sis factor α has an inflammatory and atherogenic property, whereas adiponectin has an anti-atherogenic and anti-inflammatory adipokine. Because RBP4 may be linked to insulin resistance, it could affect vascular deterioration.

In the present study we determined serum RBP4 levels in the subjects with diabetic nephropathy. Furthermore, we would clarify clinical feature of RBP4 in diabetic nephropathy and related atherosclerotic disorders.

Subjects and Methods

Subjects

One hundred forty nine subjects with type 2 diabetes mellitus were enrolled in the present study between March 2005 and January 2007. They were admitted to Jichi Medical University Saitama Medical Center for 2 weeks to learn how and why to treat diabetes mellitus, and also to control their plasma glucose levels practically. They were 78 males and 71 females, with the ages of 63.9 ± 11.7 years (mean ± SD) ranging from 18 to 88 years. Ninety-one subjects had hypertension, 90 had hyperlipidemia, and 60 had obesity. Twenty-six subjects had ischemic heart disease, 18 subjects had cerebral vascular disease, and 6 had arteriosclerosis obliterans. According to the progression of nephropathy, the diabetic subjects were divided into stages 1–4 based on the classification of diabetic nephropathy by Research Committee of the Japanese Ministry of Health, Labor and Welfare for Disorders of diabetes mellitus [23]. Namely, stage 1, no microalbuminuria; stage 2, microalbuminuria (>30 mg/g creatinine, <300 mg/g creatinine); stage 3A, macroalbuminuria (>300 mg/g creatinine) or positive urinary protein analysis (<1 g urinary protein/g creatinine/day); stage 3B, proteinuria (>1 g urinary protein/g creatinine/day) and normal serum creatinine level; stage 4, elevated serum creatinine level; and stage 5, under dialysis treatment. The numbers of subjects taking medication for diabetes mellitus, hypertension and hyperlipidemia were summarized in Table 1. Also, 19 age- and gender-matched control subjects were collected from the subjects examining their medical status. They were 11 males and 8 females, with the ages of 59.2 ± 8.5 years (mean ± SD) ranging from 46 to 72 years. Eight subjects had hypertension, 11 had hyperlipidemia and 1 had obesity. Also, 3 subjects had ischemic heart disease, 5 subjects had cerebrovascular diseases, and 1 subject had arteriosclerosis obliterans. Blood samples

Table 1. Clinical and laboratory findings in the diabetic patients at the hospitalization

	Control subjects	Diabetic patients					P value
		Stage 1	Stage 2	Stage 3A	Stage 3B	Stage 4	
N	19	85	28	10	7	19	
Sex (male/female)	11/8	42/43	12/16	9/1	3/4	12/7	
Age (years)	59.2 ± 8.5	61.4 ± 11.5	65.5 ± 11.8	59.7 ± 14.3	69.1 ± 6.0	72.5 ± 6.9	0.0006
BMI	23.3 ± 1.2	24.4 ± 4.4	25.7 ± 4.2	24.3 ± 4.3	24.6 ± 3.0	25.3 ± 2.7	0.39
Systolic blood pressure (mmHg)	132.9 ± 11.0	132.2 ± 18.0	138.1 ± 16.9	147.0 ± 20.3	139.3 ± 16.8	135.4 ± 19.7	0.14
Diastolic blood pressure (mmHg)	72.8 ± 8.6	75.1 ± 10.5	77.6 ± 9.9	78.8 ± 12.2	70.1 ± 8.8	69.6 ± 10.1	0.063
Total cholesterol (mg/dl)	203.6 ± 41.8	209.2 ± 68.8	203.8 ± 43.6	203.1 ± 39.3	235.9 ± 30.1	180.3 ± 47.0	0.31
HDL cholesterol (mg/dl)	47.3 ± 7.5	46.5 ± 13.2	50.1 ± 11.5	46.9 ± 11.0	48.6 ± 16.1	44.5 ± 14.5	0.74
Triglyceride (mg/dl)	130.9 ± 47.6	227.1 ± 427.7	157.6 ± 103.2	141.3 ± 58.9	181.1 ± 84.3	119.8 ± 46.3	0.67
Fasting plasma glucose (mg/dl)	109.3 ± 10.9	162.3 ± 56.8	179.1 ± 54.1	159.3 ± 62.9	153.0 ± 65.1	129.7 ± 50.9	0.0002
HbA1c (%)	5.6 ± 0.1	8.9 ± 1.6	9.3 ± 2.1	9.2 ± 1.5	8.9 ± 1.3	8.1 ± 1.7	0.0067
Creatinine (mg/dl)	0.70 ± 0.10	0.65 ± 0.15	0.64 ± 0.16	0.77 ± 0.12	1.00 ± 0.27	1.25 ± 0.28	<0.0001
1/creatinine	1.46 ± 0.19	1.62 ± 0.41	1.66 ± 0.43	1.32 ± 0.18	1.06 ± 0.26	0.84 ± 0.18	<0.0001
Estimated GFR (ml/min/1.73m2)	87.8 ± 14.6	84.5 ± 21.0	84.2 ± 22.3	76.7 ± 13.7	48.6 ± 8.6	40.0 ± 9.0	<0.0001
Diabetes mellitus (n) (diet only/drug/insulin)	0	5/61/19	0/15/13	2/5/3	0/5/3	1/9/9	0.074
Hypertension (n) (%)	8 (42.1)	38 (44.7)	19 (67.9)	9 (90)	7 (100)	18 (94.7)	<0.0001
Hyperlipidemia (n) (%)	11 (57.9)	55 (64.7)	15 (53.6)	3 (30)	6 (85.7)	11 (57.9)	0.22
Ischemic heart disease (IHD) (n) (%)	3 (15.8)	11 (12.9)	4 (14.3)	1 (10)	2 (28.5)	8 (42.1)	0.062
Cerebral vascular disease (CVD) (n) (%)	5 (26.3)	7 (8.2)	3 (10.7)	3 (30)	1 (14.2)	4 (21.1)	0.154
Arteriosclerosis obliterans (ASO) (n) (%)	1 (5.3)	1 (1.2)	1 (3.6)	0	0	4 (21.1)	0.006

Values are mean ± SD. Values are analyzed by one-way ANOVA or chi-square for independence test.

were collected from the subjects in the supine position after an overnight fast to determine fasting plasma glucose, hemoglobin A1c, total cholesterol, high-density lipoprotein cholesterol, triglyceride, and serum RBP4 at the time of hospitalization or visiting to the outpatient clinic. Risk factors for atherosclerosis were defined as follows: Dyslipidemia was defined as a total cholesterol of greater than 220 mg/dl, a high-density lipoprotein cholesterol level of less than 40 mg/dl, and a triglyceride level of greater than 150 mg/dl, or the subject's having taken either statins or fibrates. Hypertension was defined as systolic blood pressure of greater than 140 mmHg, diastolic pressure of greater than 90 mmHg, or the subject's having taken antihypertensive agents. The present study was approved by the ethical committee of Jichi Medical University for human studies. We obtained informed consents from the subjects who joined the present protocol.

Measurements

Blood samples were collected into tubes and centrifuged at 3,000 rpm at 4°C for 15 minutes. The supernatants were decanted and frozen at –80°C until assayed. RBP4 was measured by the method of ELISA using Human RBP4 ELISA kits (AdipoGen, Seoul, Korea). Fasting plasma glucose, hemoglobin A1c, total cholesterol, HDL-cholesterol, triglyceride and creatinine were determined by standard laboratory methods. Urine samples were collected in the morning and 24-hour urine collection was made. Renal function was calculated as the estimated glomerular filtration rate (eGFR) by the Modification of Diet in Renal Disease equation (MDRD) revised for Japanese by the Japan Society of Nephrology: eGFR (ml/min/1.73 m^2) = $0.741 \times 175 \times age^{-0.203} \times (\text{serum creatinine (mg/dl)})^{-1.154}$ × (0.742, in female).

Statistical analysis

All values are expressed as means ± SD. The values were analyzed by one-way ANOVA to compare the difference among the groups. Categorical data were analyzed by the χ^2 test. Simple regression analysis was performed to evaluate correlation between the parameters. Also, we checked the independence of parameter by multiple regression analysis. The statistical packages of Statcel Statistical Software (Second Edition, OMS Publishing Inc., Japan) and Mulcel Statistical Software (First Edition, OMS Publishing Inc., Japan) were employed for the present analysis. A p values less than 0.05 was considered significant.

Results

We compared clinical features in the diabetic subjects and control subjects (Table 1). Fasting plasma glucose and hemoglobin A1c were elevated in the diabetic subjects as compared to the control subjects (p value for trend test: 0.0002 and 0.0067). The number of hypertensive subjects was increased according to the progression of diabetic nephropathy (p value for trend test <0.0001).

Serum RBP4 levels were increased to 70.5 ± 35.3 μg/ml in all the subjects with type 2 diabetes mellitus, a value significantly greater than that of 40.1 ± 13.0 μg/ml in the control subjects ($p<0.01$). Initially, we analyzed serum RBP4 levels in the diabetic subjects according to the amount of albuminuria. Serum RBP4 was significantly increased in the subjects with macroalbuminuria as compared to that in the subjects having normoalbuminuria (Normo-: 64.6 ± 29.7, Micro-: 63.7 ± 29.4, and Macroalbuminuria: 90.3 ± 44.6 μg/ml, p value for trend test <0.01).

Fig. 1 shows serum RBP4 levels in the diabetic subjects following the stage of nephropathy. According to the progression of diabetic nephropathy, serum RBP4 levels were gradually elevated in the diabetic subjects (p value for trend test <0.001). Its elevation was significantly greater in the diabetic subjects with stages 1, 3B and 4 than the control subjects (Control: 40.1 ±

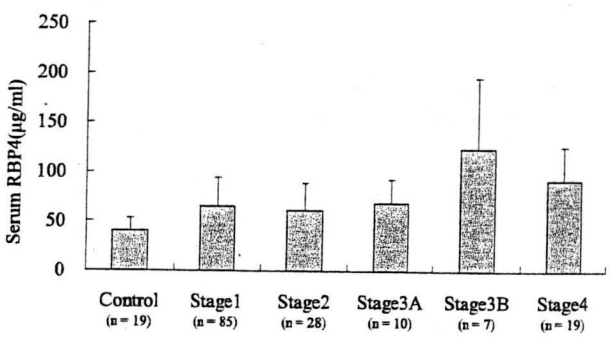

Fig. 1. Serum RBP4 levels in the diabetic patients on the classification of nephropathy and the controls. Serum RBP4 levels in the patients with stages 1, 3B and 4 were significantly elevated than the control subjects (p-value for trend test <0.001). Values are means ± SD.

Table 2. Linear regression analysis of serum RBP4 levels with varying parameters in the diabetic patients

	Parameters	r	p-value
RBP4 vs.	Age	0.199	0.015
	BMI	0.023	0.775
	Systolic blood pressure	0.008	0.927
	Diastolic blood pressure	–0.128	0.12
	Total cholesterol	0.088	0.285
	HDL-cholesterol	–0.062	0.45
	Triglyceride	–0.011	0.89
	Fasting plasma glucose	–0.092	0.264
	HbA1c	–0.113	0.17
	HOMA-R	–0.064	0.528
	Fasting serum insulin	–0.086	0.398
	Creatinine	0.377	<0.001
	1/Creatinine	–0.42	<0.001
	Estimated GFR	–0.436	<0.001
	Log (albuminuria)	0.211	0.015

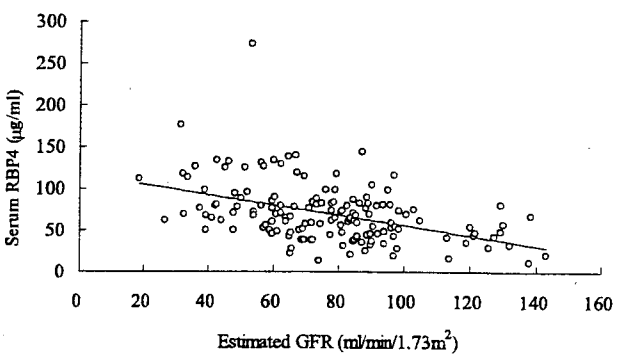

Fig. 2. The relationship of serum RBP4 levels with estimated GFR in the diabetic patients. Serum RBP4 (μg/ml) = 117.6 – 0.62 × estimated GFR, r = –0.436, p<0.001.

13.0 μg/ml vs. Stage 1: 64.6 ± 29.7, p<0.05, Stage 3B: 123.3 ± 71.8, p <0.01, and Stage 4: 91.4 ± 33.8 μg/ml, p<0.01).

Table 2 shows the correlation between serum RBP4 levels and varying parameters in the diabetic subjects with nephropathy. Serum RBP4 levels had a positive correlation with serum creatinine levels (r = 0.377, p<0.001), and a negative correlation with 1/creatinine (r = –0.420, p<0.001). Fig. 2 depicts the relationship between the estimated glomerular filtration rate (GFR) and serum RBP4 levels in the diabetic subjects. Serum RBP4 levels had a negative correlation with the estimated GFR (r = –0.436, p<0.001). In addition, serum RBP4 levels positively correlated with log-transformed albuminuria. Multiple regression analysis was performed using the following parameters, which were associated with serum RBP4 levels at the p<0.1 levels in the simple linear regression analysis (Table 2). Estimated GFR was an independent determinant for increased serum RBP4 levels (standardized coefficient = –0.466, p<0.001).

Discussion

The present study demonstrated that serum RBP4 levels were elevated in the diabetic subjects with nephropathy. eGFR was equivalent in the control subjects and the diabetic subjects with stage 1, but serum RBP4 levels were significantly increased in the stage 1 diabetic subjects compared with the controls. The marked difference was only the deterioration of plasma glucose control between the stage 1 diabetic subjects and the control. Though it is still not clear that plasma glucose could alter RBP4 level, impaired control of plasma glucose may affect the elevation of serum RBP4 in the diabetic subjects. We analyzed several parameters to clarify the association of serum RBP4 with renal impairment. A significant increase in serum RBP4 was obtained in the subjects with macroalbuminuria, but not in those with normo- and microalbuminuria. Similar study was reported by Raila *et al.* [14]. They noted that serum RBP4 was increased in patients with microalbuminuria, an early stage of diabetic nephropathy. Similarly, serum RBP4 levels were elevated according to the progression of clinical stages of diabetic nephropathy. They were further elevated in the diabetic subjects with stages 3B and 4. Furthermore, serum RBP4 levels had a negative correlation with the estimated GFR in the diabetic subjects. There was a possibility that the discrepancy of the findings from those of previous reports may result from the analysis using different assay kit of RBP4. Ziegelmeier *et al.* [24] reported a fourfold increase in serum RBP4 levels in patients with chronic hemodialysis compared with control subjects. Their patients included both diabetic and non-diabetic patients. As in the present study the diabetic subjects were admitted to learn how and why control plasma glucose, end-stage kidney disease was not contained in the analysis. Nevertheless, the finding was in concert with that of Ziegelmeier *et al.* in the end-stage kidney disease. These results indicate an increase in serum RBP4 levels in advanced renal impairment of the diabetic subjects.

The previous study has suggested that RBP4 is related to insulin resistance in diabetic subjects [4, 5]. We analyzed linear regression of serum RBP4 with other variables as shown in Table 2. Serum RBP4 did not correlate with either of fasting serum insulin levels and HOMA-R, a finding distinct from the study of Graham *et al.* [4]. The discrepancy may come from the blood samplings, that is, the blood was collected in a steady state of glucose homeostasis in the non-hospitalized subjects in the study of Graham *et al.* [4], and it was collected in the hospitalized, poorly controlled subjects in the present study. No relationship of serum RBP4 levels with insulin resistance was also reported by several investigators [8–13]. There was no difference in serum RBP4 levels between the subjects with and without macrovascular diseases. In the subjects with advanced diabetic nephropathy (stages 3 and 4) no difference in serum RBP4 was between the presence and absence of macrovascular diseases. Taken together, there was no evidence that elevation of serum RBP4 is associated with insulin resistance and atherosclerotic changes in the diabetic subjects. However, study limitation of the present analysis was come from the small number of the subjects with atherosclerotic diseases. Further study will be necessary to elucidate the exact relation of RBP4 with atherosclerotic diseases.

RBP4 is the primary carrier for vitamin A (retinol) in plasma and synthesized by the hepatocytes. RBP4 expression is also present in extrahepatic tissues including skeletal muscles and white adipose tissues [1]. It is known that kidney plays a role in maintenance of whole body retinol homeostasis [25, 26], which is regulated by glomerular filtration rate and subsequent reabsorption of RBP4 into proximal tubule. The present study showed a negative correlation of serum RBP4 with the estimated GFR, and an increase in serum RBP4 was apparently found in the advanced diabetic nephropathy. Though the clearance study was not performed, a decrease in GFR could accumulate RBP4 in the systemic circulation [27, 28]. Also, a protein complex of RBP4 bound to transthyretin homotetramer in the systemic circulation may reduce renal clearance of RBP4. Further study will have to be clarified whether kidney actually involves in plasma retinol homeostasis and whether renal-hepatic or renal-extrahepatic pathway stimulates the binding of retinol and RBP4.

As shown in Table 2, serum RBP4 levels had significant correlation with age, serum creatinine, 1/creatinine and estimated GFR. Also, the profound correlation of estimated GFR with serum RBP4 was proven by multiple regression analysis. Either of BMI, plasma glucose control, lipid metabolism and blood pressure did not affect serum RBP4 levels. Also, inflammatory alternations of CRP and IL-18 did not correlate with serum RBP4 levels (data not shown). Thus, metabolic and inflammatory changes did not affect serum RBP4 levels in the diabetic subjects with nephropathy.

In summary, the present study clarified that serum RBP4 levels were increased in the diabetic subjects. According to the progression of diabetic nephropathy, serum RBP4 was gradually elevated. There was a negative correlation between serum RBP4 levels and the estimated GFR. The alteration in serum RBP4 levels did not associate with insulin resistance and macrovascular diseases. The present study indicates an increase in serum RBP4 levels in the type 2 diabetic subjects, particularly complicated with advanced renal impairment.

References

1. Soprano DR, Soprano KJ, Goodman DS (1986) Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat. *J Lipid Res* 27: 166–171.
2. Blomhoff R, Green MH, Green JB, Berg T, Norum KR (1991) Vitamin A metabolism: new perspectives on absorption, transport, and storage. *Physiol Rev* 71: 951–990.
3. Blaner WS (1989) Retinol-binding protein: The serum transport protein for vitamin A. *Endocr Rev* 10: 308–316.
4. Graham TE, Yang Q, Blüher M, Hammarstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, Kahn BB (2006) Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med* 354: 2552–2563.
5. Janke J, Engeli S, Boschmann M, Adams F, Böhnke J, Luft FC, Sharma AM, Jordan J (2006) Retinol-binding protein 4 in human obesity. *Diabetes* 55: 2805–2810.
6. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB (2005) Serum retinol binding protein 4 contributes to insulin

resistance in obesity and type 2 diabetes. *Nature* 436: 356–362.

7. Mody N, Graham T, Tsuji Y, Yang Q, Kahn BB (2008) Decreased clearance of serum retinol binding protein and elevated levels of transthyretin in insulin-resistant ob/ob mice. *Am J Physiol Endocrinol Metab* 294: E785–E793.
8. Stefan N, Hennige AM, Staiger H, Schleicher E, Fritsche A, Häring HU (2007) Circulating retinol-binding protein-4, insulin sensitivity, insulin secretion, and insulin disposition index in obese and nonobese subjects: response to Broch *et al. Diabetes Care* 30:e91; author reply e92.
9. Promintzer M, Krebs M, Todoric J, Luger A, Bischof MG, Nowotny P, Wagner O, Esterbauer H, Anderwald C (2007) Insulin resistance is unrelated to circulating retinol binding protein and protein C inhibitor. *J Clin Endocrinol Metab* 92: 4306–4312.
10. Lewis JG, Shand BI, Elder PA, Scott RS (2008) Plasma retinol-binding protein is unlikely to be a useful marker of insulin resistance. *Diabetes Res Clin Pract* 80: e13–e15.
11. Yao-Borengasser A, Varma V, Bodles AM, Rasouli N, Phanavanh B, Lee MJ, Starks T, Kern LM, Spencer HJ 3rd, Rashidi AA, McGehee RE Jr, Fried SK, Kern PA (2007) Retinol binding protein 4 expression in humans: relationship to insulin resistance, inflammation, and response to pioglitazone. *J Clin Endocrinol Metab* 92: 2590–2597.
12. Bajzová M, Kovaciková M, Vitková M, Klimcaková E, Polak J, Kovacová Z, Viguerie N, Vedral T, Mikulášek L, Sramková P, Srp A, Hejnová J, Langin D, Stich V. Retinol -binding protein 4 expression in visceral and subcutaneous fat in human obesity. *Physiol Res* 2007 Nov 30 (Epub ahead of print).
13. von Eynatten M, Lepper PM, Liu D, Lang K, Baumann M, Nawroth PP, Bierhaus A, Dugi KA, Heemann U, Allolio B, Humpert PM (2007) Retinol-binding protein 4 is associated with components of the metabolic syndrome, but not with insulin resistance, in men with type 2 diabetes or coronary artery disease. *Diabetologia* 50: 1930–1937.
14. Raila J, Henze A, Spranger J, Möhlig M, Pfeiffer AF, Schweigert FJ (2007) Microalbuminuria is a major determinant of elevated plasma retinol-binding protein 4 in type 2 diabetic patients. *Kidney Int* 72: 505–511.
15. Takebayashi K, Suetsugu M, Wakabayashi S, Aso Y, Inukai T (2007) Retinol binding protein-4 levels and clinical features of type 2 diabetes patients. *J Clin Endocrinol Metab* 92: 2712–2719.
16. Cabré A, Lázaro I, Girona J, Manzanares J, Marimón F, Plana N, Heras M, Masana L (2007) Retinol-binding protein 4 as a plasma biomarker of renal dysfunction and cardiovascular disease in type 2 diabetes. *J Intern Med* 262:496–503.
17. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y (1999) Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 100:2473–2476.
18. Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T, Matsuzawa Y (2001) Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 103: 1057–1063.
19. Okamoto Y, Arita Y, Nishida M, Muraguchi M, Ouchi N, Takahashi M, Igura T, Inui Y, Kihara S, Nakamura T, Yamashita S, Miyagawa J, Funahashi T, Matsuzawa Y (2000) An adipocyte-derived plasma protein, adiponectin, adheres to injured vascular walls. *Horm Metab Res* 32: 47–50.
20. Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M, Nakamura T, Shimomura I, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y (2002) Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation* 105: 2893–2898.
21. Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, Kihara S, Funahashi T, Tenner AJ, Tomiyama Y, Matsuzawa Y (2000) Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* 96: 1723–1732.
22. Matsuda M, Shimomura I, Sata M, Arita Y, Nishida M, Maeda N, Kumada M, Okamoto Y, Nagaretani H, Nishizawa H, Kishida K, Komuro R, Ouchi N, Kihara S, Nagai R, Funahashi T, Matsuzawa Y (2002) Role of adiponectin in preventing vascular stenosis. The missing link of adipo-vascular axis. *J Biol Chem* 277: 37487–37491.
23. The research committee of diabetic nephropathy (2001) the renewal of classification by Research Committee of the Japanese Ministry of Health, Labor and Welfare for Disorders of diabetes mellitus. *J Jpn Diab Soc* 44: 623.
24. Ziegelmeier M, Bachmann A, Seeger J, Lossner U, Kratzsch J, Bluher M, Stumvoll M, Fasshauer M (2007) Serum levels of adipokine retinol-binding protein-4 in relation to renal function. *Diabetes Care* 30: 2588–2592.
25. Goodman DS (1984) Plasma retinol-binding protein. In: Sporn MB, Roberts AB, Goodman DS (eds) The Retinoids, (Vol 2), Academic Press: Orlando, FL,

pp. 41–88.
26. Green MH, Green JB (1994) Dynamics and control of plasma retinol. In: Blomhoff R (ed). Vitamin A in Health and Disease. Marcel Dekker: New York, Basel, Hong Kong, pp 199–133.
27. Smith FR, Goodman DS (1971) The effects of diseases of the liver, thyroid, and kidneys on the transport of vitamin A in human plasma. *J Clin Invest* 50: 2426–2436.
28. Vahlquist A, Peterson PA, Wibell L (1973) Metabolism of the vitamin A transporting protein complex. I. Turnover studies in normal persons and in patients with chronic renal failure. *Eur J Clin Invest* 3: 352–362.

Diabetes Mellitus as a Predictor of Retinopathy Associated With Acute Myocardial Infarction

Takuji Katayama, MD*; Takanori Yasu, MD*,†; Nozomi Kinoshita, MD**; Akihiro Kakehashi, MD**; Norifumi Kubo, MD*; Muneyasu Saito, MD*; Shin-ichi Momomura, MD*; Masanobu Kawakami, MD*

Background: A unique transient retinopathy characterized by soft exudates around the optic disc after percutaneous coronary intervention (PCI) for acute myocardial infarction (AMI) has been reported, so in the present study the risk factors for retinopathy associated with AMI (RAMI) were investigated.

Methods and Results: The study group comprised 62 patients with their first AMI who underwent successful PCI within 24 h of onset (48 men, 14 women; age 63±10 years). The fundus of each eye was assessed on days 3–5, and again at 4 weeks after AMI onset. New soft exudates developed in 29 patients (47%) at 4 weeks. The frequency of diabetes mellitus (DM), and the hemoglobin A_{1c} and peak creatine kinase concentrations were higher in patients with than in those without RAMI (55% vs 21%, P=0.008; 7.0±2.0% vs 5.9±1.4%, P=0.013; and 3,428±2,210 IU/L vs 2,352±1,652 IU/L, P=0.036, respectively). Multivariate analysis identified DM as an independent predictive factor for the occurrence of RAMI (odds ratio, 6.60; 95% confidence interval, 1.68–25.90; P=0.007).

Conclusions: DM might be a risk factor for RAMI. (*Circ J* 2009; **73**: 1278–1282)

Key Words: Acute myocardial infarction; Diabetes mellitus; Ischemia; Microcirculation; Reperfusion

In acute myocardial infarction (AMI), ischemia–reperfusion causes local and even systemic inflammation through various mechanisms[1–3] Clinically adverse manifestations that might be associated with systemic inflammation in AMI include systemic inflammatory response syndrome[4] and acute respiratory distress syndrome[5] accompanied by hemodynamic collapse. However, little is understood about remote organ disorders associated with AMI.

A few recent studies, including our own, have shown that a transient retinopathy develops in patients after reperfusion therapy for AMI[6–8] Retinopathy associated with AMI (RAMI) is characterized by soft exudates (cotton wool spots) around the optic disc with or without superficial hemorrhage that appear 2–8 weeks after the onset of AMI and disappear without any specific treatment. In our previous study, none of the patients with RAMI experienced impaired visual acuity, although a few patients reported visual complaints[7,8] The condition developed in approximately half of patients with a first episode AMI who underwent reperfusion therapy within 24 h of onset[8] However, details of the mechanism and risk factors for this unique retinopathy remain unknown. The present study investigated major predictors of RAMI and the impact of RAMI on long-term prognosis.

(Received August 13, 2008; revised manuscript received January 22, 2009; accepted February 10, 2009; released online April 28, 2009)
**First Department of Integrated Medicine, **Division of Ophthalmology, Saitama Medical Center, Jichi Medical University, Saitama and †Department of Clinical Pharmacology and Therapeutics, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan*
An abstract of this work was presented at the Annual Scientific Session of the American Heart Association on November 15, 2006.
Mailing address: Takanori Yasu, MD, Department of Clinical Pharmacology and Therapeutics, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan. E-mail: tyasu@shintoshin.nir.jp

Methods

Study Patients

We identified 70 consecutive patients with first AMI admitted to the coronary care unit between May 2001 and January 2002, and between October 2004 and February 2005. All patients underwent percutaneous coronary intervention (PCI) within 24 h after the onset of AMI (**Figure 1**). We excluded 1 patient with a cardiac rupture 2 days after PCI, 3 patients who refused ophthalmic fundus examination and 4 patients for whom fundus photographs were inadequate for evaluation because of cataracts or small pupils, leaving 62 patients (48 men, 14 women; average age, 63 years; range, 39–85 years) (**Figure 1**). Written informed consent was given by all patients before ophthalmic fundus examination.

Protocol

The Ethics Committee of Jichi Medical School approved the single-center prospective study protocol. Our previous studies showed that the soft exudates with or without superficial hemorrhage that characterize RAMI appear from 2 weeks after AMI onset and peak at 4 weeks[8] Based on those results, non-mydriatic funduscopy or fundus photography with a fundus camera (Nonmyd α-D; Kowa, Nagoya, Japan) was performed by an ophthalmologist (N.K.) 3–5 days after AMI onset to evaluate the baseline status of both eyes; 28 patients had funduscopy only without photos at baseline. Fundus photography was performed 4 weeks after AMI onset using the same fundus camera for both eyes. At the same time, all patients were interviewed by attending physicians of the cardiovascular division or division of ophthalmology regarding neurological symptoms and visual disturbances

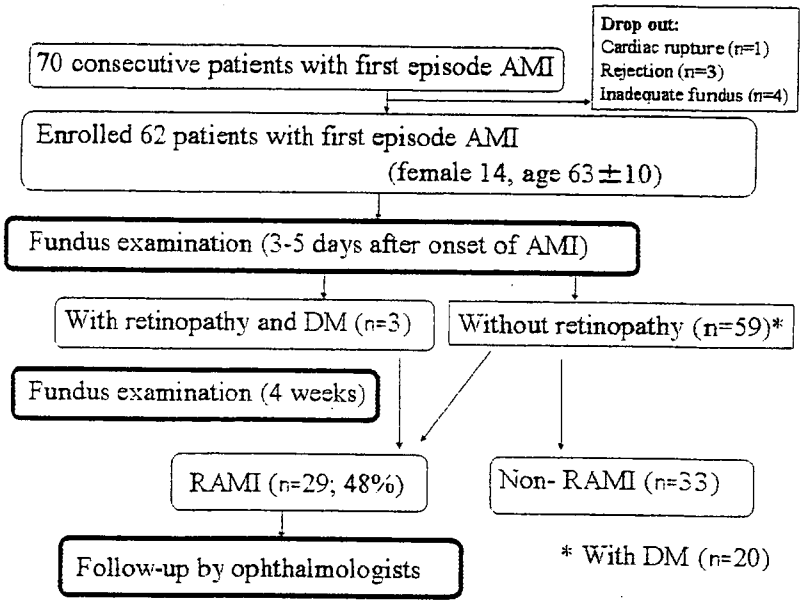

Figure 1. Study design and overall results. Study patients were 62 of 70 consecutive patients with a first acute myocardial infarction (AMI). First fundus examination 3–5 days after the onset of AMI demonstrated diabetic retinopathy in 3 patients but not in the other 59 patients. In the second fundus examination, all 3 patients who had diabetic retinopathy in the first examination showed new soft exudates, and 33 of the 59 patients who did not have diabetic retinopathy in the first examination showed new soft exudates. DM, diabetes mellitus; RAMI, retinopathy associated with AMI.

such as blurred vision. Two ophthalmologists (N.K., A.K.) evaluated all fundus photographs, and were unaware of both the patient assignment and whether the photographs were from the initial or follow-up examination. Patients with new soft exudates and/or new hemorrhages by 4 weeks after AMI onset were diagnosed as having RAMI and were periodically observed by ophthalmologists. According to our previous reports, RAMI can be easily differentiated from diabetic retinopathy by the absence of other changes that commonly coexist with cotton wool spots. Diabetic retinopathy is the most common etiology of cotton wool spots, and capillary microaneurysms, dot and blot hemorrhages, and hard exudates usually precede the appearance of such spots.

All patients received oral aspirin (162 mg) immediately after admission and intravenous heparin (10,000 units) before PCI. Tissue plasminogen activator, an aspiration catheter for thrombectomy, and a balloon and stent were used at the discretion of the operator during PCI. Left ventriculography was performed to determine wall motion abnormalities and left ventricular ejection fraction after PCI. Heparin was continued as an intravenous infusion (15,000–20,000 units/day) for the next 72 h and adjusted to achieve a target activated partial thromboplastin time of 2.0- to 3.0-fold the control level. Nicorandil was also administered as a continuous infusion (4 mg/h IV) for 72 h after admission[9] All patients received aspirin (81 mg/day) and all those with implanted bare metal stents also received ticlopidine (200 mg/day) for 4 weeks after PCI. Patients with atrial fibrillation or left ventricular aneurysm were given warfarin. Clinical reperfusion injury was defined as additional ST-segment elevation without coronary flow reduction during reperfusion therapy. Pre-infarction angina was defined as an attack within 48 h before AMI onset. Slow flow during PCI was defined as a decrease in Thrombolysis In Myocardial Infarction flow grade[10] by more than 1 grade compared with the grade before balloon dilatation or stent implantation without evidence of dissection, severe stenosis or vasospasm.

Blood samples were collected from each patient on admission and on each subsequent day until 4 days after admission to perform blood chemistry tests. The creatine kinase (CK) concentration was routinely measured every 4 h after admission to identify the peak value. Hemoglobin (Hb) A_{1c} and total cholesterol levels were also measured on admission. Levels of C-reactive protein (CRP) were measured by monoclonal antibody in an assay using latex that enabled measurement over a wide range (0.01–42 mg/dl) of concentrations (NANOPIA CRP; Daiichi Pure Chemical, Tokyo, Japan). Normal values for CRP are <0.2 mg/dl[11]

Coronary risk factors were identified from medical history or hospital data. Patients were regarded as having diabetes mellitus (DM), hypercholesterolemia or hypertension when a history of these was elicited or the patient had been on a diet or pharmacotherapy for these conditions. In addition, DM was diagnosed for HbA_{1c} levels ≥6.5%, and hypercholesterolemia was diagnosed if the total cholesterol level was ≥220 mg/dl on admission. Hypertension was diagnosed when systolic blood pressure was ≥140 mmHg and/or diastolic blood pressure was ≥90 mmHg in hospital.

Follow-up study was performed by reviewing medical records and holding telephone interviews to determine the impact of RAMI on long-term prognosis. Development of hard events was defined as a composite of all-cause death and repeat nonfatal AMI.

Statistical Analysis

Data were analyzed using StatView 5.0 software (SAS institute, Cary, NC, USA). Continuous variables are described as mean±SD and were compared using Student's t-test (variables with normal distribution) or the Mann-Whitney U-test (variables with asymmetric distribution). Categorical variables are described as frequencies and were compared using the χ^2 analysis or Fisher's exact test. Multivariate logistic regression analysis was performed for clinical factors with values of $P<0.2$ in univariate analysis. Kaplan-Meier methods and log-rank testing were used to determine hard event-free survival. Statistical significance was established at the $P<0.05$ level.

Results

All PCI procedures were successful. Tissue plasminogen activator was administered during PCI to 3 patients because of the large amount of thrombus in the infarct-related artery. Warfarin was administered to 5 patients to inhibit thrombus

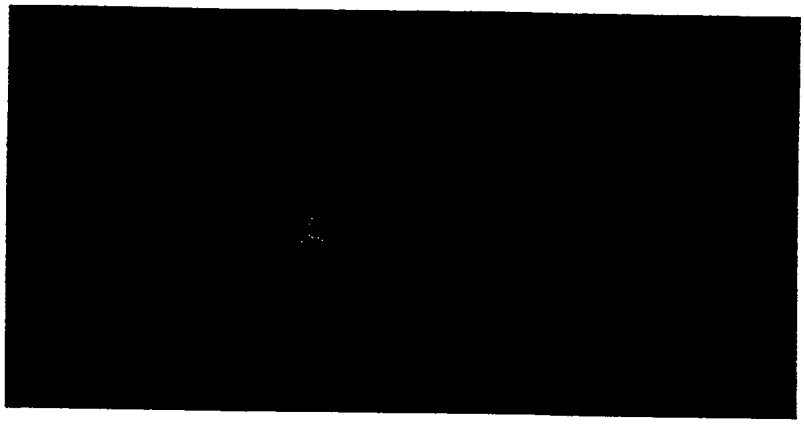

Figure 2. Fundus photographs of both eyes of a 73-year-old man 4 weeks after acute myocardial infarction onset. Soft exudates (arrows) around the optic disc in the both eyes appeared without hemorrhage. He had no history of diabetes mellitus and no retinopathy at the initial examination on day 4. Hemoglobin A_{1c} on admission was 5.2% and peak creatine kinase was 5,407 IU/L.

Table 1. Clinical Characteristics of Patients With and Without RAMI

	RAMI (n=29)	non-RAMI (n=33)	P value
Age (years)	63.0±8.5	62.7±11.4	0.906
Sex (M/F)	22/7	26/7	0.999
BMI (kg/m^2)	24.4±3.6	23.8±3.4	0.537
Current smoker (%)	18 (62%)	26 (79%)	0.171
Hypertension (%)	21 (72%)	18 (55%)	0.191
Diabetes (%)	16 (55%)	7 (21%)	0.008*
Hypercholesterolemia (%)	11 (38%)	14 (42%)	0.798
Medications before admission			
Anti-diabetic agents	6 (21%)	3 (9%)	0.196
ACEI and/or ARB	6 (21%)	6 (18%)	0.803
Statins	5 (17%)	5 (15%)	0.824
Killip IV (%)	2 (7%)	4 (12%)	0.676
IRA (RCA/LAD/LCX)	11/3/15	12/6/15	0.674
No. of lesions (1/2/3)	18/9/2	23/8/2	0.814
Time to reperfusion (min)	578±789	791±1,108	0.392
t-PA (%)	3 (10%)	0 (0%)	0.108
Thrombectomy (%)	21 (72%)	18 (55%)	0.191
Stent (%)	26 (90%)	29 (88%)	0.999
Pre-AMI angina (%)	13 (45%)	15 (45%)	0.999
Slow flow during PCI (%)	3 (10%)	4 (12%)	0.999
Reperfusion injury (%)	5 (17%)	4 (12%)	0.722
IABP (%)	12 (41%)	8 (24%)	0.181
Ejection fraction (%)	49.9±13.9	50.3±10.2	0.889
CRP (mg/dl) on admission	0.98±2.01	0.92±2.95	0.778
Peak CRP (mg/dl)	10.0±5.5	9.7±4.9	0.794
WBC (/μl) on admission	11,370±4,159	11,676±3,143	0.743
Peak WBC (/μl)	12,645±3,836	12,313±3,576	0.726
Platelets (×10^4/μl)	26.2±7.6	26.5±6.5	0.851
Total cholesterol (mg/dl)	203±36	202±34	0.910
Hemoglobin A_{1c} (%)	7.0±2.0	5.9±1.4	0.013*
BS (mg/dl) on admission	197±80	174±61	0.210
Peak CK (IU/L)	3,428±2,210	2,352±1,652	0.036*
Creatinine (mg/dl)	0.93±1.29	0.72±0.28	0.772

*P<0.05 vs non-RAMI.

RAMI, retinopathy associated with myocardial infarction; BMI, body mass index; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; IRA, infarct-related coronary artery; RCA, right coronary artery; LAD, left anterior descending artery; LCX, left circumflex artery; t-PA, tissue plasminogen activator; AMI, acute myocardial infarction; PCI, percutaneous coronary intervention; IABP, intra-aortic balloon pumping; CRP, C-reactive protein; WBC, white blood cell count; BS, blood sugar; CK, creatine kinase.

formation (3 cases of large infarct, 2 cases of atrial fibrillation). No in-hospital deaths occurred among the 62 patients.

Diabetic retinopathy was documented on initial fundus examination in 3 of the 62 patients and all 3 patients developed new soft exudates at 4 weeks after AMI onset. New soft exudates appeared in 26 of 59 patients (44%) who did not display retinopathy at the time of initial fundus examination (**Figures 1,2**). Overall, new soft exudates around the optic disc developed in 29 (47%) of 62 patients at 4 weeks after AMI onset. Five patients with RAMI experienced ophthalmologic symptoms such as blurred vision and/or metamorphopsia at 4 weeks after the onset of AMI. In contrast, none of the patients without RAMI had ophthalmologic symptoms during the study period.

The frequency of DM, as well as serum levels of HbA_{1c} and peak CK (**Table 1**, univariate analysis), were higher in the RAMI group than in the non-RAMI group. Multivariate analysis showed that DM was an independent predictive factor for the occurrence of RAMI (**Table 2**). Substituting HbA_{1c} for the frequency of DM gave very similar results in

the multivariate analysis. The occurrence of RAMI was significantly greater in patients with DM (16/23: 70%) than without DM (13/39: 33%, P=0.006 vs with DM) (**Figure 3**), although there were no significant differences in pre-AMI medications, such as antidiabetic agents, renin-angiotensin system inhibitors and statins, between the RAMI and non-RAMI groups (**Table 1**).

In the follow-up study (tracing rate, 97%; mean tracing term, 52.6 months), hard events developed in 5 patients (all-cause death, n=2; re-AMI, n=3) in the RAMI group and 3 patients (all-cause death, n=2; re-AMI, n=1) in the non-RAMI group. No significant difference in the incidence of hard events was seen between groups (**Figure 4**).

Discussion

The key findings in the present study are that RAMI developed more frequently in patients with DM compared with those without DM, and that RAMI did not exert any significant effect on long-term prognosis.

DM has been reported to be closely related to systemic inflammation and endothelial dysfunction[12,13] We and other investigators have reported that leukocyte entrapment in the retinal capillaries increases and disturbs regional capillary blood flow in animal models of DM[14–16] Even in healthy men, inflammatory mediators released as a consequence of ischemia–reperfusion activate leukocytes, platelets, and endothelial cells in remote organs[17] Hence, we postulated that extant retinal microvascular dysfunction in DM might be reinforced by systemic inflammation following AMI in itself and ischemia–reperfusion injury. Granger has reported that hypertension and hypercholesterolemia, as well as DM, can exaggerate the microcirculation in vivo elicited by ischemia–reperfusion[18] Although we could not confirm a relationship between RAMI and risk factors for atherosclerotic disease other than DM, the degree of microvascular retinal dysfunction in DM might be greater than that among patients with other risk factors before and after ischemia–reperfusion.

Soft exudates in the fundus photographs were transient, small and whitish opacities in the retina, and thought to have arisen from pathological accumulation of edematous damaged nerve axis fibers resulting from micro-infarctions of small retinal arterioles or capillaries[19] In fact, we have documented by fluorescein angiography that non-perfused areas were consistent with the area of soft exudates in patients with RAMI[8] Soft exudates occur frequently in diabetic retinopathy, hypertensive retinopathy and other systemic diseases or condition such as anemia, collagen diseases, HIV infection, and interferon treatment. However, RAMI can be easily differentiated from these other forms of retinopathy by the other changes that commonly coexist with cotton wool spots and by the unique time course from AMI onset[8] Although PCI-induced microembolic retinopathy has been reported in patients without AMI,[20,21] retinopathy did not develop in 10 patients following stent implantation for stable angina in our previous study[8] Therefore, PCI-related embolism probably does not represent an important contributor to RAMI pathogenesis. The results of the present study with a limited number of subjects show that RAMI did not affect the long-term hard-event prognosis. To clarify the clinical significance of RAMI, large-scale studies are warranted in the fields of both cardiology and ophthalmology.

Table 2. Multivariate Logistic Regression Analysis for RAMI

	OR (95%CI)	P value
Thrombectomy	2.573 (0.64–10.42)	0.186
IABP	0.573 (0.09–3.58)	0.551
Hypertension	1.813 (0.49–6.76)	0.375
Current smoking	0.261 (0.06–1.11)	0.069
Peak CK	1.000 (1.00–1.01)	0.063
Diabetes	6.598 (1.68–25.90)	0.007

OR, odds ratio; CI, confidence interval. Other abbreviations see in Table 1.

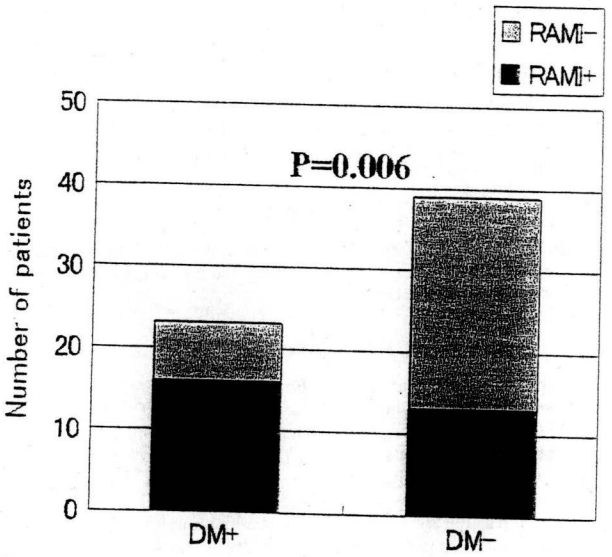

Figure 3. Frequency of retinopathy associated with acute myocardial infarction (RAMI) was significantly greater in patients with diabetes mellitus (DM) than in those without DM.

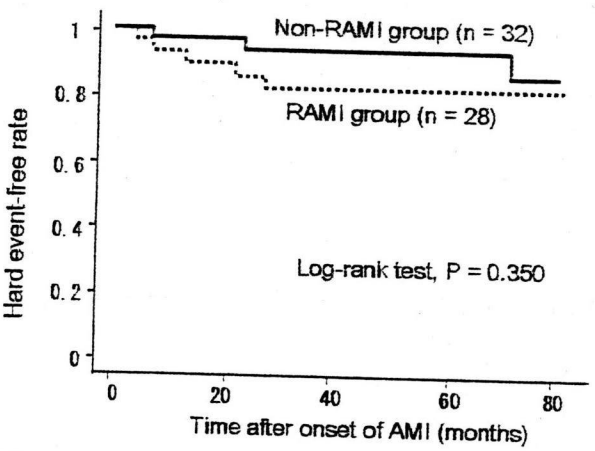

Figure 4. Kaplan-Meier estimate of hard events (all-cause death and repeat nonfatal acute myocardial infarction (AMI)) in patients with and without retinopathy associated with AMI (RAMI). No significant differences in long-term hard events were seen between the RAMI and non-RAMI groups.

Study Limitations

Firstly, pan-microvascular dysfunction can also be induced by AMI. Thus, whether AMI alone or AMI+PCI therapy has a greater impact on the occurrence of RAMI is unclear. Conducting a randomized study to compare PCI- and non-PCI-treated patients with AMI may not be ethically acceptable. Secondly, because only 3 of the patients had diabetic

retinopathy before AMI, the frequency of RAMI in patients with diabetic retinopathy and the influence of RAMI on the progress of diabetic retinopathy should be explored in the future. Thirdly, we did not measure fasting insulin levels, and so could not evaluate insulin sensitivity, which might be related to inflammation and endothelial dysfunction[22,23] It is possible that insulin resistance, as well as DM, is involved in the occurrence of RAMI. Fourthly, 28 patients had funduscopy only without photos at baseline examination. Although funduscopy is suitable for observation in the larger field of the fundus, rather than photography, this difference between methods seems not to have affected detection of RAMI caused by the occurrence of soft exudates around optic disc. Finally, the time between the onset of AMI and reperfusion by PCI was relatively long, because most patients were referred to us from other medical institutions.

In conclusion, DM should be considered as an independent risk factor for RAMI after reperfusion therapy for AMI.

Acknowledgments

We thank Professors Wilfred Y. Fujimoto and Shinichiro Ueda for their helpful comments regarding the present results, and Sachimi Jinbo for her technical assistance with the data analysis.

References

1. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res* 2002; **53:** 31–47.
2. Verma S, Fedak PW, Weisel RD, Butany J, Rao V, Maitland A, et al. Fundamentals of reperfusion injury for the clinical cardiologist. *Circulation* 2002; **105:** 2332–2336.
3. Neumann FJ, Ott I, Gawaz M, Richardt G, Holzapfel H, Jochum M, et al. Cardiac release of cytokines and inflammatory responses in acute myocardial infarction. *Circulation* 1995; **92:** 748–755.
4. Kohsaka S, Menon V, Lowe AM, Lange M, Dzavik V, Sleeper LA, et al; SHOCK Investigators. Systemic inflammatory response syndrome after acute myocardial infarction complicated by cardiogenic shock. *Arch Intern Med* 2005; **165:** 1643–1650.
5. Keren A, Klein J, Stern S. Adult respiratory distress syndrome in the course of acute myocardial infarction. *Chest* 1980; **77:** 161–164.
6. Kusano Y, Endo S, Mukai S, Yamaguchi T. Retinopathy after percutaneous transluminal coronary angioplasty and stent insertion for acute myocardial infarction. *Am J Ophthalmol* 2003; **136:** 557–560.
7. Katayama T, Yasu T, Kinoshita N, Kakehashi A, Ueba H, Hashimoto S, et al. Transient retinopathy after acute myocardial infarction treated by reperfusion therapy: Clinical background and manifestation. *J Cardiol* 2003; **42:** 23–28 (in Japanese).
8. Kinoshita N, Kakehashi A, Yasu T, Katayama T, Kuroki M, Tsurimaki Y, et al. A new form of retinopathy associated with myocardial infarction treated with percutaneous coronary intervention. *Br J Ophthalmol* 2004; **88:** 494–496.
9. Ikeda N, Yasu T, Kubo N, Hashimoto S, Tsuruya Y, Fujii M, et al. Nicorandil vs isosorbide dinitrate as adjunctive therapy to direct balloon angioplasty in acute myocardial infarction. *Heart* 2004; **90:** 181–185.
10. The TIMI Study Group. The Thrombolysis in Myocardial Infarction (TIMI) trial. *N Engl J Med* 1985; **312:** 932–936.
11. Ikeda N, Yasu T, Kubo N, Nakamura T, Sugawara Y, Ueda S, et al. Daily exercise and bone marrow derived $CD34^+/133^+$ cells after myocardial infarction treated by bare metal stent implantation. *Circ J* 2008; **72:** 897–901.
12. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 2001; **286:** 327–334.
13. Meigs JB, Hu FB, Rifai N, Manson JE. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. *JAMA* 2004; **291:** 1978–1986.
14. Kinoshita N, Kakehashi A, Inoda S, Itou Y, Kuroki M, Yasu T, et al. Effective and selective prevention of retinal leukostasis in streptozotocin-induced diabetic rats using gliclazide. *Diabetologia* 2002; **45:** 735–739.
15. Miyamoto K, Hiroshiba N, Tsujikawa A, Ogura Y. In vivo demonstration of increased leukocyte entrapment in retinal microcirculation of diabetic rats. *Invest Ophthalmol Vis Sci* 1998; **39:** 2190–2194.
16. Kim SY, Johnson MA, McLeod DS, Alexander T, Hansen BC, Lutty GA. Neutrophils are associated with capillary closure in spontaneously diabetic monkey retinas. *Diabetes* 2005; **54:** 1534–1542.
17. Kharbanda RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, et al. Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. *Circulation* 2001; **103:** 1624–1630.
18. Granger DN. Ischemia-reperfusion: Mechanisms of microvascular dysfunction and the influence of risk factors for cardiovascular disease. *Microcirculation* 1999; **6:** 167–178.
19. Albert DM, Jakobiec FA. Principle and practice of ophthalmology. WB Saunders; 1994; 27–28.
20. Bucci FA Jr, Dimitsopulos TM, Krohel GB. Branch retinal artery occlusion secondary to percutaneous transluminal coronary angiography. *Br J Ophthalmol* 1989; **73:** 309–310.
21. Teitelbaum BA. Asymptomatic unilateral microembolic retinopathy secondary to percutaneous transluminal coronary angioplasty. *J Am Optom Assoc* 1999; **70:** 587–592.
22. Kim JA, Montagnani M, Koh KK, Quon MJ. Reciprocal relationships between insulin resistance and endothelial dysfunction: Molecular and pathophysiological mechanisms. *Circulation* 2006; **113:** 1888–1904.
23. Ishikawa S, Kayaba K, Gotoh T, Nakamura Y, Kajii E. Metabolic syndrome and C-reactive protein in the general population: JMS Cohort Study. *Circ J* 2007; **71:** 26–31.

Clinical Chemistry 55:10
1801–1808 (2009)

Development of an Immunoassay for the Quantification of Soluble LR11, a Circulating Marker of Atherosclerosis

Masanao Matsuo,[1] Hiroyuki Ebinuma,[1*] Isamu Fukamachi,[1] Meizi Jiang,[2] Hideaki Bujo,[2] and Yasushi Saito[3]

BACKGROUND: Vascular smooth muscle cells (SMCs) migrate from the arterial media to the intima in the progression of atherosclerosis, and dysfunction of SMCs leads to enhanced atherogenesis. A soluble form of the LDL receptor relative with 11 ligand-binding repeats (sLR11) is produced by the intimal SMCs, and the circulating concentrations of sLR11 likely reflect the pathophysiological condition of intimal SMCs. Furthermore, polymorphism of the LR11 gene has been found to be related to the onset of Alzheimer disease. This study describes the development of a sandwich immunoassay for quantifying sLR11 in human serum and cerebrospinal fluid.

METHODS: We used synthetic peptides or DNA immunization to produce monoclonal antibodies (MAbs) A2-2–3, M3, and R14 against different epitopes of LR11.

RESULTS: sLR11 was immunologically identified as a 250-kDa protein in human serum and cerebrospinal fluid by SDS-PAGE separation, and was purified from serum by use of a receptor-associated protein and MAb M3. An immunoassay for quantification of sLR11 with a working range of 0.25–4.0 μg/L was developed using the combination of MAbs M3 and R14. Treatment of serum with 5.25% n-nonanoyl-N-methyl-d-glucamine reduced the matrix effects of serum on the absorbance detection in the ELISA system. The linear dynamic range of the ELISA spanned the variation of circulating sLR11 concentrations in individuals with atherosclerosis.

CONCLUSIONS: A sandwich ELISA was established for quantifying sLR11 in serum and cerebrospinal fluid. This technique provides a novel means for assessing the pathophysiology of atherosclerosis, and possibly neurodegenerative diseases.

The LDL receptor relative with 11 ligand-binding repeats (LR11)[4] (also known as SorLA) *(1, 2)* is a member of the LDL receptor family and is highly expressed in atheromatous plaques, particularly in the intimal smooth muscle cells (SMCs) at the border between the arterial intima and the media *(3)*. Overproduction of LR11 protein promotes the enhanced migration of SMCs via the upregulation of urokinase-type plasminogen activator receptor *(4, 5)*. LR11 plays an essential role in the angiotensin II–induced mobility of SMCs, and angiotensin II type 1 receptor blockers have been found to reduce intimal thickness through the inhibition of the LR11/urokinase-type plasminogen activator receptor–mediated pathway of intimal SMCs in cuff-injured mice *(6)*. The extracellular domain of the membrane-spanning LR11 is released to yield an active soluble form of LR11 (sLR11) *(5, 7, 8)*. Recombinant sLR11 stabilizes urokinase-type plasminogen activator receptor and enhances the activation of the integrin/FAK/Rac1 pathway in SMCs and macrophages *(6, 8)*. The concentrations of sLR11 in arteries increased 2 weeks after endothelial injury in rats *(8)*, and the neutralization of sLR11 activity by specific antibodies reduced the intimal thickness after cuff injury in mice *(5)*. Statins, as well as angiotensin II type 1 receptor blockers, have been reported to inhibit the migration of intimal SMCs via the downregulation of LR11 expression and to attenuate LR11 expression in the intimal SMCs of aortic arteriosclerotic plaques in hyperlipidemic rabbits *(9)*.

Circulating sLR11 can be immunologically detected in serum by use of specific antibodies against LR11 *(6)*. Circulating concentrations of LR11 were positively correlated with intimal-media thickness in dyslipidemic individuals, and the correlation was independent of other classical risk factors for atherosclerosis *(6)*. In addition, neuronal LR11 expression is characteristically reduced in mild cognitive impairment and in the brains of individuals with Alzheimer disease (AD) *(10–13)*. Single nucleotide polymorphism anal-

[1] Tsukuba Research Institute, Sekisui Medical, Ibaraki, Japan; [2] Department of Genome Research and Clinical Application, Graduate School of Medicine, Chiba University, Chiba, Japan; [3] Department of Clinical Cell Biology, Graduate School of Medicine, Chiba University, Chiba, Japan.
* Address correspondence to this author at: Tsukuba Research Institute, Sekisui Medical, 3-3-1, Koyodai, Ryugasaki, Ibaraki 301-0852, Japan. Fax +81-297-62-8635; e-mail ebinuma002@sekisui.jp.

Received March 9, 2009; accepted July 9, 2009.
Previously published online at DOI: 10.1373/clinchem.2009.127027

[4] Nonstandard abbreviations: LR11, LDL receptor relative with 11 ligand-binding repeats; SMC, smooth muscle cells; sLR11, soluble form of LR11; AD, Alzheimer disease; MAb, monoclonal antibody; CSF, cerebrospinal fluid; RAP, receptor-associated protein; PBST, PBS with Tween.

ysis of the gene for LR11 [sortilin-related receptor, L(DLR class) A repeats-containing (*SORL1*)] has been used to predict AD onset *(14, 15)*.

In this study, we produced specific monoclonal antibodies (MAbs) that bind to intact sLR11 without prior purification. Using these antibodies, we developed a novel sandwich ELISA method to quantify circulating sLR11 concentrations in both serum and cerebrospinal fluid (CSF). This technique provides a means for quantifying sLR11 to be used potentially as a marker for atherosclerosis and a predictor of AD and other neurodegenerative diseases.

Materials and Methods

BIOLOGICAL SAMPLES

Human and animal sera were purchased from Tennessee Blood Services and Cosmo Bio, respectively. Commercial human CSF samples (n = 13) were obtained from Scipac. To evaluate the normal concentration range of circulating sLR11, human serum was obtained from 87 healthy normolipidemic individuals (41 males and 46 females), who gave informed consent for participation in this study, which was approved by the Human Investigation Review Committee of the Chiba University Graduate School of Medicine.

EXTRACTION OF sLR11 WITH A RECEPTOR-ASSOCIATED PROTEIN AFFINITY RESIN

Recombinant human receptor-associated protein (RAP) was prepared as a glutathione *S*-transferase fusion protein *(16)* and applied to a glutathione-Sepharose resin (GE Healthcare), which was used to extract sLR11 from serum. Briefly, samples were incubated overnight at 4 °C at a RAP affinity resin–to–sample volume ratio of 1:20, and the resin was packed into a separation column. The column was washed with 20 mmol/L Na,K-phosphate buffer (pH 7.2) containing 150 mmol/L NaCL, and sLR11 was eluted with 50 mmol/L sodium citrate buffer (pH 5.0) containing 150 mmol/L NaCl. The sLR11 from cultured human IMR32 cells was extracted with a RAP affinity resin as described previously *(5)*.

PREPARATION OF MAb BY SYNTHETIC PEPTIDE IMMUNIZATION

Anti-LR11 MAb for use in the immunoblot analyses of human and animal sera was prepared by immunizing mice with a synthetic peptide (SMNEENMRSVITFDKG) corresponding to amino acid residues 432–447 of LR11 *(2, 17)* coupled to keyhole-limpet hemocyanin. The peptide–keyhole-limpet hemocyanin complex was emulsified with complete Freund's adjuvant (Gibco) and subcutaneously injected into BALB/c mice, 4 times at 2-week intervals. The spleen cells extracted from the immunized mice were fused with mouse myeloma cells (Sp2/0) in the presence of 50% polyethylene glycol. A single clone was selected to yield MAb A2-2-3 (IgG1,k), which reacted with both human and rabbit sLR11 in immunoblot analyses.

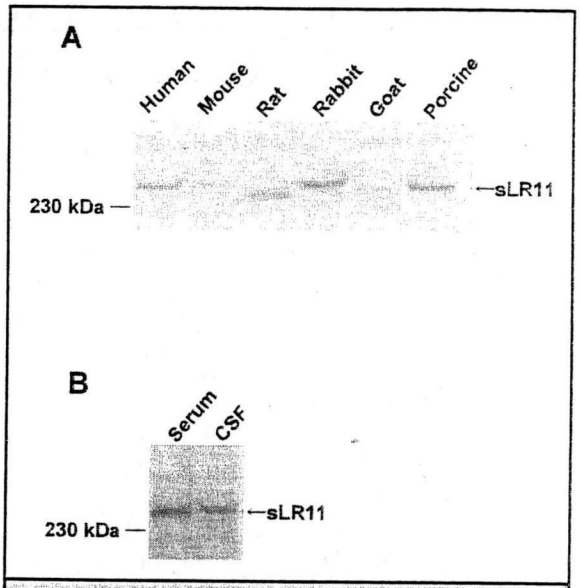

Fig. 1. Identification of sLR11 in serum and CSF.
(A), Samples (50 μL) extracted from human and animal sera by RAP affinity resin were separated by SDS-PAGE (2%–15% gradient) under reducing conditions. The sLR11 was detected by using an immunoblot assay with MAb A2-2–3. (B), Human CSF (10 μL) was separated by SDS-PAGE (2%–15% gradient) under reducing conditions, with RAP affinity-treated human serum as a control. The sLR11 protein was detected as above. A representative photo is shown. A 230-kDa marker is shown at the left in both panels.

IMMUNOBLOT ANALYSIS

Before immunoblot analysis, serum proteins were boiled in SDS-Tris buffer, with or without β-mercaptoethanol (reducing or nonreducing condition, respectively), and then separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 1% BSA in PBS containing 0.05% Tween 20 (PBST), incubated with MAb A2-2–3, reacted with horseradish peroxidase–conjugated rabbit antimouse IgG using a VECTASTAIN ABC kit (Vector Laboratories) according to the manufacturer's instructions, and subsequently stained with diaminobenzidine.

PREPARATION OF MAbs BY DNA IMMUNIZATION

Anti-LR11 MAbs for the sandwich ELISA were prepared via DNA immunization at Nosan Corporation *(18–20)*. Briefly, cDNA encoding amino acid resi-

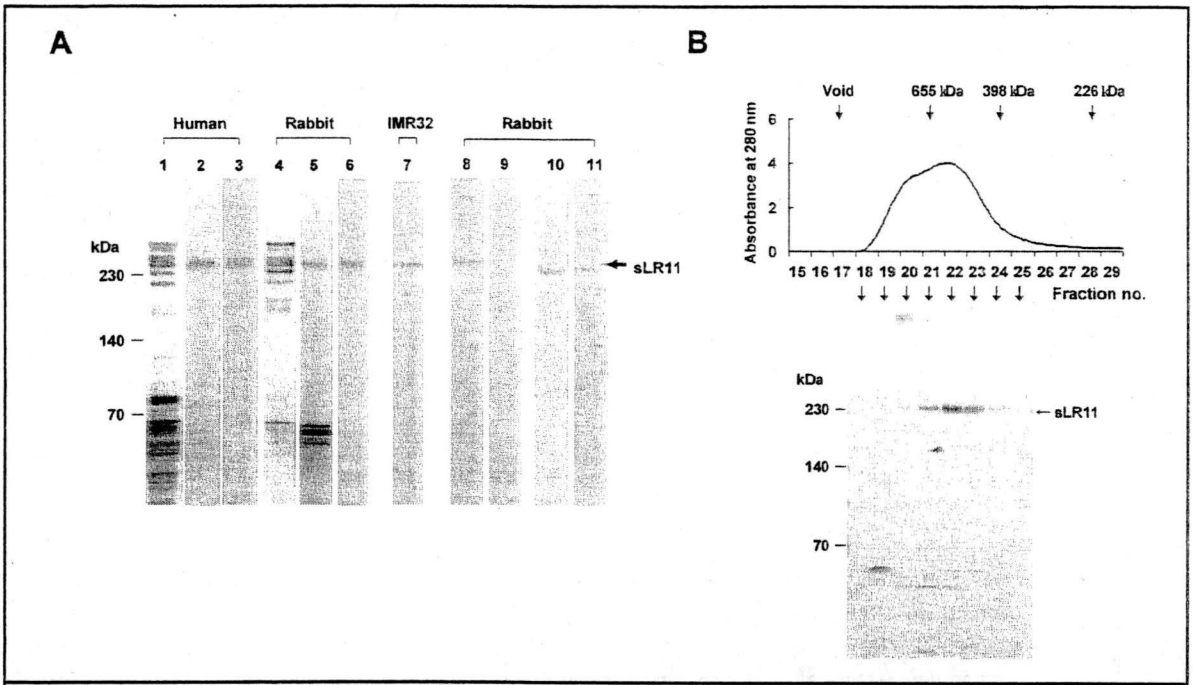

Fig. 2. Purification of sLR11 from human and rabbit serum.
(A), Samples extracted from a RAP affinity column with (lanes 2, 3, 5, 6, and 8–11) or without (lanes 1, 4, and 7) subsequent purification using an MAb-M3 affinity column were separated by SDS-PAGE (2%–15% gradient) under reducing (lanes 1–9) or nonreducing (lanes 10 and 11) conditions. Following electrophoresis, the samples were silver stained (lanes 1, 2, 4, and 5), or subjected to immunoblot analysis using MAb A2-2–3 (lanes 3, 6, and 7), R14 (lanes 8 and 10), or M3 (lanes 9 and 11). Lanes 1–3, human serum; lanes 4–6 and 8–11, rabbit serum; lane 7, human IMR32 cells. (B), Purified rabbit sLR11 protein was fractionated by gel filtration chromatography (HiLoad Superdex 200), and absorbance was monitored at 280 nm. The eluted proteins in each fraction were separated by SDS-PAGE under nonreducing conditions, and then silver stained. Markers at 230, 140, and 70 kDa are shown at the left.

dues 1000–1550 of LR11 *(2, 17)* was cloned into an expression plasmid (in-house vector, Nosan), and we immunized BALB/c mice or Wistar rats by intradermal application of DNA-coated gold particles, using a hand-held device for particle bombardment (Gene Gun, Bio-Rad). Antibody-producing cells were isolated and fused with Sp2/0 myeloma cells by use of polyethylene glycol, according to standard procedures. Five mouse and 5 rat MAbs were selected based on their reactivity with extracted rabbit sLR11, and preliminary sandwich ELISAs were performed using various combinations of these MAbs and A2-2–3. Mouse MAb M3 (IgG2a,k) and rat MAb R14 (IgG2b,k) were identified as the most sensitive for rabbit and human sLR11, respectively, and the combination of these antibodies gave the strongest reactivity against serum sLR11 in our ELISA system. Rat MAb R14 was then conjugated with sulfo-NHS-LC-biotin (Pierce), according to the manufacturer's instructions.

PURIFICATION OF sLR11 FROM HUMAN AND RABBIT SERA

The RAP affinity resin described above was used to extract sLR11 from 2.5 L of human serum or 1.0 L of rabbit serum. The eluted proteins were concentrated and applied to a HiLoad Superdex 200 gel filtration column (GE Healthcare) equilibrated with PBS. The fractions containing immunologically detected sLR11 were pooled, concentrated, and incubated overnight at room temperature with anti-LR11 MAb M3-Sepharose resin. After the resin was rinsed with PBS, immunologically bound sLR11 was eluted with 100 mmol/L sodium citrate buffer (pH 3.0). The sLR11 content was quantified by comparison with BSA standards on silver-stained gels.

SANDWICH ELISA

The wells of a polystyrene microtiter plate (Nunc) were coated with 100 μL of MAb M3 (10 mg/L in PBS) and incubated for 2 h. After extensive washing with PBST, the wells were blocked by incubation with 200 μL of

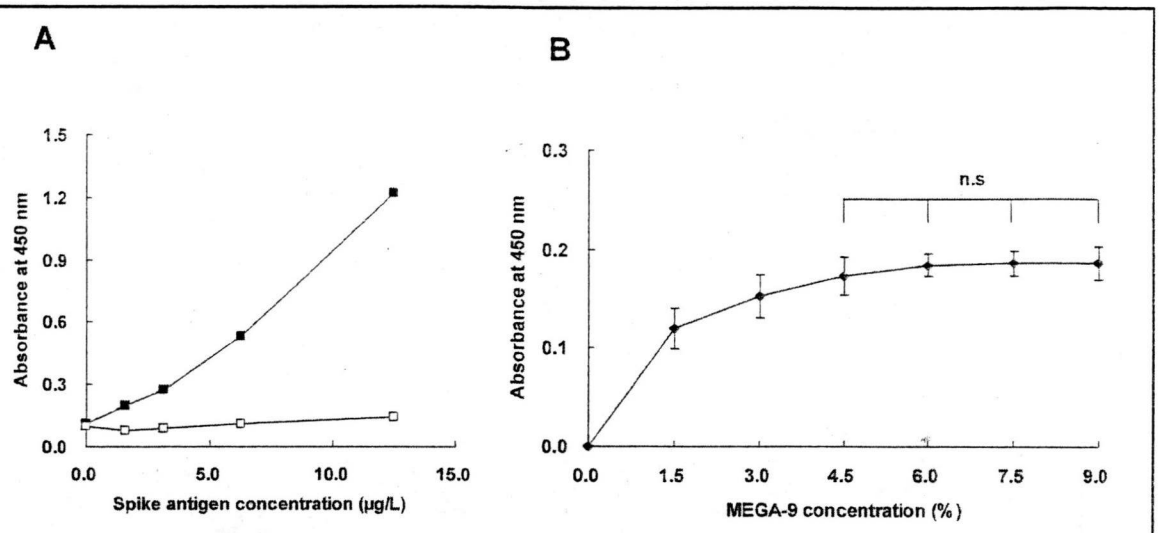

Fig. 3. The effect of MEGA-9 on ELISA measurements of sLR11 in the presence or absence of human serum.
(A), Extracted human sLR11 was measured by ELISA; sLR11 samples were diluted with PBS in the presence (□) or absence (■) of 10% human serum. (B), The sLR11 concentrations were quantified by ELISA (n = 8) in the sample buffer containing 10% human serum in the presence of various concentrations of MEGA-9. n.s., not significant.

1% BSA-PBST for 1 h. The samples (10 μL) were diluted with 100 μL of sample buffer, which consisted of 5.25% n-nonanoyl-N-methyl-d-glucamine (MEGA-9; Dojindo) and 25% heterophilic blocking reagent (Scantibodies Laboratory) in PBS. The calibration samples (0–4.0 μg/L rabbit sLR11) serially diluted in sample buffer together with the above diluted samples (100 μL) were placed into wells and then incubated for 6–16 h. After extensive washing with PBST, 100 μL of biotinylated MAb R14 was added to each well, and the plate was subsequently incubated for 4 h. After extensive washing with PBST, the LR11-MAb complex was reacted with horseradish peroxidase–conjugated streptavidin (Pierce) for 1 h. The trapped complexes were washed and incubated with 100 μL of substrate solution (tetramethyl-benzidine in citrate buffer, pH 3.65, containing hydrogen peroxide) for 30 min. The chromogenic reaction was stopped with 100 μL H_2SO_4, and the absorbance of each sample was determined at 450 nm. All steps were performed at room temperature. ELISA data (μg/L) were significantly and positively correlated with the immunoblotting data (U) previously observed following purification with RAP affinity chromatography ($r = 0.781$, $P < 0.001$, $y = 1.31\,x + 8.34$) *(6)*.

STATISTICAL ANALYSIS

Statistical analyses were performed with commercial software (Stat Flex, Ver. 5.0). The effect of sample dilution with various concentrations of MEGA-9 on the ELISA results was examined using a paired *t*-test, with $P < 0.05$ considered significant. The correlation between variables was evaluated using Pearson correlation analysis. Furthermore, sLR11 concentrations in individuals with atherosclerosis vs those in normal individuals were compared by use of box plot analysis.

Results

IDENTIFICATION OF sLR11 IN VARIOUS SERA AND HUMAN CSF

sLR11 was isolated as a 250-kDa protein from rabbit SMCs and human IMR32 cells by use of immunoblot techniques under reducing conditions and an antibody against a recombinant protein corresponding to a partial amino acid sequence of rabbit LR11 *(5)*. For comparison, sLR11 was extracted from both human and animal sera, using RAP–glutathione *S*-transferase resin. A single 250-kDa protein was detected in human serum by use of MAb A2-2–3 (Fig. 1A). The migration distance of the protein during electrophoresis was consistent with that of sLR11 from rabbit SMCs and human IMR32 cells *(5)*. A single protein band, similar in size to that obtained from human serum, was detected immunologically by MAb A2-2–3 in mouse, rat, rabbit, goat, and porcine sera. The relative intensities of the immunological signals suggested that sLR11 was most abundant in rabbit serum.

We also assessed the presence of sLR11 in human CSF (Fig. 1B), where it was identified by MAb A2-2–3 without the need for RAP extraction. Although sLR11

obtained from CSF was slightly larger than that in serum, the protein appeared as a single band at 250-kDa in both cases.

PURIFICATION OF sLR11 FROM HUMAN AND RABBIT SERA

Using DNA immunization, we established 2 MAbs, M3 and R14, against different epitopes of human sLR11; these MAbs were then used to purify intact sLR11 from serum and construct a sandwich ELISA assay. Intact sLR11 protein was first purified from human and rabbit sera using RAP affinity resin and was released from the resin with an eluting buffer, without decoupling from RAP–glutathione *S*-transferase (Fig. 2A, lanes 1 and 4). The eluted samples were treated with anti-LR11 MAb M3-Sepharose resin. Silver staining after electrophoresis indicated that the M3-reactive samples contained sLR11 as a single protein at 250 kDa as well as low molecular weight proteins, in both human and rabbit sera (lanes 2 and 5). The purified sLR11, but no other low molecular weight protein, was specifically bound to MAb A2-2–3 (lanes 3 and 6). The migration distance of sLR11 from human and rabbit sera was not different from that of sLR11 in the culture medium of IMR32 cells (lane 7). Therefore, 2-step affinity chromatography with RAP and MAb M3 can be used to specifically purify serum sLR11 as a soluble protein identical to that released from cultured cells. R14, as well as A2-2–3 and M3, showed reactivity against the purified sLR11, but did not bind any other low molecular weight protein (lanes 8–11). Notably, R14 reacted with sLR11 under both reducing and nonreducing conditions, whereas M3 reacted with sLR11 under nonreducing condition only.

Silver staining of the purified protein after gel filtration chromatography showed that the position to which purified sLR11 eluted corresponded to an estimated molecular weight >398 kDa (Fig. 2B). Notably, no other distinct protein proportional to the level of the stained sLR11 protein was detected in these fractions. The apparent molecular weight of sLR11 estimated from gel filtration was greater than that determined by use of gel electrophoresis (see Figs. 1 and 2A).

PREPARATION OF SAMPLES WITH MEGA-9 FOR SANDWICH ELISA

Sample conditions for the sandwich ELISA were determined using the above MAbs and purified samples. The absorbance level of immunologically detected sLR11 was proportional to the volume of extracted human sLR11 when diluted with PBS. However, the expected change in absorbance was not observed when samples were diluted with human serum instead of PBS (Fig. 3A). To measure sLR11 in human serum accurately, the matrix effects were mitigated by the addition of MEGA-9 detergent. The effects of MEGA-9 on absorbance recovery increased with increasing amounts of MEGA-9, up to 4.5%. No significant differences were observed at higher concentrations (Fig. 3B). These results suggest that human serum contains unknown factors that interfere with sLR11 quantification, and that this interference could be diminished by the presence of MEGA-9. Therefore, samples were diluted with 5.25%, which was chosen as the middle concentration of 4.5% and 6.0%.

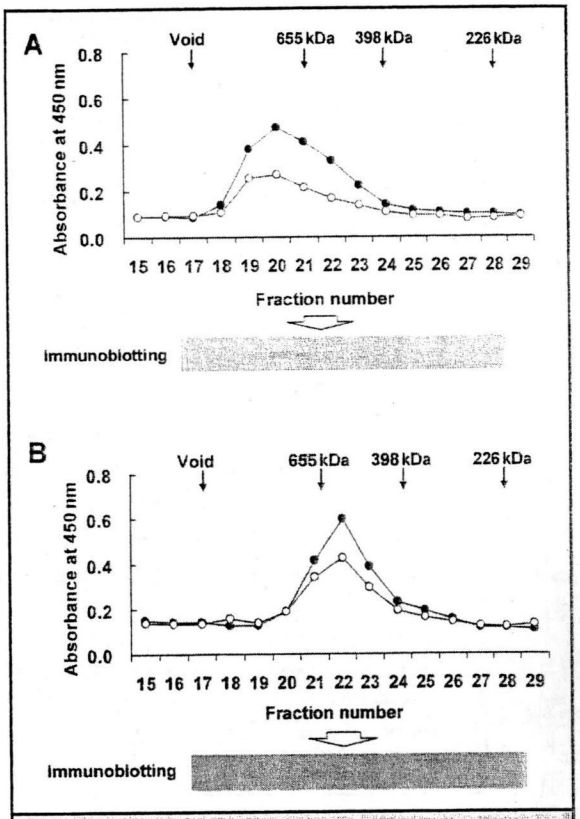

Fig. 4. Gel filtration elution profiles of sLR11 in human serum and CSF.

Human serum (A) and pooled CSF (B) samples (2 mL each) were fractionated by gel filtration chromatography (HiLoad Superdex 200), and the sLR11 concentration in each fraction was measured by ELISA in the presence (●) or absence (○) of MEGA-9. The amount of sLR11 in each fraction was also visualized by immunoblotting after extraction with the RAP affinity resin.

CHARACTERIZATION OF sLR11 IN SERUM AND CSF BY GEL FILTRATION

To assess whether ELISA can specifically detect naturally occurring sLR11, each fraction of human serum and CSF was analyzed for sLR11, following to separation by gel filtration chromatography; the results were then compared with the immunologically purified

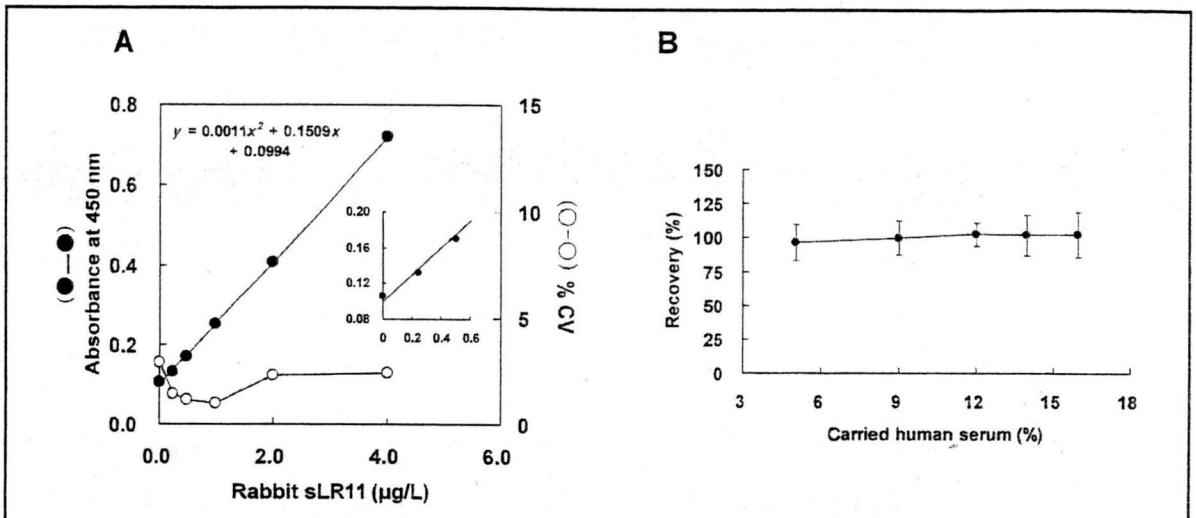

Fig. 5. Calibration curve and sample dilution test.

(A), Using the ELISA system developed in this study, we constructed a typical calibration curve (●) for sLR11 extracted from rabbit serum and the % CV of each point (○). Imprecision was assessed based on 5 replicates of the calibration curve. The insert graph is a close-up of the low-concentration area of the calibration curve. (B), Percentage recovery of purified sLR11 (2 μg/L) measured in the presence of various concentrations of human serum (5%–16%) in sample buffer. The concentration of each sample was measured using ELISA, and the percentage recoveries were calculated as a ratio of the actual-to-theoretical sLR11 concentrations.

sLR11 protein as a quantitative calibrator. The ELISA of serum and CSF samples that had been diluted with or without MEGA-9 showed abundant sLR11 in fractions with molecular weights >398 kDa (Fig. 4), similar to the results of the purified protein (see Fig. 2B). Immunoblot analyses showed that the concentration of sLR11 was proportional to the signal intensity of the gel-filtered 250-kDa proteins in both the serum and CSF samples. These results strongly suggest that this ELISA based on the immunologically purified 250-kDa sLR11 protein is also appropriate for quantifying the naturally occurring sLR11 in serum and CSF, although the gel filtration analyses suggested that the naturally occurring protein may be involved in a high molecular weight complex.

ELISA PERFORMANCE: ASSAY CHARACTERISTICS

A representative calibration curve is shown in Fig. 5A. The working range of this ELISA was 0.25–4.0 μg/L. A quadratic equation was applied to the calibration curve in the working range. The sensitivity, defined as the mean back-fit value for the lowest standard giving acceptable precision (CV = 10%), was 0.25 μg/L. With this ELISA method the lower limit of detection for sLR11 was 0.1 μg/L, which corresponds to the mean blank signal plus 3 SDs. The intraassay CVs (n = 10) were 3.0% and 3.7% at sLR11 concentrations of 7.6 μg/L in serum and 4.4 μg/L in CSF, respectively. The interassay CVs (n = 4) were 3.9% and 10.5% at sLR11 concentrations of 7.6 μg/L in serum and 4.1 μg/L in CSF, respectively. When we used samples containing 5%–16% human serum, percentage recovery ranged from 96.5% to 102.6% (Fig. 5B).

VARIATIONS OF sLR11 IN SERUM AND CSF

Measurements of sLR11 in 87 serum samples and 13 CSF samples obtained from normal individuals gave mean (SD) sLR11 concentrations of 8.7 (2.1) μg/L (range, 4.5–14.2 μg/L) and 8.5 (3.5) μg/L (range, 3.7–13.0 μg/L) in serum and CSF, respectively. We observed no significant difference in serum sLR11 concentrations between males [8.4 (1.9) μg/L, n = 41] and females [9.8 (5.8) μg/L, n = 46].

sLR11 CONCENTRATIONS IN INDIVIDUALS WITH ATHEROSCLEROSIS

To evaluate whether this ELISA method is useful for detecting variation in circulating sLR11 under pathophysiological conditions, we measured sLR11 concentrations in individuals with atherosclerosis. The sLR11 concentrations determined by immunoblotting after RAP affinity chromatography were positively correlated with the degrees of atherosclerosis in the carotid arteries of individuals with dyslipidemia *(6)*. The sLR11 concentrations in individuals with atherosclerosis were compared to those of healthy individuals (see

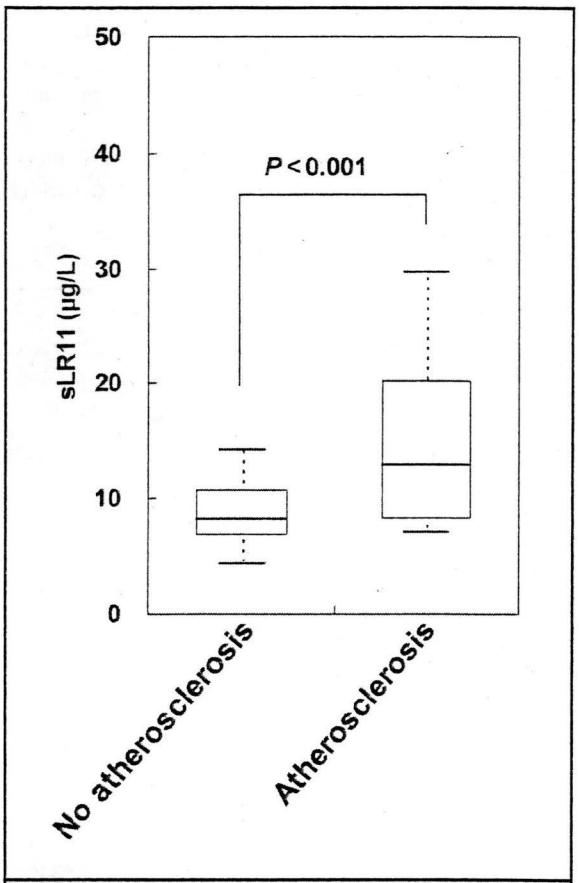

Fig. 6. Circulating sLR11 concentrations in individuals with atherosclerosis vs those in individuals without atherosclerosis.

Center horizontal lines indicate median values. Upper and lower edges of boxes, mean ± 1 SD; upper and lower bars, maximum and minimum.

previous section on variation of sLR11 in serum and CSF). The sLR11 concentrations [14.2 (6.0) μg/L] in individuals with atherosclerosis were significantly higher than those in healthy individuals (Fig. 6). The variation in circulating sLR11 concentrations in individuals with atherosclerosis was within the dynamic range of the ELISA.

Discussion

Three MAbs were established against different epitopes of human sLR11, and an ELISA method was developed for the quantitative measurement of sLR11 in serum and CSF. One of the MAbs (M3), in combination with RAP affinity extraction, enabled the purification of sLR11 from human and rabbit sera. The purified human and rabbit sLR11 was immunologically identical to sLR11 released from cultured cells, strongly suggesting that circulating sLR11 corresponds to the soluble form of membrane-bound LR11. This soluble form has been identified in the media of IMR32 and SMC cultures *(5, 6, 9)*. The combination of MAb M3 and MAb R14 yielded an ELISA that is highly specific for sLR11 in serum and CSF, without the need for prior RAP affinity extraction.

Strong matrix effects interfered with the accurate determination of sLR11 in serum by ELISA. However, these effects were diminished by pretreatment with MEGA-9 detergent (see Fig. 3). This pretreatment may dissociate complexes of sLR11 and serum components or may induce a conformational change in sLR11 such that it more efficiently interacts with the MAbs. Previous studies have shown that several serum components, including apolipoprotein E–containing lipoproteins, urokinase plasminogen activator–plasminogen activator inhibitor type 1 complex, and amyloid-β, can interact with membrane-bound LR11 *(1, 16, 21)*. The observation that MEGA-9 increased the absorbance of the isolated protein at 450 nm in gel filtration fractions obtained from both serum and CSF, and did so in proportion to the signal intensity of the sLR11 protein detected immunologically in the absence of MEGA-9 (see Fig. 4), suggests that epitope recognition by MAbs was strengthened by MEGA-9. The mechanism of MEGA-9–mediated absorbance enhancement requires further elucidation, specifically with regard to the interaction between naturally occurring sLR11 and various matrices in serum and with homomeric or heteromeric complexes under various column conditions (see Fig. 2B).

Using the established ELISA conditions, we investigated the mean sLR11 concentrations in serum and CSF. In 74% of healthy individuals, serum sLR11 concentrations were <10 μg/L. The sLR11 concentrations in the sera of individuals with atherosclerosis ranged from 6 to 30 μg/L. Therefore, the ELISA technique described here provides sufficient sensitivity for detecting circulating sLR11 concentrations in individuals with atherosclerosis and in normal populations.

Given that sLR11 is abundantly expressed in intimal SMCs *(3)* and that circulating sLR11 concentrations are positively correlated with the carotid intima-media thickness in dyslipidemic individuals *(6)*, variation in the circulating sLR11 concentration may be indicative of the condition of intimal SMCs. Metabolic disorders such as dyslipidemia and diabetes can cause pathological changes in intimal SMC function, possibly leading to accelerated progression of atherosclerosis *(22–24)*. The expression level of LR11 is drastically higher in intimal SMCs relative to that in medial SMCs *(3)*, and a large proportion of the LR11 in the cell membrane is released into the culture medium of

SMCs (*5*). Therefore, the concentration of circulating sLR11, rather than the LR11 expression level in intimal SMCs, may be more effective as a novel marker for pathogenic changes in SMCs.

Recent studies have highlighted the pathological function of sLR11 in neurodegenerative diseases. Immunological analyses indicate that sLR11 exists in CSF at concentrations similar to those in serum. Neuronal LR11 expression is significantly reduced in individuals with mild cognitive impairment and AD (*10–13*), and polymorphism of the gene for LR11 is highly associated with the onset of AD (*14*). Therefore, methods for determining sLR11 concentrations in CSF may be vital for future research into neuronal diseases, particularly AD.

In conclusion, we established a sensitive ELISA method for determining sLR11 concentrations in serum and CSF. This ELISA method constitutes a useful tool for monitoring the pathological condition of intimal SMCs and the progression of atherosclerosis (*25*). Use of this ELISA method to measure sLR11 as an indicator of intimal SMC function may enable novel strategies for treating atherosclerosis and help to determine risk factors for vascular disease. Furthermore, the ELISA described here has adequate sensitivity and dynamic range for determining sLR11 concentrations in CSF and may allow significant progress in AD-related research.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

1. Yamazaki H, Bujo H, Kusunoki J, Seimiya K, Kanaki T, Morisaki N, et al. Elements of neural adhesion molecules and a yeast vacuolar protein sorting receptor are present in a novel mammalian low density lipoprotein receptor family member. J Biol Chem 1996;271:24761–8.
2. Jacobsen L, Madsen P, Moestrup SK, Lund AH, Tommerup N, Nykjaer A, et al. Molecular characterization of a novel human hybrid-type receptor that binds the α2-macroglobulin receptor associated protein (RAP). J Biol Chem 1996;271:31379–83.
3. Kanaki T, Bujo H, Hirayama S, Ishii I, Morisaki N, Schneider WJ, et al. Expression of LR11, a mosaic LDL receptor family member, is markedly increased in atherosclerotic lesions. Arterioscler Thromb Vasc Biol 1999;19:2687–95.
4. Zhu Y, Bujo H, Yamazaki H, Hirayama S, Kanaki T, Takahashi K, et al. Enhanced expression of the LDL receptor family member LR11 increases migration of smooth muscle cells in vitro. Circulation 2002;105:1830–6.
5. Zhu Y, Bujo H, Yamazaki H, Ohwaki K, Jiang M, Hirayama S, et al. LR11, an LDL receptor gene family member, is a novel regulator of smooth muscle cell migration. Circ Res 2004;94:752–8.
6. Jiang M, Bujo H, Ohwaki K, Unoki H, Yamazaki H, Kanaki T, et al. Ang II-stimulated migration of vascular SMC is dependent on LR11. J Clin Invest 2008;118:2733–46.
7. Böhm C, Seibel NM, Henkel B, Steiner H, Haass C, Hampe W. SorLA signaling by regulated intramembrane proteolysis. J Biol Chem 2006;281:14547–53.
8. Ohwaki K, Bujo H, Jiang M, Yamazaki H, Schneider WJ, Saito Y. A secreted soluble form of LR11, specifically expressed in intimal smooth muscle cells, accelerates formation of lipid-laden macrophages. Arterioscler Thromb Vasc Biol 2007;27:1050–6.
9. Jiang M, Bujo H, Zhu Y, Yamazaki H, Hirayama S, Kanaki T, et al. Pitavastatin attenuates the PDGF-induced LR11/uPA receptor-mediated migration of smooth muscle cells. Biochem Biophys Res Commun 2006;348:1367–77.
10. Andersen OM, Reiche J, Schmidt V, Gotthardt M, Spoelgen R, Behlke J, et al. Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein. Proc Natl Acad Sci USA 2005;102:13461–6.
11. Scherzer CR, Offe K, Gearing M, Rees HD, Fang G, Heilman CJ, et al. Loss of apolipoprotein E receptor LR11 in Alzheimer disease. Arch Neurol 2004; 61:1200–5.
12. Dodson SE, Gearing M, Lippa CF, Montine TJ, Levey AI, Lah JJ. LR11/SorLA expression is reduced in sporadic Alzheimer disease but not in familial Alzheimer disease. J Neuropathol Exp Neurol 2006;65:866–72.
13. Sager KL, Wuu J, Leurgans SE, Rees HD, Gearing M, Mufson EJ, et al. Neuronal LR11/sorLA expression is reduced in mild cognitive impairment. Ann Neurol 2007;62:640–7.
14. Rogaeva E, Meng Y, Lee JH, Gu Y, Kawarai T, Zou F, et al. The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease. Nat Genet 2007;39:168–77.
15. Cuenco KT, Lunetta KL, Baldwin CT, McKee AC, Guo J, Cupples LA, et al. Association of distinct variants in SORL1 with cerebrovascular and neurodegenerative changes related to Alzheimer disease. Arch Neurol 2008;65:1640–8.
16. Taira K, Bujo H, Hirayama S, Yamazaki H, Kanaki T, Takahashi K, et al. LR11, a mosaic LDL receptor family member, mediates the uptake of ApoE-rich lipoproteins in vitro. Arterioscler Thromb Vasc Biol 2001;21:1501–6.
17. Mörwald S, Yamazaki H, Bujo H, Kusunoki J, Kanaki T, Seimiya K, et al. A novel mosaic protein containing LDL receptor elements is highly conserved in humans and chickens. Arterioscler Thromb Vasc Biol 1997;17:996–1002.
18. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. Nature 1992;356:152–4.
19. Krause S, Behrends J, Borowski A, Lohrmann J, Lang S, Myrtek D, et al. Blockade of interleukin-13-mediated cell activation by a novel inhibitory antibody to human IL-13 receptor alpha1. Mol Immunol 2006;43:1799–807.
20. Ikawa Y, Ng PS, Endo K, Kondo M, Chujo S, Ishida W, et al. Neutralizing monoclonal antibody to human connective tissue growth factor ameliorates transforming growth factor-b-induced mouse fibrosis. J Cell Physiol 2008;216:680–7.
21. Gliemann J, Hermey G, Nykjaer A, Petersen CM, Jacobsen C, Andreasen PA. The mosaic receptor sorLA/LR11 binds components of the plasminogen-activating system and platelet-derived growth factor-BB similarly to LRP1 (low-density lipoprotein receptor-related protein), but mediates slow internalization of bound ligand. Biochem J 2004;381:203–12.
22. Inaba T, Yamada N, Gotoda T, Shimano H, Shimada M, Momomura K, et al. Expression of M-CSF receptor encoded by c-fms on smooth muscle cells derived from arteriosclerotic lesion. J Biol Chem 1992;267:5693–9.
23. Tamura K, Kanzaki T, Tashiro J, Yokote K, Mori S, Ueda S, et al. Increased atherogenesis in Otsuka Long-Evans Tokushima fatty rats before the onset of diabetes mellitus: association with overexpression of PDGF beta-receptors in aortic smooth muscle cells. Atherosclerosis 2000;149:351–8.
24. Seki N, Bujo H, Jiang M, Tanaga K, Takahashi K, Yagui K, et al. LRP1B is a negative modulator of increased migration activity of intimal smooth muscle cells from rabbit aortic plaques. Biochem Biophys Res Commun 2005;331:964–70.
25. Bujo H, Saito Y. Modulation of smooth muscle cell migration by members of the low-density lipoprotein receptor family. Arterioscler Thromb Vasc Biol 2006;26:1246–52.

Atherosclerosis 204 (2009) 595–600

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Plasma preβ1-HDL level is elevated in unstable angina pectoris

Jun Tashiro[a,c], Osamu Miyazaki[b,*], Yoshitake Nakamura[c], Akira Miyazaki[c], Isamu Fukamachi[b], Hideaki Bujo[d], Yasushi Saito[e]

[a] Department of Internal Medicine, Matsudo City Hospital, 4005, Kamihongo, Matsudo, Chiba 271-8511, Japan
[b] Tsukuba Research Institute, Sekisui Medical Co. Ltd. (Daiichi Pure Chemicals Co. Ltd.), 3-3-1, Koyodai, Ryugasaki, Ibaraki 301-0852, Japan
[c] Department of Cardiology, Chiba Cardiovascular Center, 575, Tsurumai, Ichihara, Chiba 290-0512, Japan
[d] Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, 1-8-1, Inohana, Chuo-ku, Chiba 260-8670, Japan
[e] Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8670, Japan

ARTICLE INFO

Article history:
Received 30 June 2008
Received in revised form 8 October 2008
Accepted 10 October 2008
Available online 1 November 2008

Keywords:
Preβ1-HDL
Acute coronary syndrome
HDL
Reverse cholesterol transport
LCAT

ABSTRACT

Preβ1-HDL, a minor HDL subfraction consisting of apolipoprotein A-I (apoA-I), phospholipids and unesterified cholesterol, plays an important role in reverse cholesterol transport. Plasma preβ1-HDL levels have been reported to be increased in patients with coronary artery disease (CAD) and dyslipidemia. To clarify the clinical significance of measuring plasma preβ1-HDL levels, we examined those levels in 112 patients with CAD, consisting of 76 patients with stable CAD (sCAD) and 36 patients with unstable angina pectoris (uAP), and in 30 patients without CAD as controls. The preβ1-HDL levels were determined by immunoassay using a specific monoclonal antibody (Mab55201) that we established earlier. The mean preβ1-HDL level in the CAD patients was significantly higher than the level in the controls (34.8 ± 12.9 mg/L vs. 26.6 ± 6.9 mg/L, $p < 0.001$). In addition, the mean preβ1-HDL level was markedly higher in the uAP subgroup than in the sCAD subgroup (43.1 ± 11.5 mg/L vs. 30.9 ± 11.7 mg/L, $p < 0.0001$). These tendencies remained even after excluding dyslipidemic subjects.

These results suggest that elevation of the plasma preβ1-HDL level is associated with the atherosclerotic phase of CAD and may be useful for identifying patients with uAP.

1. Introduction

Preβ1-HDL, an HDL subfraction consisting of one or two molecules of apolipoprotein A-I (apoA-I), small amounts of phospholipids and unesterified cholesterol, plays an important role in reverse cholesterol transport, although it comprises only 1–5% of total apoA-I in blood plasma [1–4]. The initial step of reverse cholesterol transport, called cholesterol efflux, is a reaction by which excessively accumulated cholesterol in peripheral tissues is removed by HDL. Preβ1-HDL is known as the initial plasma acceptor of cell-derived cholesterol [1–5].

Three pathways have been suggested as routes by which preβ1-HDL is generated [6–11]. The first is a pathway in which preβ1-HDL is formed when lipid-free apoA-I or lipid-poor apoA-I removes cell-derived, unesterified cholesterol, mediated by ATP-binding cassette transporter A1 (ABCA1) located on cell membranes [6–8]. The second is a pathway in which preβ1-HDL is directly secreted from the liver [9,10], and the third is a pathway in which preβ1-HDL is released from α-migrating HDL during its remodeling [11]. As the catabolic pathway of preβ1-HDL, a lecithin–cholesterol acyltransferase (LCAT)-dependent conversion pathway has been suggested. The unesterified cholesterol on the preβ1-HDL is esterified by LCAT, and the preβ1-HDL removes cellular cholesterols, increases in size and is converted to an α-migrating HDL [12–14].

Thus, preβ1-HDL is proposed to be a key component of reverse cholesterol transport. We previously reported development of a monoclonal antibody (Mab55201) specifically recognizing an epitope of apoA-I that is exposed only in preβ1-HDL, and we established an ELISA system for direct measurement of preβ1-HDL using Mab55201 [15]. The method provides a way to investigate the clinical significance of measuring plasma preβ1-HDL levels.

Plasma preβ1-HDL levels have been reported to be elevated in patients with coronary artery disease (CAD) and dyslipidemia [15–17]. However, the mechanism responsible for elevation of the preβ1-HDL level has not been clarified. In this study we examined whether the preβ1-HDL level is elevated in normolipidemic CAD patients and whether the levels differ between patients with unstable angina pectoris (uAP) and those with stable CAD (sCAD), including stable effort angina pectoris and old myocardial infarction. In addition, we studied the relationship between

* Corresponding author. Tel.: +81 297 62 6425; fax: +81 297 62 8635.
E-mail address: miyazaki084@sekisui.jp (O. Miyazaki).

0021-9150/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2008.10.015