative atherogenicity of remnants and LDL cholesterol are more precisely determined. In the case of TGs of greater than 500 mg/dL, the risk of pancreatitis should be carefully considered as a potential acute complication.

Treatment of Hypertriglyceridemia Based upon Non-HDL Cholesterol Level

Treatment of patients with hypertriglyceridemia for primary prevention should be initiated with lifestyle modifications, especially reducing weight and increasing physical activity. Lifestyles exacerbating hypertrigylyceridemia, such as overweight, obesity, physical inactivity, cigarette smoking, excess alcohol intake, and very high carbohydrate diets, need to be improved. Other disorders and drugs that cause secondary hypertriglyceridemia, including diabetes, chronic renal filure, nephrotic syndrome, and steroid therapy, should also be treated first. In the event that lifestyle modification for at least three months is not effective to achieve the goal of non-HDL cholesterol, medication should be considered. Currently, due to lack of evidence to fully justify the use of fibrates for high TGs prior to statins, it is recommended to use a statin as the first line choice for high non-HDL cholesterol. If statin therapy is already used to control LDL cholesterol, management of non-HDL should be targeted by increasing the dose of the statin or switching to a stronger form. This is based upon the notion that remnant lipoproteins, as well as LDL, are taken up through LDL receptors that are up-regulated by statins. In the case of type III hyperlipidemia, or if high non-HDL cholesterol is much more prominent than LDL cholesterol because of hypertriglyceridemia, fibrates could be considered as they specifically reduce plasma TGs and are effective against type III hyperlipidemia. However, LDL cholesterol should be carefully monitored since fibrates occasionally raise LDL cholesterol following a decrease in TGs (VLDL cholesterol). In case the goal for LDL cholesterol is not attainable, the addition of cholestimide and/or ezetimibe to statin could be considered, whereas EPA could be considered for hypertriglyceridemia. A positive result from a recent large scale Japanese study using both EPA and pravastatin to estimate the prevention of atherosclerotic events, justifies superimposing EPA on statin therapy, although the contribution of the plasma TG-lowering effect of EPA to the prevention of cardiovascular events is not yet determined[25]. The complexity of the choice of medication for high non-HDL cholesterol is currently inevitable because no agents specifically decrease non-HDL cholesterol. Drug information strongly warns against the use of both statins and fibrates because of increasing the risk of the life-threatening side effect of rhabdomyolysis. Joint use is justified only when the benefit exceeds the risk, which requires expertise in this field; however, considering the very few reports of rhabdomyolysis as a severe side effect in recent post-market studies in Japan, carefully prescribing both agents for high-risk patients such as those with type IIb hyperlipidemia could be re-considered. Joint use might be restricted in the elderly or renal compromised patients. In addition, monitoring mus-

cle symptoms and plasma creatine phosphokinase is necessary in patients prescribed either statins or fibrates.

Conclusions and Future Prospect of the Guidelines

Non-HDL cholesterol containing both LDL cholesterol and remnant cholesterol, is an excellent predictor of atherosclerotic risk, and should be a treatment target. Non-HDL cholesterol is simple, convenient, and free from dietary variations. These advantages are crucial for nation-wide use of the guidelines and health check activity. This simple measurement could also make it possible to re-evaluate previous clinical studies using this parameter to offer a good chance of estimating the usefulness and importance of this marker in a large meta-analytical scale.

In the current study, we propose that LDL cholesterol is the primary target and non-HDL cholesterol should be the secondary target for elevated TG. Considering that non-HDL and LDL cholesterol are partially redundant, non-HDL could replace LDL as the primary target and as a general marker for both elevated cholesterol and TG. As **Table 1** shows, non-HDL cholesterol could be used as a general and convenient lipid marker for type IIb hyperlipidemia.

This proposal still faces the recent problem of selecting lipid markers for the initial assessment for dyslipidemia. The recent GL focus has been on LDL cholesterol rather than TC, while LDL cholesterol has a problem the lower reliability for direct measurement. In addition, a considerable portion of hypertriglyceridemia is not applicable to this equation. For subjects with hypertriglyceridemia, application of this new GL eventually requires all TC, TG, HDL, and LDL cholesterol measurements to assess both LDL and non-HDL cholesterol. Currently, however, the Japanese medical system covers only three out of four lipid measurements as healthcare services provided by health insurance. Further Japanese clinical studies and careful evaluation of the data, as well as technical improvements of reliable LDL cholesterol measurements, are required to determine the most efficient protocol to select lipid measurements as the initial assessment of dyslipidemia to prevent CVD in Japan. Furthermore, guidelines for HDL cholesterol should also be established, although the relative importance and positioning of non-HDL and HDL is yet to be determined.

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References

1) Assmann G, Schulte H, Funke H, and von Eckardstein A: The emergence of triglycerides as a significant independent risk factor in coronary artery disease. Eur Heart J, 1998; 19 Suppl M:M8-14
2) Austin MA, Hokanson JE, and Edwards KL: Hypertriglyceridemia as a cardiovascular risk factor. Am J Cardiol, 1998; 81:7B-12B
3) Krauss RM: Atherogenicity of triglyceride-rich lipoproteins. Am J Cardiol, 1998; 81:13B-17B
4) Grundy SM: Hypertriglyceridemia, atherogenic dyslipidemia, and the metabolic syndrome. Am J Cardiol, 1998; 81:18B-25B
5) Havel RJ: Role of triglyceride-rich lipoproteins in progression of atherosclerosis. Circulation, 1990; 81:694-696
6) Breslow JL: Mouse models of atherosclerosis. Science, 1996; 272:685-688
7) Elkeles RS, Diamond JR, Poulter C, Dhanjil S, Nicolaides AN, Mahmood S, Richmond W, Mather H, Sharp P, and Feher MD: Cardiovascular outcomes in type 2 diabetes. A double-blind placebo-controlled study of bezafibrate: the St. Mary's, Ealing, Northwick Park Diabetes Cardiovascular Disease Prevention (SENDCAP) Study. Diabetes Care, 1998; 21:641-648
8) Tenenbaum A, Motro M, Fisman EZ, Tanne D, Boyko V, and Behar S: Bezafibrate for the secondary prevention of myocardial infarction in patients with metabolic syndrome. Arch Intern Med, 2005; 165:1154-1160
9) Keech A, Simes RJ, Barter P, Best J, Scott R, Taskinen MR, Forder P, Pillai A, Davis T, Glasziou P, Drury P, Kesäniemi YA, Sullivan D, Hunt D, Colman P, d'Emden M, Whiting M, Ehnholm C, and Laakso M: Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): randomised controlled trial. Lancet, 2005; 366:1849-1861
10) Grundy SM: Non-high-density lipoprotein cholesterol level as potential risk predictor and therapy target. Arch Intern Med, 2001; 161:1379-1380
11) Packard CJ and Saito Y: Non-HDL cholesterol as a measure of atherosclerotic risk. J Atheroscler Thromb, 2004; 11:6-14
12) Liu J, Sempos CT, Donahue RP, Dorn J, Trevisan M, and Grundy SM: Non-high-density lipoprotein and very-low-density lipoprotein cholesterol and their risk predictive values in coronary heart disease. Am J Cardiol, 2006; 98:1363-1368
13) Pischon T, Girman CJ, Sacks FM, Rifai N, Stampfer MJ, and Rimm EB: Non-high-density lipoprotein cholesterol

and apolipoprotein B in the prediction of coronary heart disease in men. Circulation, 2005; 112:3375-3383
14) Kawamoto R, Oka Y, Tomita H, and Kodama A: Non-HDL cholesterol as a predictor of carotid atherosclerosis in the elderly. J Atheroscler Thromb, 2005; 12:143-148
15) Jiang R, Schulze MB, Li T, Rifai N, Stampfer MJ, Rimm EB, and Hu FB: Non-HDL cholesterol and apolipoprotein B predict cardiovascular disease events among men with type 2 diabetes. Diabetes Care, 2004; 27:1991-1997
16) Nishizawa Y, Shoji T, Kakiya R, Tsujimoto Y, Tabata T, Ishimura E, Nakatani T, Miki T, and Inaba M: Non-high-density lipoprotein cholesterol (non-HDL-C) as a predictor of cardiovascular mortality in patients with end-stage renal disease. Kidney Int Suppl, 2003; 84:S117-120
17) Frost PH and 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
18) Cui Y, Blumenthal RS, Flaws JA, Whiteman MK, Langenberg P, Bachorik PS, and Bush TL: Non-high-density lipoprotein cholesterol level as a predictor of cardiovascular disease mortality. Arch Intern Med, 2001; 161:1413-1419
19) Vega GL and Grundy SM: Does measurement of apolipoprotein B have a place in cholesterol management? Arteriosclerosis, 1990; 10:668-671
20) Simon A, Chironi G, Gariepy J, Del Pino M, and Levenson J: Differences between markers of atherogenic lipoproteins in predicting high cardiovascular risk and subclinical atherosclerosis in asymptomatic men. Atherosclerosis, 2005; 179:339-344
21) Sugimoto K, Isobe K, Kawakami Y, and Yamada N: The relationship between non-HDL cholesterol and other lipid parameters in Japanese subjects. J Atheroscler Thromb, 2005; 12:107-110
22) Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation, 2002; 106:3143-3421
23) Goldstein JL, Schrott HG, Hazzard WR, Bierman EL, and Motulsky AG: Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. J Clin Invest, 1973; 52:1544-1568
24) Heiss G, Tamir I, Davis CE, Tyroler HA, Rifkand BM, Schonfeld G, Jacobs D, and Frantz ID Jr.: Lipoprotein-cholesterol distributions in selected North American populations: the lipid research clinics program prevalence study. Circulation, 1980; 61:302-315
25) Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, Kita T, Kitabatake A, Nakaya N, Sakata T, Shimada K, and Shirato K: Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet, 2007; 369:1090-1098

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Coexpression of CLA-1 and Human PDZK1 in Murine Liver Modulates HDL Cholesterol Metabolism

Hidenori Komori, Hidenori Arai, Terumi Kashima, Thierry Huby, Toru Kita and Yukihiko Ueda

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Coexpression of CLA-1 and Human PDZK1 in Murine Liver Modulates HDL Cholesterol Metabolism

Hidenori Komori, Hidenori Arai, Terumi Kashima, Thierry Huby, Toru Kita, Yukihiko Ueda

Objective—In rodents scavenger receptor class B type I (SR-BI) is a key molecule for selective uptake of cholesteryl ester from high-density lipoprotein (HDL). This study was aimed to clarify the role of the human SR-BI/CD36 and LIMP-II Analogues-1 (CLA-1) as a molecular target of selective uptake of cholesteryl ester from HDL in vivo.

Methods and Results—To clarify the function and regulation of CLA-1 in vivo we produced *CLA-1* BAC transgenic mice. In spite of abundant hepatic RNA expression of *CLA-1*, *CLA-1* BAC transgenic mice had no significant effect on mouse HDL cholesterol. Although coexpression of a human scaffolding protein PDZK1 along with CLA-1 enhanced hepatic CLA-1 expression, it did not affect mouse HDL cholesterol levels, either. However, in the presence of human apoA-1, HDL cholesterol level and size were significantly reduced in CLA-1 transgenic mice, and its reduction was more pronounced in *CLA-1*/human *PDZK1* double transgenic mouse.

Conclusions—We established a mouse model to study human reverse cholesterol transport by expressing *CLA-1*, human *PDZK1*, and human *apoA-I* gene. Our results imply that enhancing CLA-1 expression by human PDZK1 in the liver can modulate HDL cholesterol metabolism and possibly enhance reverse cholesterol transport to prevent the progression of atherosclerosis in human. (***Arterioscler Thromb Vasc Biol.* 2008;28:1298-1303**)

Key Words: lipoproteins ■ receptor ■ transgenic model ■ apolipoproteins ■ genetically altered mice

The inverse correlation between plasma high-density lipoprotein (HDL) cholesterol levels and the risk of the coronary heart disease has been established.[1,2] HDL is an antiatherogenic lipoprotein involved in the reverse cholesterol transport (RCT) system where HDL removes excess cholesteryl ester (CE) from peripheral tissues and carries it back to the liver. Therefore, establishing a new therapeutic strategy which can enhance RCT is of great benefit to prevent the development of coronary heart disease.

One of the candidate molecules for RCT is scavenger receptor class B type I (SR-BI). SR-BI was first cloned in 1994 as a modified low-density lipoprotein (LDL) receptor.[3,4] In 1996 this receptor was found to be an HDL receptor that mediates selective uptake of CE from HDL to the liver.[5,6] SR-BI interacts with HDL via apoA-I, which is a major apolipoprotein on HDL molecules,[7] and is necessary for mediating CE uptake.[8,9]

We and others showed that increasing the expression of SR-BI in the liver enhances RCT resulting in a decrease of atheromatous lesion formation in spite of the decreased plasma levels of HDL cholesterol.[10–13] In contrast, decreased expression of SR-BI stimulates the lesion formation in spite of increased HDL cholesterol levels.[14–16] Thus, in rodents SR-BI is a key molecule as an HDL receptor in the liver affecting RCT and the pathogenesis of atherosclerosis. These observations suggest SR-BI as a molecular target for antiatherosclerosis therapy.

However, the RCT system in human is more complicated than in rodents, and it has been hard to study the role of SR-BI in human because of the presence of CE transfer protein (CETP). The human homologue of SR-BI was independently cloned as CD36 and LIMPII analogous-1 (CLA-1).[17,18] CLA-1 functions as a receptor for HDL as well as LDL, VLDL, modified LDL, hepatitis C virus (HCV) in vitro.[19–21] In human CLA-1 is expressed in liver, adrenal gland, testis, and macrophages in the atherosclerotic lesion.[20,22,23] However, the physiological function and the regulation of this molecule still remain unknown.

Tissue expression of SR-BI is regulated both transcriptionally and posttransscriptionally. PDZK1 was cloned as an associating protein with SR-BI stabilizing SR-BI protein on the hepatocyte.[24] PDZK1 is found in liver, kidney, small intestine, pancreas, adrenal cortex, gastrointestinal tract, and testis in human tissue samples.[25] PDZK1 is a 70-kDa protein composed of 4 functional PDZ domains that play an important role in the transport, localization, assembly, and scaffolding of membrane proteins. Coexpression of hamster SR-BI and rat PDZK1 in CHO cells increases SR-BI expression.[24] In

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contrast, hepatic downregulation of PDZK1 with fibrates, MAP17 transgenic mouse, and *PDZK1*-deleted mice showed decreased hepatic SR-BI protein expression posttranscriptionally.[26–28] Interestingly, *PDZK1*-deletion mice show dramatically reduced hepatic SR-BI expression along with increased plasma cholesterol levels.[26,29] Thus in the liver, but not in steroidogenic tissues,[26] posttranscriptional control of SR-BI proteins expression depends on the presence of PDZK1 in rodents.

To explore the function and the regulatory mechanism of CLA-1 in vivo, we generated *CLA-1* transgenic and human *PDZK1* transgenic mouse by introducing human BAC clones. Then we produced double transgenic mouse coexpressing CLA-1 and human PDZK1 to enhance hepatic CLA-1 expression. We investigated CLA-1 expression in the liver along with PDZK1 and their effect on HDL metabolism.

Methods

Bacterial Artificial Chromozome Isolation and Generating Transgenic Mouse

Bacterial artificial chromozome (BAC) clone 265I19 containing full length of the 75-Kb *CLA-1* gene as well as 70-Kb upstream and 25-Kb downstream sequence (Figure 1A) was obtained from Research Genetics by polymerase chain reaction (PCR) screening. BAC was isolated by alkali method and the backbone, pBELOBAC11, was removed by pulsed field gel (PFG) electrophoresis. The linearized fragment was purified by phenol-chloroform extraction and then by dialyzing against an injection buffer (10 mmol/L Tris·HCl pH 7.5; 1 mmol/L EDTA; 100 mmol/L NaCl) through Millipore type VS 0.025-mm membrane (Millipore). Purified DNA was diluted to 5.0 μg/mL for pronuclear injection into FVB mouse fertilized embryos (B.L. Hogan, *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Springs Harbor Express). By the same method the 180-kb genomic DNA fragment containing the full-length sequence of human *PDZK1* with both 34-kbp upstream of exon 1 and 109 kbp downstream of exon 10 (Figure 2A) isolated as BAC clone 354F1 was used to generate human *PDZK1* transgenic mouse.

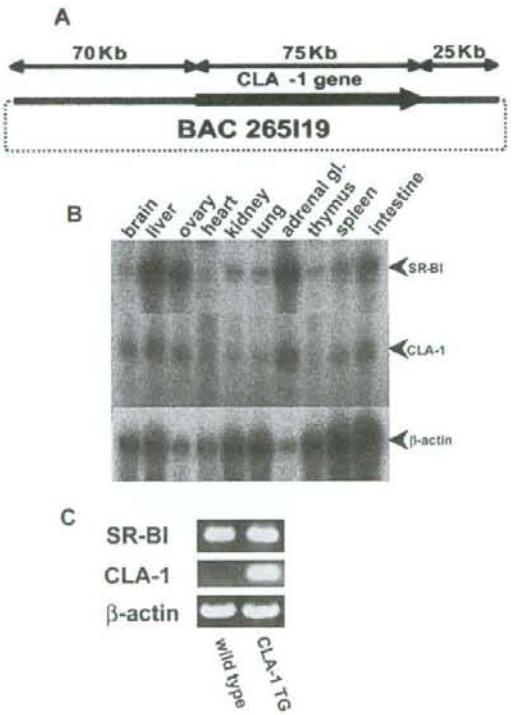

Figure 1. Analysis of CLA-1 BAC transgenic mouse. A, Schema of BAC 265I19 containing the CLA-1 gene. B, RNase protection assay for CLA-1 RNA expression. Ten μg of total RNA in each organ were subjected for the analysis to detect the expression of the first exon of CLA-1 or mouse-SR-BI. C, SR-BI and CLA-1 expression in macrophages by RT-PCR.

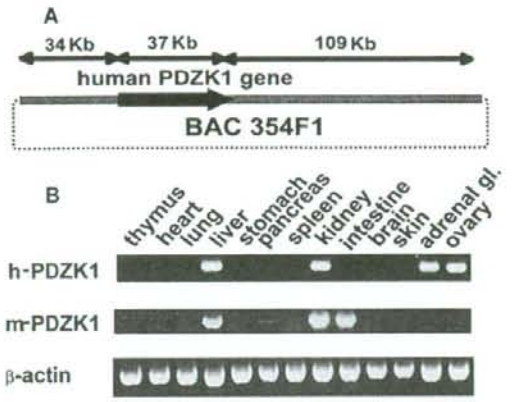

Figure 2. Analysis of human PDZK1 transgenic mouse. A, Schema of BAC 354F1 containing the human *PDZK1* gene. B, Expression pattern of PDZK1 in human *PDZK1* BAC transgenic mouse by RT-PCR. After digestion by DNaseI, 0.5 μg of total RNA were transcribed to subject for PCR amplification. Mouse β-actin was served as an internal control. h-PDZK1 indicates human PDZK1; m-PDZK1, mouse PDZK1.

Other Genetically Engineered Mice

SR-BI-deleted (*SR-BI*$^{-/-}$) mouse was generated as described.[30] Human *apoA-I* transgenic mouse (*h-apoAI*^{tg})[31] and *apoA-I*–deleted mouse (*m-apoAI*$^{-/-}$)[32] were obtained from the Jackson Laboratory (Bar Harbor, Me). All transgenics were established in an FVB strain, and *SR-BI*$^{-/-}$ and *m-apoAI*$^{-/-}$ mice were back-crossed with FVB mice at least 6 generations, respectively, so that all mice used in the current study have FVB genetic background. All animals in the current study were caged in Kyoto University Animal Facility, which is air-conditioned with controlled light-cycle, and were manipulated along with the Animal Welfare Regulations of Japanese government.

Plasma Lipid and HDL Particle Analysis

At the age of 8 weeks after fed normal chow, blood samples of female mouse were collected by tail vein bleeding in EDTA-coated Microtainer tube (Becton Dickinson and Company). Samples were centrifuged at 7500*g* for 5 minutes and stored at −80°C until analysis. Plasma total (TC) and free cholesterol (FC) was determined by cholesterol oxidase method using Cholesterol E-test Wako (Wako) for TC and Free Cholesterol E-test Wako for FC. Esterified cholesterol (CE) concentration was calculated by subtracting FC from TC. Plasma levels of HDL cholesterol were evaluated by measuring cholesterol after prepicitating the apolipoprotein B containing fraction with 6.5% polyethylene glycol (PEG).[33]

Cholesterol profile in plasma lipoproteins was analyzed by a dual detection high-performance liquid chromatography (HPLC) system with 2 tandem TSKgel LipopropakXL columns according to the method of Usui et al (Lipidsearch System, Skylight Biotech Inc).[34]

Genotyping of transgenic mice, RNA isolation and analysis, macrophage RNA isolation, Western blotting for liver lysates, and statistical analysis are described under Materials and Methods section in the online Data Supplement available at http://atvb.ahajournals.org.

Results

Gene Expression and Lipid Profile of CLA-1 BAC Transgenic Mouse

To clarify the function of CLA-1 in vivo we generated *CLA-1* transgenic mouse. The inclusion of endogenous regulatory elements within the BAC transgene allows assessment of physiological tissue distribution and regulation of *CLA-1* (Figure 1A). The CLA-1 BAC clone does not contain any other genes. To confirm the expression of the transgene in various tissues of *CLA-1* transgenic mice (*CLA-1 TG*), RNase protection assays were performed (Figure 1B). Under physiological regulation by the human promoter, the expression pattern of *CLA-1* was quite similar to that of the mouse *SR-BI* gene. Expression of the *CLA-1* gene was high in liver, adrenal gland, intestine, and ovary. CLA-1 was also expressed in macrophages of *CLA-1 TG* (Figure 1C).

To investigate the effect of the transgene on lipid metabolism, plasma levels of total cholesterol (TC) and HDL cholesterol (HDL-C) were determined. Plasma TC levels of 10 transgenic mice were not significantly decreased compared with those of the littermate (WT) animals (*CLA-1 TG* 86.5±12.1 mg/dL versus WT 99.5±9.5 mg/dL; n.s.). No significant difference was found in plasma HDL-C levels (*CLA-1 TG* 53.1±23.0 mg/dL versus WT 63.9±10.8 mg/dL; n.s.), either. Although we have established 2 independent transgenic lines, there was no difference in cholesterol levels in both lines. There was no gender difference of cholesterol levels in these mice. Thus CLA-1 expression in addition to endogenous SR-BI did not affect plasma cholesterol levels.

CLA-1 Protein Expression Was Increased by Coexpression of Human PDZK1 in Liver

Because PDZK1 is an important regulator for SR-BI expression, we assumed that expression of human PDZK1 can be effective for the expression and function of CLA-1. To this end we first produced human *PDZK1* BAC transgenic mouse (*h-PDZK1*). The BAC fragment including the whole human *PDZK1* gene with 34-kb upstream and 110-kb downstream sequences was prepared for microinjection (Figure 2A). To evaluate the expression pattern of the human *PDZK1* transgene and to distinguish it from the endogenous *PDZK1* gene, we performed RT-PCR in various tissues of *h-PDZK1* mice. Similar to endogenous PDZK1 expression in liver, kidney, and intestine, the human *PDZK1* transgene was expressed in liver, kidney, adrenal gland, and ovary (Figure 2B), which is consistent to the previous report.[25] Neither human nor mouse PDZK1, however, was not expressed in macrophages (data not shown).

To assess the effect of human PDZK1 expression on hepatic CLA-1 expression, we then produced *CLA-1/h-PDZK1* double transgenic mouse and performed immunoblotting analysis of hepatic CLA-1 in these mice (Figure 3A). To exclude the effect of endogenous SR-BI and its cross-reaction to the SR-BI/CLA-1 antibody, we introduced the *CLA-1* transgene in *SR-BI*$^{-/-}$ mice, producing *SR-BI*$^{-/-}$/*CLA-1* and *SR-BI*$^{-/-}$/*CLA-1/h-PDZK1* mice. Because the PDZK1 antibody does not distinguish between human and mouse PDZK1, the band corresponding to PDZK1 in *SR-BI*$^{-/-}$/*CLA-1/h-PDZK1* mouse represent the sum of endogenous and transgenic expression. Compared with SR-BI expression in WT mice, *SR-BI*$^{-/-}$/*CLA-1* mouse expressed a significantly lower level of CLA-1 protein in the liver (Figure 3B). However, introduction of the *PDZK1* transgene (*SR-BI*$^{-/-}$/*CLA-1/h-PDZK1* mice) significantly increased expression of human PDZK1 protein along with CLA-1 protein expression. Thus human PDZK1 expression in the liver enhanced hepatic CLA-1 protein expression.

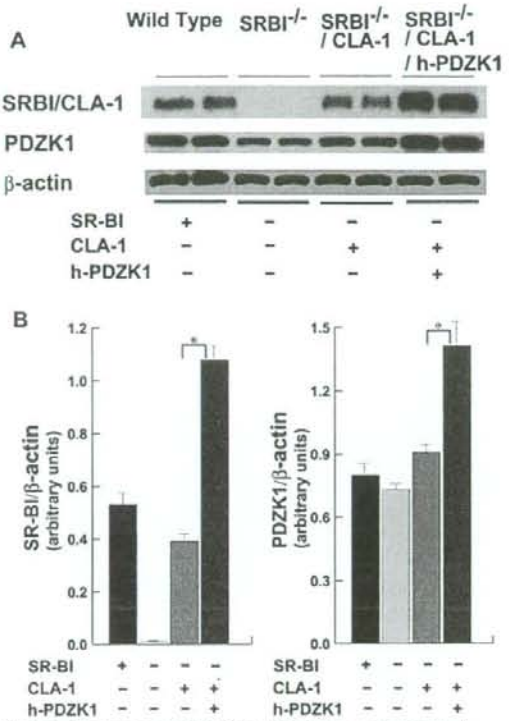

Figure 3. Expression of PDZK1 and CLA-1 in each tissue. A, SR-BI/CLA-1 protein expression in liver. Fifteen micrograms of liver lysates from each 2 mice were subjected for blotting. The PDZK1 band in the h-PDZK1 mouse represents the sum of mouse and human PDZK1. B, Quantitative analysis of SR-BI and PDZK1 protein expression in liver by Optical densitometry. *$P<0.05$.

Species Difference in apoA-I–SR-BI Interaction on CE Uptake

To determine whether CLA-1 can regulate HDL-C metabolism in the presence of human PDZK1, we measured cholesterol levels in these mice (Table). HDL-C was increased by approximately 2 folds in the absence of SR-BI or CLA-1 (WT: 96.3±17.3 mg/dL versus *SR-BI*$^{-/-}$: 209.0±17.8 mg/dL; $P<0.05$). However, introducing lower levels of CLA-1 (*SR-*

Table. Plasma Cholesterol Levels in Transgenic Mice

ApoA-I Genotype	SR-BI$^{+/+}$	SR-BI$^{+/-}$	SR-BI$^{-/-}$/CLA-1	SR-BI$^{-/-}$/CLA-1/h-PDZK1
mapoAI$^{+/+}$				
TC	111.3±15.1* (−53%)	234.0±18.8	215.8±20.5 (−8%)	206.7±12.3* (−12%)
HDL-C	96.3±17.3* (−54%)	209.0±17.8	194.7±22.8 (−7%)	185.6±19.3 (−11%)
CE	79.9±6.7* (−35%)	123.7±32.6	143.1±14.0 (+16%)	141.3±14.8 (+14%)
FC	31.4±2.6* (−72%)	110.3±29.1	72.7±7.1 (−34%)	65.0±6.8* (−41%)
mapoAI$^{-/-}$/hapoAI^{tg}				
TC	162.1±16.5† (−72%)	580.2±85.5	359.8±42.5† (−38%)	286.6±32.2† (−51%)
HDL-C	147.9±15.4† (−73%)	557.3±38.3	339.8±36.9† (−39%)	247.4±45.9† (−56%)
CE	114.4±5.5† (−67%)	342.6±90.9	235.2±40.9† (−31%)	179.3±31.2† (−48%)
FC	47.7±2.3† (−80%)	237.6±63.0	124.6±21.7† (−48%)	107.3±18.7† (−55%)

Values are expressed as means ± SD (mg/dl).

TC indicates total cholesterol; HDL-C, HDL cholesterol; CE, esterified cholesterol; FC, free cholesterol; mapoAI, mouse apoA-I; hapoAI, human apoA-I.

*$P<0.05$ vs mapoAI$^{+/+}$/SR-BI$^{-/-}$; †$P<0.05$ vs mapoAI$^{-/-}$/hapoAI/SR-BI$^{-/-}$.

(−%) percent reduction compared to the value of SR-BI$^{-/-}$ in each apoA-I genotype.

BI$^{-/-}$*/CLA-1*) or higher levels of CLA-1 in the presence of PDZK1 (*SR-BI*$^{-/-}$/CLA-1/*h-PDZK1*) did not show any change in HDL-C.

Therefore, we presumed that CLA-1 requires human apoA-I for its sufficient interaction with HDL. To address this issue we introduced human *apoA-I* in the absence of mouse *apoA-I*, producing mouse *apoA-I*–deleted/human *apoA-I* transgenic mice (*m-apoAI*$^{-/-}$/h-apoAI ^{tg}). Introducing human *apoA-I* resulted in a 1.5-fold increase in HDL-C. In the absence of *SR-BI* or *CLA-1*, HDL-C was increased by approximately 3.5 folds (Table). In contrast to the results in mouse *apoA-I*, introducing *CLA-1* lowered HDL-C by 39% (*SR-BI*$^{-/-}$*/CLA-1*: 339.8±36.9 mg/dL versus *SR-BI*$^{-/-}$: 557.3±38.3 mg/dL; $P<0.05$). Addition of the human *PDZK1* transgene further reduced HDL-C by 56% (*SR-BI*$^{-/-}$*/CLA-1/h-PDZK1*: 247.4±45.9 mg/dL; $P<0.05$ compared to *SR-BI*$^{-/-}$*/CLA-1*). We also determined the plasma CE and FC levels in these mice. Plasma CE levels were not different among the 3 groups of *SR-BI*$^{-/-}$ mice in the presence of mouse *apoA-I* (Table). However, plasma FC levels significantly decreased in the presence of *CLA-1* and *h-PDZK1*. Meanwhile, in the presence of human *apoA-I*, CE and FC decreased significantly along with HDL-C by the addition of *CLA-1* and *h-PDZK1*. By HPLC analysis the HDL particle size was also decreased along with the expression level of CLA-1 (Figure 4). However, the pattern of HDL particle size is somewhat different in mice with mouse or human *apoA-I*. The HDL particle size is almost the same between *SR-BI*$^{+/+}$ and *SR-BI*$^{-/-}$*/CLA-1/h-PDZK1* mice with human *apoA-I*, whereas the HDL particle size is larger in *SR-BI*$^{-/-}$*/CLA-1/h-PDZK1* mice than in WT mice with mouse *apoA-I*. Thus, effects of CLA-1 expression on plasma CE/FC and HDL particles size were consistent in mice with mouse or human *apoA-I*. These results suggest that CLA-1 requires human apoA-I to interact efficiently with HDL particles in vivo.

Discussion

In this study we have established a mouse model to study human RCT by coexpressing of *CLA-1*, human *PDZK1*, and human *apoA-I* gene and found that CLA-1 expression plays an important role in plasma HDL cholesterol metabolism in vivo. Therefore, this model would be a useful tool to study the function of human CLA-1 and its effect on HDL metabolism. Because we have used BAC clones for transgenic mice to obtain physiological levels of expression, this could be another advantage for in vivo study.

Upregulating hepatic SR-BI can prevent atherosclerosis by accelerating RCT in rodents. However, RCT is more complicated in human. Human has another mechanism to transfer

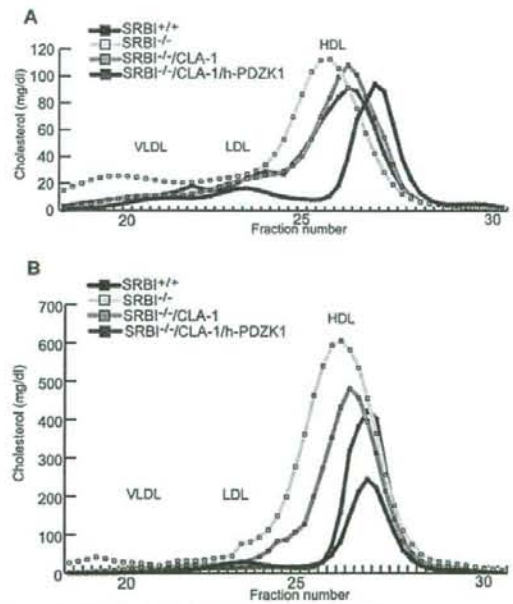

Figure 4. Plasma lipoprotein analyses by HPLC. Plasma samples obtained from 8-week-old chow-fed mice of the indicated genotypes were fractionated by HPLC. A, Plasma obtained from mice with mouse apoA-I. B, Plasma obtained from mice with human apoA-I transgene in the absence of mouse apoA-I (m-apoAI$^{-/-}$/h-apoAI^{tg}).

CE in HDL particles to the liver, which is mediated by CETP. In CETP-deficient patients,[35,36] HDL-C levels are higher than people with CETP, suggesting that SR-BI–dependent RCT is less important in human lipid metabolism than in rodents. However, there is a huge variability in HDL cholesterol levels in those patients, also suggesting a role of CLA-1 and other molecules in RCT in human. Therefore, by introducing CETP in this model we might be able to produce a model that resembles human RCT.

Ikemoto et al show that PDZK1 can bind its N-terminal PDZ domain to the C terminus of SR-BI in vitro and in vivo and suggest that PDZK1 contributes to the proper sorting and delivery of SR-BI to the plasma membrane in hepatocytes as well as to its stability and function in the liver.[24] We have shown that expression of human PDZK1 enhanced CLA-1 protein expression compared with the mice expressing only CLA-1. Our animal model coexpressing both CLA-1 and human PDZK1 demonstrates that human PDZK1 is an important enhancer of CLA-1 expression in the liver. For the interaction between SR-BI and PDZK1 4 C-terminal amino acids of SR-BI are essential, which are preserved completely in various species.[37] Despite containing the same 4 C-terminal amino acids mouse PDZK1 could not fully stabilize CLA-1 in the liver. These species difference may be caused by a higher constructive change of protein for the molecular interaction. Similarly different SR-BI reaction to the drugs between human and mouse[38] might be explained by the species-specific involvement of PDZK1.

Our data indicate that CLA-1 can selectively uptake CE from HDL containing human apoA-I, suggesting a species difference in apoA-I–SR-BI interaction in the CE uptake mechanism, in spite of its high levels of homology by 80% amino acid identity between mouse SR-BI and CLA-1. It is not clearly known which site of apoA-I is critical for the binding to SR-BI because apoA-I contains multiple regions with amphipatic α-helical repeats, which can bind to SR-BI.[7] Comparing amino acids sequences between human and mouse apoA-I, the carboxyl-terminal region (residue 185 to 243) shows 60% identity between human and mouse, whereas the amino-terminal region (residue 1 to 43) and the central core region (residue 68 to 185) have 72% identity. The carboxyl terminus of apoA-I is involved in protein-lipid interaction, which is critical for the initial rapid binding to HDL, cholesterol efflux from the plasma membrane, and binding to the receptor.[39] Gong et al have reported that the difference of mean hydrophobicity in the helical segment may be responsible for lower efficiency of mouse apoA-I in its stability and lipid binding than of human apoA-I.[40] Thus the constructive difference in the carboxyl terminus may affect the conformation of HDL and contribute to species-specific binding.

We have demonstrated that FC was decreased by the expression of CLA-1 and human-PDZK1, but not CE in mice with mouse *apoA-I*. SR-BI has been reported to mediate not only selective uptake of HDL-CE[5] but also bidirectional transfer of FC between HDL and cells.[41,42] SR-BI–mediated cellular uptake of FC and CE correlates with the expression level of SR-BI in different cell lines.[41] In vivo SR-BI promotes the uptake of HDL-FC and facilitates the rapid clearance of HDL-FC by the liver into bile.[43] However, FC efflux mediated by SR-BI seems to occur independently of acceptor binding to SR-BI.[44] Therefore, it is conceivable that CLA-1 could mediate the uptake of FC from HDL particles independent of species-specific apoA-I. In vitro study is necessary to address this issue.

Hepatitis C virus (HCV) is known to infect hepatocytes via CLA-1. However, mouse SR-BI is not able to bind E2, the envelope glycoprotein of HCV.[21] Because of the species specificity of HCV infection as we found in apoA-I interaction, HCV infection can be studied in vivo only in a chimpanzee model. Therefore, our murine model expressing CLA-1 can be a useful model to study the mechanism of HCV infection.

In conclusion our data implicate that CLA-1 could modulate HDL cholesterol metabolism in human as an HDL receptor in the liver and that CLA-1 and PDZK1 are possible molecular targets for athero-therapeutical strategies in human.

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Disclosures

None.

References

1. Castelli WP, Doyle JT, Gordon T, Hames CG, Hjortland MC, Hulley SB, Kagan A, Zukel WJ. HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation*. 1977;55:767–772.
2. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am J Med*. 1977;62:707–714.
3. Acton SL, Scherer PE, Lodish HF, Krieger M. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J Biol Chem*. 1994; 269:21003–21009.
4. Rigotti A, Acton SL, Krieger M. The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. *J Biol Chem*. 1995; 270:16221–16224.
5. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science*. 1996;271:518–520.
6. Landschulz KT, Pathak RK, Rigotti A, Krieger M, Hobbs HH. Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. *J Clin Invest*. 1996;98: 984–995.
7. Williams DL, de La Llera-Moya M, Thuahnai ST, Lund-Katz S, Connelly MA, Azhar S, Anantharamaiah GM, Phillips MC. Binding and cross-linking studies show that scavenger receptor BI interacts with multiple sites in apolipoprotein A-I and identify the class A amphipathic alpha-helix as a recognition motif. *J Biol Chem*. 2000;275:18897–18904.
8. Temel RE, Walzem RL, Banka CL, Williams DL. Apolipoprotein A-I is necessary for the in vivo formation of high density lipoprotein competent for scavenger receptor BI-mediated cholesteryl ester-selective uptake. *J Biol Chem*. 2002;277:26565–26572.
9. Williams DL, Temel RE, Connelly MA. Roles of scavenger receptor BI and APO A-I in selective uptake of HDL cholesterol by adrenal cells. *Endocr Res*. 2000;26:639–651.
10. Arai T, Wang N, Bezouevski M, Welch C, Tall AR. Decreased atherosclerosis in heterozygous low density lipoprotein receptor-deficient mice expressing the scavenger receptor BI transgene. *J Biol Chem*. 1999;274: 2366–2371.

11. Ueda Y, Gong E, Royer L, Cooper PN, Francone OL, Rubin EM. Relationship between expression levels and atherogenesis in scavenger receptor class B, type I transgenics. *J Biol Chem*. 2000;275:20368–20373.
12. Kozarsky KF, Donahee MH, Glick JM, Krieger M, Rader DJ. Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse. *Arterioscler Thromb Vasc Biol*. 2000;20:721–727.
13. Zhang Y, Da Silva JR, Reilly M, Billheimer JT, Rothblat GH, Rader DJ. Hepatic expression of scavenger receptor class B type I (SR-BI) is a positive regulator of macrophage reverse cholesterol transport in vivo. *J Clin Invest*. 2005;115:2870–2874.
14. Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc Natl Acad Sci U S A*. 1997;94:12610–12615.
15. Huszar D, Varban ML, Rinninger F, Feeley R, Arai T, Fairchild-Huntress V, Donovan MJ, Tall AR. Increased LDL cholesterol and atherosclerosis in LDL receptor-deficient mice with attenuated expression of scavenger receptor B1. *Arterioscler Thromb Vasc Biol*. 2000;20:1068–1073.
16. Braun A, Trigatti BL, Post MJ, Sato K, Simons M, Edelberg JM, Rosenberg RD, Schrenzel M, Krieger M. Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice. *Circ Res*. 2002;90:270–276.
17. Calvo D, Vega MA. Identification, primary structure, and distribution of CLA-1, a novel member of the CD36/LIMPII gene family. *J Biol Chem*. 1993;268:18929–18935.
18. Calvo D, Dopazo J, Vega MA. The CD36, CLA-1 (CD36L1), and LIMPII (CD36L2) gene family: cellular distribution, chromosomal location, and genetic evolution. *Genomics*. 1995;25:100–106.
19. Calvo D, Gomez-Coronado D, Lasuncion MA, Vega MA. CLA-1 is an 85-kD plasma membrane glycoprotein that acts as a high-affinity receptor for both native (HDL, LDL, and VLDL) and modified (OxLDL and AcLDL) lipoproteins. *Arterioscler Thromb Vasc Biol*. 1997;17:2341–2349.
20. Murao K, Terpstra V, Green SR, Kondratenko N, Steinberg D, Quehenberger O. Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. *J Biol Chem*. 1997;272:17551–17557.
21. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *Embo J*. 2002;21:5017–5025.
22. Nakagawa-Toyama Y, Hirano K, Tsujii K, Nishida M, Miyagawa J, Sakai N, Yamashita S. Human scavenger receptor class B type I is expressed with cell-specific fashion in both initial and terminal site of reverse cholesterol transport. *Atherosclerosis*. 2005;183:75–83.
23. Hirano K, Yamashita S, Nakagawa Y, Ohya T, Matsuura F, Tsukamoto K, Okamoto Y, Matsuyama A, Matsumoto K, Miyagawa J, Matsuzawa Y. Expression of human scavenger receptor class B type I in cultured human monocyte-derived macrophages and atherosclerotic lesions. *Circ Res*. 1999;85:108–116.
24. Ikemoto M, Arai H, Feng D, Tanaka K, Aoki J, Dohmae N, Takio K, Adachi H, Tsujimoto M, Inoue K. Identification of a PDZ-domain-containing protein that interacts with the scavenger receptor class B type I. *Proc Natl Acad Sci U S A*. 2000;97:6538–6543.
25. Kocher O, Comella N, Tognazzi K, Brown LF. Identification and partial characterization of PDZK1: a novel protein containing PDZ interaction domains. *Lab Invest*. 1998;78:117–125.
26. Kocher O, Yesilaltay A, Cirovic C, Pal R, Rigotti A, Krieger M. Targeted disruption of the PDZK1 gene in mice causes tissue-specific depletion of the high density lipoprotein receptor scavenger receptor class B type I and altered lipoprotein metabolism. *J Biol Chem*. 2003;278:52820–52825.
27. Mardones P, Pilon A, Bouly M, Duran D, Nishimoto T, Arai H, Kozarsky KF, Altayo M, Miquel JF, Luc G, Clavey V, Staels B, Rigotti A. Fibrates down-regulate hepatic scavenger receptor class B type I protein expression in mice. *J Biol Chem*. 2003;278:7884–7890.
28. Silver DL, Wang N, Vogel S. Identification of small PDZK1-associated protein, DD96/MAP17, as a regulator of PDZK1 and plasma high density lipoprotein levels. *J Biol Chem*. 2003;278:28528–28532.
29. Kocher O, Pal R, Roberts M, Cirovic C, Gilchrist A. Targeted disruption of the PDZK1 gene by homologous recombination. *Mol Cell Biol*. 2003;23:1175–1180.
30. Huby T, Doucet C, Dachet C, Ouzilleau B, Ueda Y, Afzal V, Rubin E, Chapman MJ, Lesnik P. Knockdown expression and hepatic deficiency reveal an atheroprotective role for SR-BI in liver and peripheral tissues. *J Clin Invest*. 2006;116:2767–2776.
31. Rubin EM, Krauss RM, Spangler EA, Verstuyft JG, Clift SM. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature*. 1991;353:265–267.
32. Williamson R, Lee D, Hagaman J, Maeda N. Marked reduction of high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I. *Proc Natl Acad Sci U S A*. 1992;89:7134–7138.
33. Chiba H, Eto M, Fujisawa S, Akizawa K, Intoh S, Miyata O, Noda K, Matsuno K, Kobayashi K. Increased plasma apolipoprotein E-rich high-density lipoprotein and its effect on serum high-density lipoprotein cholesterol determination in patients with familial hyperalphalipoproteinemia due to cholesteryl ester transfer activity deficiency. *Biochem Med Metab Biol*. 1993;49:79–89.
34. 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;43:805–814.
35. Inazu A, Jiang XC, Haraki T, Yagi K, Kamon N, Koizumi J, Mabuchi H, Takeda R, Takata K, Moriyama Y, et al. Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J Clin Invest*. 1994;94:1872–1882.
36. Hirano K, Yamashita S, Nakajima N, Arai T, Maruyama T, Yoshida Y, Ishigami M, Sakai N, Kameda-Takemura K, Matsuzawa Y. Genetic cholesteryl ester transfer protein deficiency is extremely frequent in the Omagari area of Japan. Marked hyperalphalipoproteinemia caused by CETP gene mutation is not associated with longevity. *Arterioscler Thromb Vasc Biol*. 1997;17:1053–1059.
37. Silver DL. A carboxyl-terminal PDZ-interacting domain of scavenger receptor B, type I is essential for cell surface expression in liver. *J Biol Chem*. 2002;277:34042–34047.
38. Hirano K, Ikegami C, Tsujii K, Zhang Z, Matsuura F, Nakagawa-Toyama Y, Koseki M, Masuda D, Maruyama T, Shimomura I, Ueda Y, Yamashita S. Probucol enhances the expression of human hepatic scavenger receptor class B type I, possibly through a species-specific mechanism. *Arterioscler Thromb Vasc Biol*. 2005;25:2422–2427.
39. Schmidt HH, Remaley AT, Stonik JA, Ronan R, Wellmann A, Thomas F, Zech LA, Brewer HB, Jr, Hoeg JM. Carboxyl-terminal domain truncation alters apolipoprotein A-I in vivo catabolism. *J Biol Chem*. 1995;270:5469–5475.
40. Gong EL, Tan CS, Shoukry MI, Rubin EM, Nichols AV. Structural and functional properties of human and mouse apolipoprotein A-I. *Biochim Biophys Acta*. 1994;1213:335–342.
41. Ji Y, Jian B, Wang N, Sun Y, Moya ML, Phillips MC, Rothblat GH, Swaney JB, Tall AR. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J Biol Chem*. 1997;272:20982–20985.
42. Jian B, de la Llera-Moya M, Ji Y, Wang N, Phillips MC, Swaney JB, Tall AR, Rothblat GH. Scavenger receptor class B type I as a mediator of cellular cholesterol efflux to lipoproteins and phospholipid acceptors. *J Biol Chem*. 1998;273:5599–5606.
43. Ji Y, Wang N, Ramakrishnan R, Sehayek E, Huszar D, Breslow JL, Tall AR. Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile. *J Biol Chem*. 1999;274:33398–33402.
44. de la Llera-Moya M, Rothblat GH, Connelly MA, Kellner-Weibel G, Sakr SW, Phillips MC, Williams DL. Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface. *J Lipid Res*. 1999;40:575–580.