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雑誌

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Sone H, Yoshimura Y, Tanaka S, Iimuro S, Ohashi Y, Ito H, Seino H, Ishibashi S, Akanuma Y, Yamada N; Japan Diabetes Complications Study (JDCS) Group.	Cross-sectional association between BMI, glycemic control and energy intake in Japanese patients with type 2 diabetes. Analysis from the Japan Diabetes Complications Study.	Diabetes Res Clin Pract.	;77	Suppl 1:S23-9. Epub 2007 May 23	2007 Sep
Ishigaki N, Yamamoto T, Shimizu Y, Kobayashi K, Yatoh S, Sone H, Takahashi A, Suzuki H, Yamagata K, Yamada N, Shimano H.	Involvement of glomerular SREBP-1c in diabetic nephropathy.	Biochem Biophys Res Commun.	364	502-508	2007
Arai H, Yamada N, Kita T, et al	Polymorphisms of apolipoprotein E and methylenetetrahydrofolate reductase in the Japanese populatio n	J Arterioscl Thromb	14	167-171	2007
Kanamori H, Matsubara T, **Arai H,** et al	Inhibition of MCP-1/CCR2 pathway ameliorates the development of diabetic nephropathy	Biochem Biophys Res Commun	360	772-777	2007
Ohwaki K, Bujo H, Jiang M, Yamzaki H, Schneider WJ, Saito Y.	A secreted soluble form of LR11, specifically expressed in intimal smooth muscle cells, accelerates a formation of lipid-accumulated macrophages.	Arterioscler Thromb Vasc Biol.	27(5)	1050-6	2007
Kubota Y, Unoki H, Bujo H, Rikihisa N, Udagawa A, Yoshimoto S, Ichinose M, Saito Y.	Low-dose GH supplementation reduces the TLR2 and TNF-alpha expressions in visceral fat.	Biochem Biophys Res Commun.	368	81-87	2008
Murakami K, Bujo H, Unoki H. Saito Y.	Effect of PPARalpha activation of macrophages on the secretion of inflammatory cytokines in cultured adipocytes.	Eur. J. Pharmacol.	561(1-3)	206-13	2007

Oka R, Kobayashi J, Asano A, Tani i H, Hagishita T, Mori M, Moriuchi T, Kobayashi M, Katsuda S, Kawa shiri M, Nohara A, Yagi K, Yama gishi M, Mabuchi H,Miyamoto S.	Reassessment of the cuto ff values of waist circu mference and visceral fat area for identifying Jap anese subjects at risk for the metabolic syndrome.	Diabetes Res Clin Pract.			2007 Nov 19;
Kobayashi J, Nishi mura K, Matoba M, Maekawa N, Mabuchi H,	Generation and gender di fferences in the compone nts contributing to the di agnosis of the metabolic syndrome according to the Japanese criteria.	Cir J.	71:	1734-1737.	2007;

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Importance of Lipid Levels in Elderly Diabetic Individuals
— Baseline Characteristics and 1-Year Survey of Cardiovascular Events —

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Background The respective incidences of ischemic heart and cerebrovascular disease (IHD, CVD) are high in diabetic individuals. Complications of dyslipidemia increase the risk, but direct evidence is limited, so a cohort prospective study (Japan-CDM) was conducted.

Methods and Results The study group comprised 4,014 subjects with type 2 diabetes (1,936 women, 2,078 men; mean age 67.4±9.5 years) who were divided into dyslipidemic patients (79.1%) with or without medication (medicated, 50.9%; not medicated, 28.2%) and normo-lipidemic patients (20.9%). The incidence of IHD, CVD, arteriosclerosis obliterans (ASO), congestive heart failure (CHF) and death was assessed. IHD and CVD occurred in 0.82 and 0.67%, respectively, during the first year following registration. CHF, ASO and sudden death occurred in 0.27%, 0.12% and 0.12%, respectively. There was a significant statistical difference in the relation of elevated levels of high-density lipoprotein-cholesterol to lower rates of IHD and CVD. IHD and CVD in males were dependent on the level of low-density lipoprotein-cholesterol (LDL-C): 0.45%, 1.56%, 1.78%, 1.91% and 2.34% were observed in less than 2.11, 2.11–2.62, 2.63–3.15, 3.16–3.67, and more than 3.68 mol/L of LDL-C. In the lowest LDL-C group, death other than from vascular diseases was increased. Age, sex (male) and complicated hypertension increased the risk of events. Patients who were prescribed antihyperlipidemic agents suffered less events than patients who were not being treated, which suggests direct effects of therapy.

Conclusion Strict lipid control may be effective for reducing the incidence of vascular events in Japanese diabetic individuals. (*Circ J* 2008; **72:** 218–225)

Key Words: Diabetes mellitus; Elderly; HDL-cholesterol; Ischemic heart disease; LDL-cholesterol

Investigators in Western countries have reported that patients with both hypercholesterolemia and type 2 diabetes mellitus (DM) have a higher risk of coronary events (ie, acute myocardial infarction and sudden cardiac death) than patients with hypercholesterolemia alone[1] The incidence of ischemic heart and cerebrovascular diseases (IHD, CVD) in patients with type 2 DM is reported to be high in Japan[2] so it has been speculated that when hypercholesterolemia is complicated with DM, the risk of IHD (and perhaps CVD) is also increased in Japanese patients, but direct evidence is limited because there have been few large-scale epidemiological surveys. Recently, the J-LIT study reported a relationship between plasma lipid and coronary events[3] but it is a study of hypercholesterolemic patients and important information about DM (such as glycohemoglobin) was not studied. Furthermore, only individuals aged 69 years or younger were assessed, and no data were available for the elderly; in addition, all patients were medicated with simvastatin. Therefore, it is worthwhile analyzing the data from the Japan Cholesterol and Diabetes Mellitus investigation (Japan-CDM), which was a nationwide observational cohort study of a large number of diabetic individuals who were treated in clinical practice; it was designed to assess the relationship between lipid levels and the incidence of cardiovascular diseases in Japanese diabetic individuals[4] In Japan, cholesterol-lowering therapy is well established, but evidence from large-scale studies is not available in relation to plasma levels of low-density lipoprotein (LDL) in diabetic individuals, and at the beginning of the present study (2004), atrovastatin was the only

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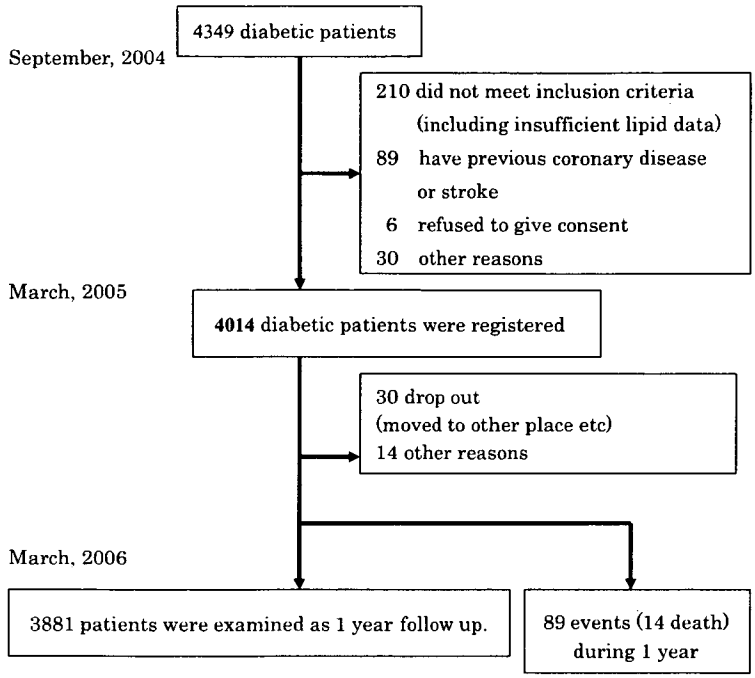

Fig 1. Trial profile.

Table 1 Clinical Background of the Patients With Diabetes

Age (years)	*67.7±9.5*	*<65*	*≥65*
n	*4,014*	*1,701*	*2,313*
Gender ratio (M/F)	*1.055*	*1.260*	*0.932*
Fasting plasma glucose (mmol/L)	*8.81±3.05*	*8.84*	*8.79*
Glycated hemoglobin (%)	*7.30±1.25*	*7.33*	*7.28*
TC (mmol/L)	*5.28±0.96*	*5.43*	*5.21*
Triglyceride (mmol/L)	*1.54±1.19*	*1.77*	*1.46*
HDL-C (mmol/L)	*1.45±0.43*	*1.44*	*1.45*
Systolic BP (mmHg)	*134.6±27.4*	*132.3*	*135.6*
Diastolic BP (mmHg)	*74.0±16.0*	*77.0*	*72.8*
Hyperlipidemic patients	*87.0*	*84.0*	*90.1*
Antihyperlipidemic agents	*49.2*	*46.0*	*50.0*
Hypertensive patients	*61.6*	*63.7*	*60.9*
Diabetic therapy (diet/oral agents/insulin, %)	*15.4/49.5/28.6*	*19.8/52.2/27.7*	*14.8/48.1/29.2*

TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; BP, blood pressure.

strong statin available and it is reported to possibly affect glucose metabolism[5,6] In short, such a randomized control study was not run for ethical and practical reasons. In the Japan-CDM, individuals had no history of prior IHD (myocardial infarction or angina pectoris), so we analyzed the incidence of IHD, CVD and arteriosclerosis obliterans (ASO). We also assessed the relationship between the incidence of IHD, CVD and dyslipidemia and its treatment during the study period.

Methods

We recruited diabetic individuals through 40 institutions throughout Japan between September 2004 and March 2005 (Fig 1). Patients with coronary artery disease, which was defined as previous myocardial infarction, coronary intervention, or confirmed angina pectoris and recent stroke with admission within the past 24 months were excluded, as were patients whose medical records about plasma lipid (total cholesterol, triglyceride and high-density lipoprotein (HDL)-cholesterol) were not provided. Other exclusion criteria were a history or complication of serious heart disease (eg, severe arrhythmia, heart failure, cardiomyopathy, valvular disease, or congenital disease), CVD within the past 24 months, serious hepatic or renal disease, malignant disease, intention to undergo surgery, any illness with a poor prognosis, and judgment by the physician in charge that the patient was not suitable for the study.

The study group comprised 4,014 diabetic individuals enrolled on a consecutive outpatient basis (1,936 women, 2,078 men; M/F ratio 1.073; mean age 67.4±9.5 years, range 35–80). A single-center prospective longitudinal cohort study with an extensive screening program at baseline was carried out[4] The pursuit rate for 1 year was 98.8%. The group was divided into those with dyslipidemia with or without medication (79.1%, n=3,175; HM: 50.9% are medicated, n=2,043, 28.2%; HN: n=1,132 are not medicated) and those without dyslipidemia (H–: 20.9%, n=839). Primary endpoints were incidence of cardiovascular or CVD (fatal and non-fatal myocardial infarction, and other non-fatal events including unstable angina pectoris, angioplasty, stenting, or coronary artery bypass grafting and stroke).

Table 2 Events in the 1st Year in the Total Patient Group (4,014 Subjects)

	Fatal (n)	Non-fatal (n)	Fatal (%)	Non-fatal (%)
AMI	2	8	0.05	0.20
AP	0	11	0	0.27
CHF	1	11	0.02	0.27
Intervention	0	12	0	0.30
CVD	1	26	0.02	0.65
Renal failure	0	–	0	–
Sudden death	5	–	0.12	–
ASO	0	5	0	0.12
Internal carotid artery narowing	0	2	0	0.05
Death from other causes	5	–	0.12	–
Subtotal of IHD	2	31	0.05	0.77
Subtotal of CVD	1	26	0.02	0.65
Total events	14	75	0.33	1.91

AMI, acute myocardia infarction; AP, angina pectoris; CHF, congestive heart failure; CVD, cerebrovascular disease; ASO, arteriosclerosis obliterans; IHD, ischemic heart disease.

Table 3 Event and Lipid Profile in Relation to Plasma LDL-C Concentration on Registration (%)

	<2.11 mmol/L	2.11–2.62	2.63–3.15	3.16–3.67	≥3.68	Total
CVD	0.53	0.61	0.86	0.88	0.76	0.73
IHD (AMI+AP)	0.26	0.92	0.89	0.89	1.19	0.89
CHF	0.53	0.15	0.10	0.49	0.32	0.31
Sudden death	0.53	0.00	0.20	0.00	0.11	0.13
ASO	0.00	0.15	0.10	0.12	0.22	0.13
IHD+CVD	0.79	1.53	1.76	1.77	1.95	1.62
TVE	1.32	1.84	1.96	2.23	2.38	2.04
TVE+sudden death	1.85	1.84	2.16	2.23	2.49	2.17
Other death	0.26	0.15	0.00	0.00	0.23	0.10
TVE+total death	2.12	1.99	2.16	2.23	2.70	2.27

Data adjusted for age.
LDL-C, low-density lipoprotein-cholesterol; TVE, total vascular events. Other abbreviations see in Table 1.

Secondary endpoints were sudden cardiac death other than myocardial infarction, incidence of onset of ASO, CVD mortality, hospitalization because of CVD or arteriosclerosis thrombosis, and all-cause mortality. Events were defined as all of the primary and secondary endpoints. Detailed definitions of each event are shown in Appendix 1.[7] We evaluated the changes in plasma lipid levels, blood glucose and hemoglobinA_{1c} levels measured during the same month of each year. DM and hyperlipidemia, and the progression of diabetic complications (microangiopathy: retinopathy, neuropathy, nephropathy) were also investigated.

Lipid levels, adverse events and coronary events were monitored at least annually. The patients were to be treated according to the guidelines of the Japan Atherosclerosis Society (2002): LDL <3.16 mmol/L in diabetic individuals[8] Each patient was informed of the study purpose, as well as drug efficacy and treatment, and gave written agreement. We used the criteria for the diagnosis of type 2 DM that were established in 1999 by the Japan Diabetes Society (JDS), which are similar to those of the American Diabetes Association[8,9]

All events (clinical endpoints and severe adverse events) during the study period reported by local physicians were checked by members of then organizing committee and endpoints adjudication was also confirmed annually by the organizing committee. The study was approved by external data and safety monitoring boards, and by the institutional review boards at all hospitals.

Statistical Analysis

The results are presented as mean±SD. All statistical analyses were performed using JMP software (version 6, SAS Institute Inc, Cary, NC, USA). The incidence of events was age-standardized by direct method, in which our whole sample population was supposed as a standard population. The incidence was analyzed in relation to lipid levels on registration, and the differences among categorical variables were assessed by the Mantel-Haenszel chi-square test. Values of $p<0.05$ were considered to indicate statistical significance. The relative risk and its 95% confidence interval (CI) were calculated using logistic regression analyses with adjustment for baseline characteristics such as sex and age. As a result, adjusted odds ratios (OR) were calculated.

Results

Patients' profiles are shown in Fig 1 and Table 1. Triglyceride levels were higher in younger diabetic individuals than in the older ones. Table 2 shows the detailed information of events occurring during the first year. There were 85 vascular events and 4 deaths of other etiologies. IHD and CVD occurred in 0.82% and 0.67%, respectively, in the first year (Tables 2,3). When the events were divided according to the LDL level at registration, the lowest incidence was in individuals with LDL-cholesterol <2.11 mol/L, especially in males after adjustment for age. In other words, 0.45%, 1.56%, 1.78%, 1.91% and 2.34% of IHD or CVD events occurred in males with <2.11, 2.11–2.62, 2.63–3.15, 3.16–3.67, and >3.68 mol/L LDL-cholesterol, respectively, with significant difference after adjustment for age ($p=0.032$, Fig 2). However, total events (cardiovascular, cerebrovascular and peripheral vascular disease, congestive heart failure,

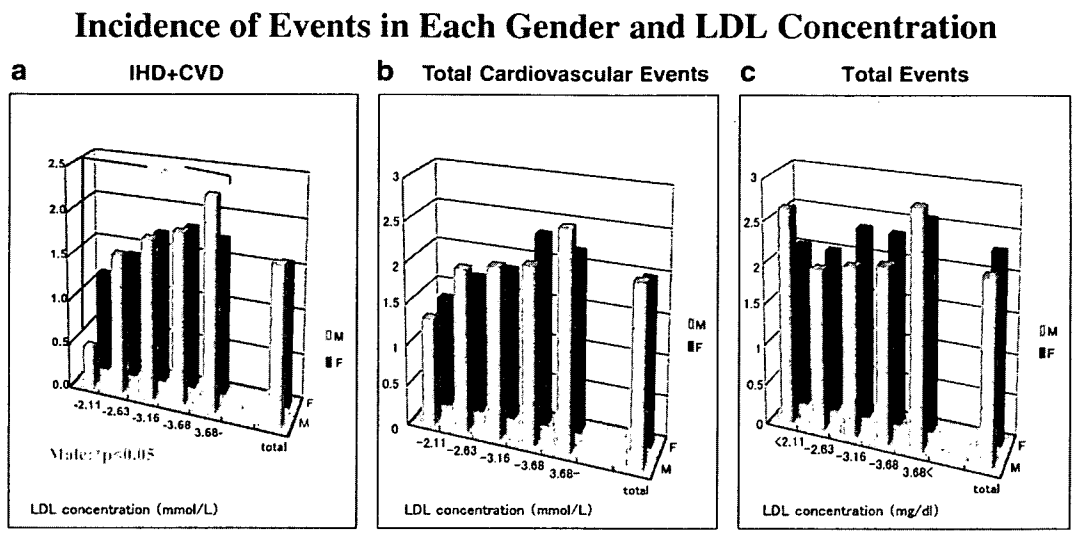

Fig 2. Incidence of events: gender and low-density lipoprotein (LDL) concentration. (a) Incidence of ischemic heart disease (IHD) and stroke (CVD). (b) Incidence of total cardiovascular, cerbrovascular and peripheral vascular events. (c) Incidence of total events (cardiovascular, cerebrovascular and peripheral vascular disease, congestive heart failure, sudden death and death by other etiology). *p<0.05; data adjusted for age.

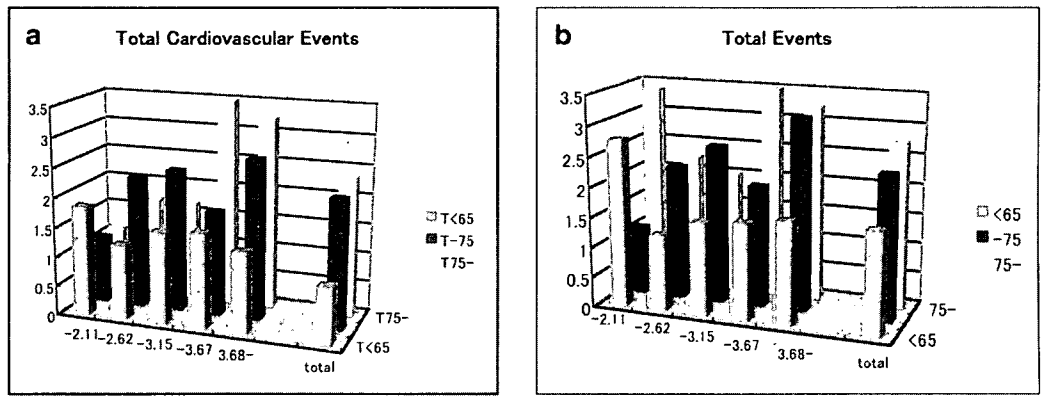

Fig 3. Incidence of events: age and low-density lipoprotein (LDL) concentration. (a) Incidence of ischemic heart disease and stroke. (b) Incidence of total events (cardiovascular, cerebrovascular and peripheral vascular disease, congestive heart failure, sudden death and death of other etiology). <65, 65–74, ≥75, age groups. Data adjusted for age and sex.

sudden death and death of other etiology) were increased in the individuals with the lowest LDL level (<2.11 mol/L, Fig 2, Table 3). Age (>75 years) and complicated hypertension, but not gender, tended to increased the risk of events despite the level of LDL (Figs 2,3, data partially not shown). Interestingly, the patients who were prescribed antihyperlipidemic agents tended to suffer fewer events than the patients who were not being treated, regardless of their LDL levels (Fig 4).

Significant statistical difference was observed in relation to elevated levels of HDL-cholesterol and lower cardiovascular and cerebrovascular events after adjustment for age (p=0.0004; Table 4, Fig 5). The OR was 3.97 (95% CI 1.91–8.20, <1.05 mmol/L vs >1.58 mmol/L, p=0.011). The same tendencies were also observed for the total vascular events in males after adjustment for age (p=0.0011, OR 3.01, 95% CI 1.15–4.03, <1.05 mmol/L vs >1.58 mmol/L, p=0.03) and total events in males after adjustment for age (p=0.0012, OR 2.91, 95% CI 1.11–3.23, <1.05 mmol/L vs >1.58 mmol/L, p=0.041) (Fig 5). Fig 6 compares the frequency of IHD and CVD events reported in a recent, large clinical trial in Japan and in other countries. Subjects with type 2 DM had significantly more IHD events than those without type 2 DM (eg, Hisayama vs JEDIT, J-DCS and J-CDM). The ratio of CVD events was not significantly different. So, the incidence was approximately 2-fold that of the Hisayama study, which is the Japanese-equivalent community cohort study. Interestingly, the frequency is low in J-LIT, based on the assumption that the individuals were 65 years of age, male and diabetic. The significance is the magnitude of the difference of incidence because of their LDL or HDL levels. Ideally, if the patient's LDL or HDL is well controlled, the incidence is comparable with the mean of that in a normal cohort (60 years of age). Compared with the Japanese data, the data available from other countries (particularly that for elderly diabetic individuals)

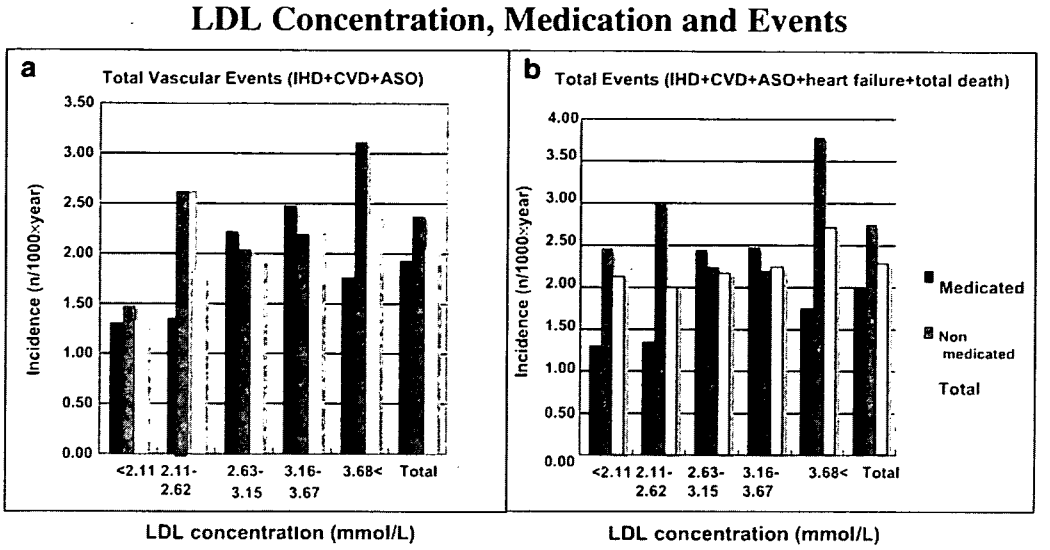

Fig 4. Incidence of events: medication and low-density lipoprotein (LDL) concentration. (a) Incidence of total cardiovascular, cerbrovascular and peripheral vascular events. (b) Incidence of total events (cardiovascular, cerebrovascular and peripheral vascular disease, congestive heart failure, sudden death and death by other etiology). (Left) Events in medicated patients; (Middle) events in non-medicated patients; (Right) events in total patients. Data adjusted for age and sex. IHD, ischemic heart disease; CVD, cerebrovascular disease; ASO, atherosclerosis obliterans.

Table 4 Event and Plasma Lipid Profile in Relation to Plasma HDL-C Concentration on Registration (%)

	<1.05 mmol/L	*1.05–1.30*	*1.31–1.57*	*1.58–*	*Total*
*CVD**	*1.28*	*0.79*	*0.97*	*0.30*	*0.73*
*IHD (AMI+AP)**	*2.19*	*0.79*	*0.65*	*0.60*	*0.89*
CHF	*0.73*	*0.30*	*0.32*	*0.15*	*0.31*
Sudden death	*0.36*	*0.10*	*0.11*	*0.07*	*0.13*
ASO	*0.18*	*0.10*	*0.22*	*0.07*	*0.13*
*IHD+CVD****	*3.47*	*1.58*	*1.61*	*0.90*	*1.62*
*TVE**	*4.38*	*2.00*	*2.04*	*1.12*	*2.04*
TVE+sudden death	*4.74*	*2.08*	*2.15*	*1.20*	*2.17*
Other death	*0.18*	*0.10*	*0.11*	*0.07*	*0.10*
*TVE+total death**	*4.93*	*2.17*	*2.26*	*1.27*	*2.27*

**p<0.05, ***p<0.001, difference in relation of elevated level of HDL-C to lower event rate.*
See Tables 1–3 for abbreviations.

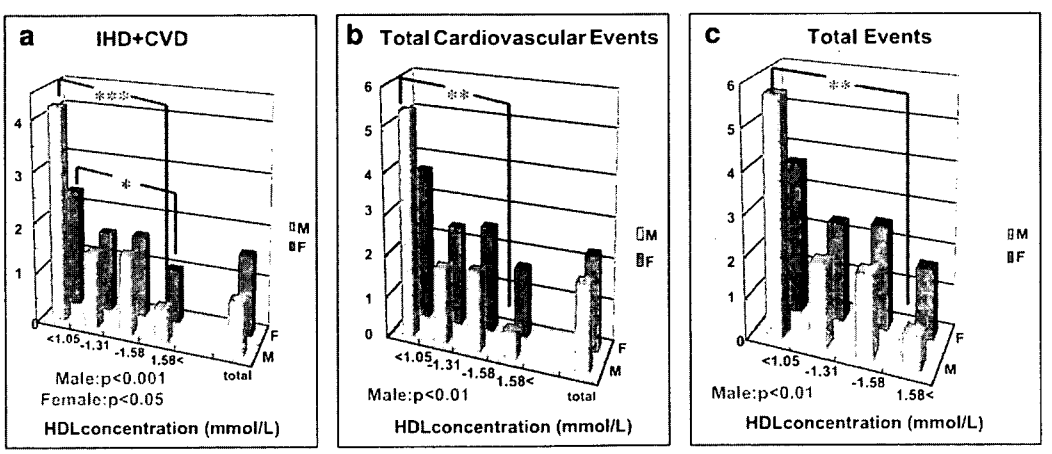

Fig 5. Incidence of events: gender and high-density lipoprotein (HDL) concentration. (a) Incidence of ischemic heart disease (IHD) and stroke (CVD). (b) Incidence of total cardiovascular, cerbrovascular and peripheral vascular events. (c) Incidence of total events (cardiovascular, cerebrovascular and peripheral vascular disease, congestive heart failure, sudden death and death of other etiology). **p<0.01, ***p<0.001. Data adjusted for age.

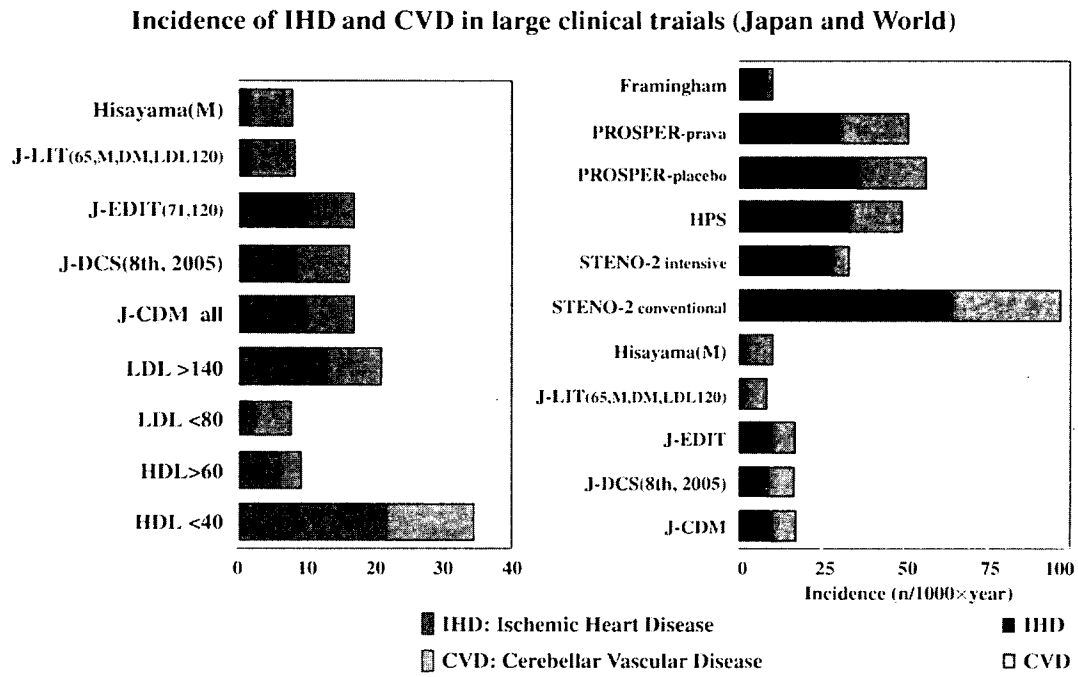

Fig 6. Comparison of the incidence of ischemic heart disease (IHD) and cerebellar vascular disease (CVD) in large-scale clinical trials in Japan and Western countries. (Left) Comparison of Japanese studies: Hisayama (M): Hisayama study, males;[27] J-LIT: 65-year-old male diabetic individuals, low-density lipoprotein (LDL)=3.16 mmol/L;[3] J-EDIT: 71-year-old diabetic individuals, low-density lipoprotein = 3.16 mmol/L;[28] J-DCS: 8th year;[2] J-CDM: all patients: LDL ≥3.68 mmol/L, LDL <2.11 mmol/L, high-density lipoprotein (HDL) ≥1.58 mmol/L, HDL <1.05 mmol/L. (Right) Comparison of Western and Japanese studies: Framingham;[29] PROSPER;[15] HPS study;[16] STENO-2 Intervention group, Control group[30]

show that the incidence of events in Japan is comparable with that in the most well-known community cohort, the Framingham study.

Discussion

Diabetes is a common cause of morbidity and premature loss of life! People with DM are 4-fold more likely to have IHD as those without DM and IHD accounts for a large proportion of the mortality related to DM![10,11] Furthermore, CVD, peripheral vascular disease (such as ASO) and congestive heart failure are reportedly increased in diabetic individuals, especially in elderly Japanese diabetics, compared with individuals without DM. The present study may be 1 of the largest cohort studies carried out for Japanese diabetic individuals with or without hypercholesterolemia. Evidence suggests that even in the absence of preexisting vascular disease, middle-aged people with type 2 DM have a similar risk of IHD to those in Western countries without DM but who have had a myocardial infarction![12] However, this is not evident in Japan, and the Japan Atherosclerosis Society suggests that the ideal level of LDL in diabetic individuals is <3.16 mmol/L compared with that after a previous myocardial infarction, which is <2.63 mmol/L[8] The idea of DM as a coronary equivalent has led to widespread changes in the approach to reducing CVD risk in this population![13–15] but that may not be the case in Japanese, as the mean LDL level was approximately 3.16 mmol/L in the present study. An issue that concerns many practitioners is the age and levels at which vascular protection strategies should be initiated in people with DM. Recent randomized controlled trials on this topic have included participants older than 70 years of age and 3 large-scale clinical studies of the effect of statins on hyperlipidemic elderly individuals (ie, Prosper (pravastatin), HPS (simvastatin) and ASCOT-LLA) have been reported![16–18] In all them hyperlipidemic patients <75 years of age were treated, and the effect of statins on cardiovascular events (including diabetic individuals) were reported. However, the effect was not as large (eg, 16% decrease in IHD without a change of CVD in Prosper) compared with previous studies on the primary and secondary prevention of IHD in diabetic individuals![15,19] To date, there are no comparable studies in Japan that we are aware of, so implementation of the results of the present study may potentially lead to a decrease in CVD and IHD by controlling LDL and HDL levels. However, many clinical practice guidelines recommend the application of existing evidence when treating these individuals, so further studies are necessary to elucidate this.

National cholesterol guidelines in several countries recommend using the same therapeutic targets for people with type 2 DM as those recommended for secondary prevention of coronary artery disease![12] In this respect, all adults with type 2 DM, irrespective of their age, are regarded as being at high risk of fatal or non-fatal coronary events. In 2005, the International Diabetes Federation published global guidelines suggesting that people with type 2 DM should be judged as being at a high risk of developing CVD if they were older than 40 years, even in the absence of preexisting CVD or coronary risk factors![20] The American Diabetes Association takes a similar approach![21] The UK National Institute for Health and Clinical Excellence uses risk assessment tables to select individuals with type 2 DM for primary prevention strategies![22] However, the relationship between age and risk of CVD in people with DM has not been fully elucidated.

On the other hand, predictive algorithms created from diabetic cohorts have shown that age is a strong predictor of IHD, but little is known about the absolute risk of these events in younger people with DM[23] Moreover, the appropriateness of existing age thresholds for the identification of people with DM who are at high risk of CVD is not known. Furthermore, in the present study, gender differences were not observed in relation to the incidence of events. In almost all clinical studies on hyperlipidemic patients, males are reported to suffer more from CVD than females. Although the high incidence in female diabetic individuals has been reported previously, there are few reports of such data in Japan. Furthermore, the incidence in females occurs from the sexagenarian onward.

In the present study patients who were prescribed antihyperlipidemic agents tended to suffer less from CVD or IHD at the same LDL levels. It is possible that medicated patients may be more conscious of their disease and their lifestyle. This is usually advocated in the interpretation of the preventive effect on IHD in postmenopausal women taking hormone replacement therapy (HRT) compared with women without HRT[24] However, it is also possible that drugs such as statins have a direct atheroprotective effect in patients with DM. Pleiotropic effects of antihyperlipidemic agents are often reported, and this may continue for several years[25,26] As the data are insufficient for further analyses, we cannot conclude what might affect the tendency of differences occurring in the incidence between medicated patients and non-medicated patients. However, we intend that this study will be carried out for more than 4 years, as planned.

Conclusion

A relationship was found between all HDL levels, or LDL levels and atherosclerotic events (cardiovascular, cerebrovascular and ASO events) especially in the elderly. The prescribed antihyperlipidemic agents tended to decrease the event ratio without any relationship with lipid levels, which suggests that vascular mechanisms, as well as lipid control, may be responsible for vascular events in diabetic individuals. The present study has the potential to provide evidence for lipid control in diabetic individuals, and may show what is important for the development of hyperlipidemia in the elderly. It also highlights possible future treatments of hyperlipidemia in Japanese diabetic individuals.

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References

1. Gotto AM. Lipid management in diabetic patients: Lessons from prevention trials. *Am J Med* 2002; **112**(Suppl 8A): 19S–26S.
2. Sone H, Mizuno S, Ohashi Y, Yamada N. Type 2 diabetes prevalence in Asian subjects. *Diabetes Care* 2004; **27:** 1251–1252.
3. Oikawa S, Kita T, Mabuchi H, Matsuzaki M, Matsuzawa Y, Nakaya N, et al; J-LIT Study Group. Risk of coronary events in Japanese patients with both hypercholesterolemia and type 2 diabetes mellitus on low-dose simvastatin therapy: Implication from Japan Lipid Intervention Trial (J-LIT). *Atherosclerosis* 2007; **191:** 440–446.
4. UMIN Clinical Trials Registry ID: UMIN000000516 Japan CDM. Available at: http://www.umin.ac.jp/ctr/index.htm.
5. Sasaki J, Iwashita M, Kono S. Statins: Beneficial or adverse for glucose metabolism. *J Atheroscler Thromb* 2006; **13:** 123–129.
6. Nakata M, Nagasaka S, Kusaka I, Matsuoka H, Ishibashi S, Yada T. Effects of statins on the adipocyte maturation and expression of glucose transporter 4 (SLC2A4): Implications in glycaemic control. *Diabetologia* 2006; **49:** 1881–1892.
7. Management of Elevated Cholesterol in the Primary Prevention Group of Adult Japanese (MEGA) Study Group. Design and baseline characteristics of a study of primary prevention of coronary events with pravastatin among Japanese with mildly elevated cholesterol levels. *Circ J* 2004; **68:** 860–867.
8. Hata Y, Mabuchi H, Saito Y, Itakura H, Egusa G, Ito H, et al; Working Committee on JAS Guideline for Diagnosis and Treatment of Hyperlipidemias. Report of the Japan Atherosclerosis Society (JAS) Guideline for Diagnosis and Treatment of Hyperlipidemia in Japanese adults. *J Atheroscler Thromb* 2002; **9:** 1–27.
9. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 1997; **20:** 1183–1197.
10. Kodama K, Sasaki H, Shimizu Y. Trend of coronary heart disease and its relationship to risk factors in a Japanese population: A 26-year follow-up: Hiroshima/Nagasaki study. *Jpn Circ J* 1990; **54:** 414–421.
11. Wakugami K, Iseki K, Kimura Y, Okumura K, Ikemiya Y, Muratani H, et al. Relationship between serum cholesterol and the risk of acute myocardial infarction in a screened cohort in Okinawa, Japan. *Jpn Circ J* 1998; **62:** 7–14.
12. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of the 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). *JAMA* 2001; **285:** 2486–2497.
13. Keech A, Simes RJ, Barter P, Best J, Scott R, Taskinen MR, et al; FIELD study investigators. Effects of long-term fenofibrate therapy on cardiovascular events in 9795 people with type 2 diabetes mellitus (the FIELD study): Randomized controlled trial. *Lancet* 2005; **366:** 1849–1861.
14. Kawai T, Tokui M, Funae O, Meguro S, Yamada S, Tabata M, et al. Efficacy of pitavastatin, a new HMG-CoA reductase inhibitor, on lipid and glucose metabolism in patients with type 2 diabetes. *Diabetes Care* 2005; **28:** 2980–2981.
15. Colhoun HM, Betteridge DJ, Durrington PN, Hitman GA, Neil HAW, Livingstone SJ, et al; CARDS Investigators. Rapid emergence of effect of atorvastatin on cardiovascular outcomes in the Collaborative Atorvastatin Diabetes Study (CARDS). *Diabetologia* 2005; **48:** 2482–2485.
16. Shepherd J, Blauw G, Murphy M, Bollen E, Buckley B, Cobbe S, et al; PROSPER study group. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): A randomized controlled trial. *Lancet* 2002; **360:** 1623–1630.
17. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: A randomized placebo-controlled trial. *Lancet* 2002; **360:** 7–22.
18. Sever PS, Poulter NR, Dahlöf B, Wedel H, Collins R, Beevers G, et al. Reduction in cardiovascular events with atorvastatin in 2,532 patients with type 2 diabetes: Anglo-Scandinavian Cardiac Outcomes Trial-lipid-lowering arm (ASCOT-LLA). *Diabetes Care* 2005; **28:** 1151–1157.
19. Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: The Scandinavian Simvastatin Survival Study (4S). *Lancet* 1994; **344:** 1383–1389.
20. IDF Clinical Guidelines Task Force. Global Guideline for Type 2 Diabetes: Recommendations for standard, comprehensive, and minimal care. *Diabet Med* 2006; **23:** 579–593.
21. American Diabetes Association. Consensus Panel: Guidelines for computer modeling of diabetes and its complications (Consensus Statement). *Diabetes Care* 2004; **27:** 2262–2265.
22. Marshall SM, Flyvbjerg A. Prevention and early detection of vascular complications of diabetes. *BMJ* 2006; **333:** 475–480.
23. Valensi P, Pariès J, Brulport-Cerisier V, Torremocha F, Sachs RN, Vanzetto G, et al. Predictive value of silent myocardial ischemia for cardiac events in diabetic patients: Influence of age in a French multicenter study. *Diabetes Care* 2005; **28:** 2722–2727.
24. Manson JE, Martin K. postmenopausal hormone-replacement therapy. *N Engl J Med* 2001; **345:** 34–40.
25. Tsunekawa T, Hayashi T, Kano H, Sumi D, Matsui-Hirai H, Thakur NK, et al. Cerivastatin, a hydroxymethylglutaryl coenzyme a reductase inhibitor, improves endothelial function in elderly diabetic patients within 3 days. *Circulation* 2001; **104:** 376–379.
26. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol* 2001; **21:** 1712–1719.

27. Kubo M, Kiyohara Y, Kato I, Tanizaki Y, Arima H, Tanaka K, et al. Trends in the incidence, mortality, and survival rate of cardiovascular disease in a Japanese community: The Hisayama study. *Stroke* 2003; **34:** 2349–2354.
28. Akisaki T, Sakurai T, Takata T, Umegaki H, Araki A, Mizuno J, et al. Cognitive dysfunction associates with white matter hyperintensities and subcortical atrophy on magnetic resonance imaging of the elderly diabetes mellitus Japanese elderly diabetes intervention trial (J-EDIT). *Diabetes Metab Res Rev* 2006; **22:** 376–3784.
29. Fox CS, Coady S, Sorlie PD, Levy D, Meigo JB, D'Agostino RB Sr, et al. Trends in cardiovascular complications of diabetes. *JAMA* 2004; **292:** 2495–2499.
30. Gaede P, Vedel P, Larsen N, Jensen GV, Parving HH, Pedersen O. Multifactorial intervention and cardiovascular disease in patients with type 2 diabetes. *N Engl J Med* 2003; **348:** 383–393.

Appendix 1

Major events are defined as follows.

1. Definite fatal and nonfatal myocardial infarction (1 or more of the following criteria must be met).
   (a) Diagnostic ECG at the time of the event.
   (b) Ischemic cardiac pain (and/or unexplained acute left ventricular failure) and diagnostic enzyme levels.
   (c) Ischemic cardiac pain and/or unexplained acute left ventricular failure with both equivocal enzyme levels and equivocal ECG.
   (d) Diagnostic enzyme levels and equivocal ECG.
   (e) Angiographic evidence of occlusion of a major artery with appropriate ventriculographic wall motion abnormality where previous angiogram since randomization showed no such abnormality.
   (f) Postmortem examination.
2. Angina pectoris (stable or unstable, both of the following criteria must be met).
   (a) Ischemic cardiac pain, relieved by nitrates.
   (b) Equivocal ECG.
3. Ischemic stroke (1 of the following conditions must be met).
   (a) Rapid onset of focal neurologic deficit lasting at least 24 h or leading to death, plus evidence from neuroimaging (computed tomography or magnetic resonance imaging) showing cerebral/cerebellar infarction or no abnormality, or postmortem examination showing cerebral and/or cerebellar infarction).
   (b) Rapid onset of global neurological deficit (eg, coma) lasting at least 24 h or leading to death, plus evidence from neuroimaging showing infarction, or postmortem examination showing infarction.
   (c) Focal neurological deficit (mode of onset uncertain) lasting at least 24 h or leading to death, plus evidence from neuroimaging showing infarction, or postmortem examination showing infarction.
4. Primary intracerebral hemorrhage (1 of the following conditions must be met).
   (a) Rapid onset of focal neurological deficit lasting at least 24 h or leading to death, plus neuroimaging or postmortem examination showing primary intracerebral and/or cerebellar hemorrhage.
   (b) Rapid onset of global neurologic deficit (eg, coma) lasting at least 24 h or leading to death, plus evidence from neuroimaging or postmortem examination showing primary intracerebral and cerebellar hemorrhage.
   (c) Focal neurologic deficit (mode of onset uncertain) lasting at least 24 h or leading to death, plus evidence from neuroimaging or postmortem examination showing primary intracerebral and/or cerebellar hemorrhage.
5. Atherosclerosis obliterans:
   (a) at least Fontaine Classification II
   (b) <0.9 (ankle–brachial pressure index)
   (c) positive vascular imaging.

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Involvement of glomerular SREBP-1c in diabetic nephropathy

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Abstract

The role of glomerular SREBP-1c in diabetic nephropathy was investigated. PEPCK-promoter transgenic mice overexpressing nuclear SREBP-1c exhibited enhancement of proteinuria with mesangial proliferation and matrix accumulation, mimicking diabetic nephropathy, despite the absence of hyperglycemia or hyperlipidemia. Isolated transgenic glomeruli had higher expression of TGFβ-1, fibronectin, and SPARC in the absence of marked lipid accumulation. Gene expression of P47phox, p67phox, and PU.1 were also activated, accompanying increased 8-OHdG in urine and kidney, demonstrating that glomerular SREBP-1c could directly cause oxidative stress through induced NADPH oxidase. Similar changes were observed in STZ-treated diabetic mice with activation of endogenous SREBP-1c. Finally, diabetic proteinuria and oxidative stress were ameliorated in SREBP-1-null mice. Adenoviral overexpression of active and dominant-negative SREBP-1c caused consistent reciprocal changes in expression of both profibrotic and oxidative stress genes in MES13 mesangial cells. These data suggest that activation of glomerular SREBP-1c could contribute to emergence and/or progression of diabetic nephropathy.

Keywords: Fatty acids; Triglycerides; Diabetes; Proteinuria; Kidney; TGF; Oxidative stress; NADPH oxidase

Diabetic nephropathy is an important and devastating consequence of diabetes, determining prognosis and quality of life of the affected patients. Recent epidemiological studies [1] have demonstrated that, in addition to glycemic control, serum cholesterol level should be a therapeutic target for prevention of diabetic nephropathy. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, transforming growth factor (TGF), and interleukin(IL)-1β, are induced in glomeruli by oxidized LDL and lipoprotein(a), and are involved in the transformation and matrix expansion of mesangial cells to leading to fibrosis [2]. These data suggest that disturbed lipid metabolism could impair renal function and be related to formation of diabetic nephropathy.

Increased oxidative stress has been also implicated in the development and progression of diabetic microvascular complications, including diabetic nephropathy. Oxidative stress has been shown to be enhanced in the kidneys of diabetic rats [3], and its blockade with antioxidants improved diabetes-induced renal injury [4]. It has been established that NADPH oxidase is the main source of reactive oxygen species (ROS) in non-phagocytic cells such as endothelial cells, smooth muscle cells, mesangial cells, podocytes, and fibroblasts [5–8]. Considering that accumulation of tissue lipids could be a source of oxidative stress, this lipotoxic process in the glomerulus could contribute to the patho-

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physiology in diabetic nephropathy. Furthermore, lipid synthesis per se also could contribute to the pathogenesis.

Sterol regulatory element-binding protein (SREBP)-1 is a transcription factor regulating lipid synthesis. The potential involvement of SREBP-1 in kidney diseases has been described in transgenic mice with PEPCK-driven expression of SREBP-1a, and in mice with obesity-related diabetes such as FVBdb/db, and diet-induced mice [9–13]. Activation of renal SREBP-1c and the resultant accumulation of TGs in the kidney was associated with increased expression of profibrotic growth factors, enhanced mesangial expansion with accumulation of extracellular matrix proteins, and proteinuria. These reports suggest contribution of SREBP-1c-mediated lipotoxicity to obesity-related nephropathy. However, the role of kidney SREBP-1c in diabetic nephropathy mainly caused by hyperglycemia has not been fully studied. We have reported that SREBP-1c is upregulated by hyperglycemia and insulin resistance, contributing to the pathophysiology of diabetes both in liver and pancreatic beta cells [14,15]. In the present study, we investigated effects of glomerular nuclear SREBP-1c on diabetic nephropathy using PEPCK-Tg-SREBP-1c and STZ-treated diabetic mice. Our data demonstrate that glomerular SREBP-1c enhanced oxidative stress through NADPH oxidase, and contributed to formation of diabetic nephropathy without marked lipid accumulation in glomeruli.

Methods

Animals. SREBP transgenic mice overexpressing human SREBP-1c under the control of the rat phosphoenolpyruvate carboxykinase promoter (PEPCK-TgSREBP-1c) were established as described [16]. SREBP-1 knockout mice were generated as previously described [17]. Mice were housed in colony cages and maintained on a 12-h light/12-h dark cycle. Mice were kept in metabolic cages for collection of 24 h urine for measurement of albumin and creatinine.

Streptozotocin (STZ)-induced diabetes. Insulin-depleted diabetes was induced by streptozotocin (100 mg/kg body weight, Sigma–Aldrich, St. Louis, USA). Mice in the STZ group received intraperitoneal injections of STZ twice weekly. Mice with blood glucose of greater than 300 mg/dl were considered diabetic. Animals were sacrificed 2 months after the induction of diabetes in the 12-h fasting state to induce the transgene of the PEPCK promoter. Mice were anesthetized and left atrium was cannulated and the kidneys perfused with ice-cold Hanks' Balanced Salt Solution (Invitrogen, NY, USA). Renal glomeruli were isolated by sieving with stainless steel and nylon meshes as previously described [18], for extraction of RNA. The remaining kidneys were used for staining with periodic acid Schiff (PAS) and periodic acid silver methenamine (PAM), and determination of TG content and 8-OHdG level. The animal experiments were approved by Animal Care Committee of the University of Tsukuba.

Histological analysis. Sections were stained with PAS for light microscopic observation. Thirty glomeruli from the cortical area of each kidney were observed and images were taken by a digital microscopy, BZ-8100 (Keyence, Osaka, Japan) and analyzed by using BZ-analyzer. The mesangial matrix areas excluding nuclei, which occupied the glomerular tuft, were analyzed and averaged.

Urine chemistries. Urine albumin concentration was determined using an ELISA Albuwell M kit (Exocell, Philadelphia, PA). Urine creatinine concentration was determined by creatinine via a Creatinine Companion kit (Exocell).

Determination of urinary 8-OHdG excretion and 8-OHdG levels in mitochondria DNA from kidney. Urinary 8-OHdG excretion and renal 8-OHdG content in mitochondrial DNA were determined as described [19] using competitive an ELISA kit (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan). Mitochondrial DNA were isolated from kidney with mtDNA Extractor CT Kit (Wako, Osaka, Japan).

Preparation of recombinant adenovirus. Adenoviral vectors of cDNAs encoding the active nuclear human SREBP-1c and dominant-negative SREBP-1 were as described [14].

Northern blot analysis. Total RNA was isolated from mouse livers and kidneys and culture cells using TRIzol reagent (Life Technologys, Inc.). Thirty-six hours after adenovirus infection, the mRNA of the mouse mesangial cell line MES13 (ATCC, Manassasu, USA) was extracted.

Real-time PCR. The expression of mRNA in isolated glomeruli was quantified by real-time PCR. The first strand cDNA was synthesized with ThermoScript (Invitrogen). Real-time fluorescent detection PCR was performed using ABI solute SYBR Green ROX Mix (NIPPON Genetics, Japan) on the ABI Prism 7000 PCR instrument (Applied Biosystems) to amplify samples. Primers of human SREBP-1c (transgene), mouse SREBP-1c, fatty acid synthase (FAS), stearoyl CoA desaturase (SCD)-1 [15], transforming growth factor-β1 (TGFβ1), fibronectin [20], type4 collagen α1 (4colα1) [21], p47phox and p67phox [22], and endothelial nitric oxide synthase (eNOS) [23] were previously described. The relative mRNA revels in each sample were normalized to 36B4 gene expression.

Results

PEPCK-TgSREBP-1c mice exhibit renal abnormalities mimicking diabetic nephropathy

Transgenic mice overexpressing nuclear (active) SREBP-1c under control of the PEPCK promoter (PEPCK-TgSREBP-1c) had urinary albumin excretion greater than control wild-type mice, indicating renal dysfunction due to activation of SREBP-1c (Fig. 1A). Light microscopic examination revealed that PAS staining and PAM staining were both enhanced in glomeruli of PEPCK-TgSREBP-1c mice (Fig. 1B). Mesangial matrix volume was quantified and significantly increased by overexpression of nSREBP-1c (Fig. 1C). These data demonstrate that SREBP-1c transgenic mice exhibit pathological changes in glomeruli with mesangial proliferation and mesangial matrix accumulation, mimicking diabetic nephropathy. Importantly, plasma glucose and glycohemoglobin were not changed. Plasma TGs and cholesterol were significantly decreased (Table 1). Thus, it is unlikely that the glomerular changes were attributed to alterations in plasma glucose or lipids, which have been implicated to be contributors for chronic kidney diseases [1,24].

As previously reported, expression of nuclear SREBP-1c from the PEPCK promoter was primarily in liver and weakly in kidney [16]. These mice exhibited hepato-steatosis caused by increased expression of SREBP-1c target lipogenic enzymes in the liver. In contrast, the SREBP-1c transgene is only modestly expressed in the kidney and activation of FAS is minimal (Fig. 1D). In contrast to the liver, TG content was decreased in kidneys of PEPCK-TgSREBP-1c mice. However, immuno-reactive human SREBP-1c was enhanced specifically in glomeruli (Fig. 1E). Gene expression analysis on isolated glomeruli

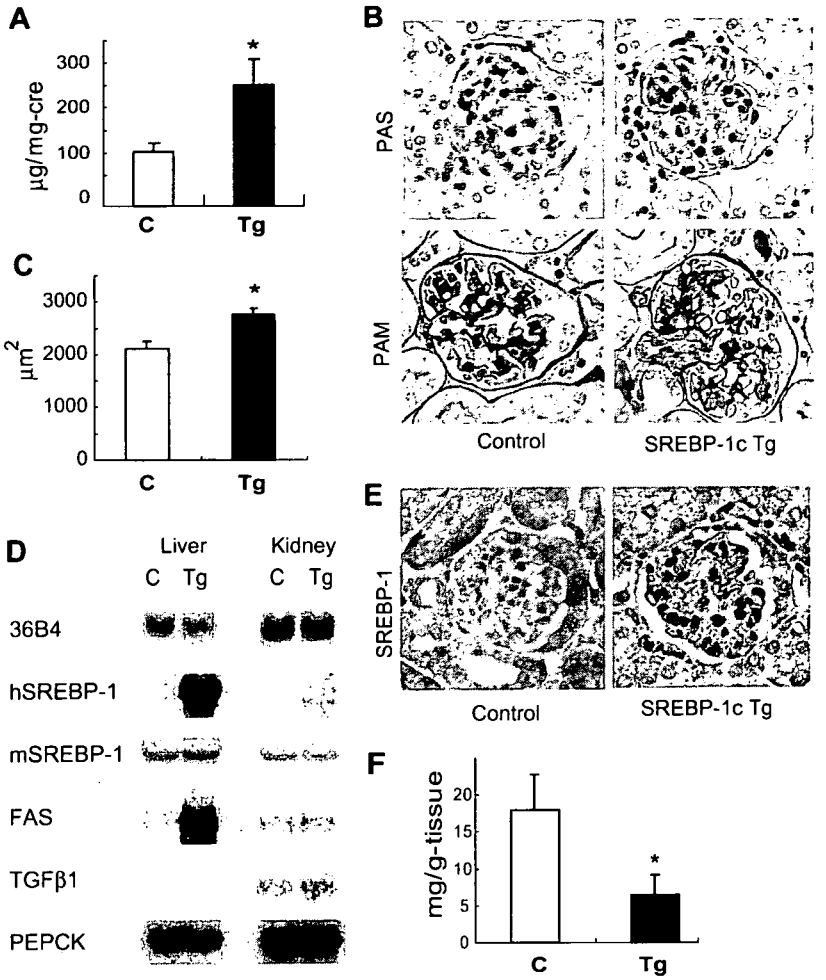

Fig. 1. Diabetic nephropathy-like glomeruli in in PEPCK-nSREBP-1cTg mice. (A) Urinary albumin excretion of control (C) and SREBP-1c transgenic mice (Tg). Data are means ± SE ($n = 16$). $^{*}P < 0.05$ vs. control mice. (B) Representative light microscopic appearance of glomeruli (PAS and PAM staining; original magnification 400×) for control and SREBP-1c transgenic mice. (C) Increase in mesangial matrix area in SREBP-1c transgenic mice. Data are means ± SE ($n = 30$). $^{*}P < 0.01$ vs. control mice. (D) Gene expression of 36B4, human SREBP-1, mouse SREBP-1, FAS, PEPCK, and TGFβ1 in liver and kidney as estimated by Northern blotting. (E) Immunoblotting of SREBP-1 in kidney. (original magnification 400×). (F) TG content of kidneys in SREBP-1c transgenic mice and control mice (eight samples at 12-h fasting in each group). $^{*}P < 0.05$ vs. control mice.

Table 1
Plasma metabolic parameters in STZ-treated and non-treated WT and PEPCK-TgSREBP-1c mice

	WT		PEPCK-TgSREBP-1c	
	Non-treat	STZ	Non-treat	STZ
TG (mg/dl)	213.9 ± 45	116.5 ± 40**	126.9 ± 72*	68.1 ± 12**
Choresterol (mg/dl)	148.0 ± 26	138.8 ± 19	117.1 ± 25	109.2 ± 32
NEFA (mEq/l)	1.18 ± 0.16	0.82 ± 0.23**	1.05 ± 0.27	0.65 ± 0.19**
Glucose (mg/dl)	168.3 ± 18	366.1 ± 110**	191.1 ± 14	356.2 ± 120**
HbA1c (%)	3.86 ± 0.33	8.5 ± 0.84**	3.66 ± 0.36	7.8 ± 1.40*

Data are means ± SE, $n = 8$ in each groups.
* $P < 0.05$ vs. WT.
** $P < 0.05$ vs. mice treated with STZ.

using RT-PCR confirmed overexpression of the transgene accompanied by increases in expression of target genes such as FAS and SCD-1 (Fig. 2). Consistent with glomerular proliferation and matrix accumulation, incremental increases in TGF-1beta, fibronection, and SPARC were also observed.

Overexpression of SREBP-1c induces NADPH oxidase in glomeruli

NADPH oxidase, a contributor to cellular oxidative stress has been reported to be increased in diabetic nephropathy of STZ-treated rats as a potential mechanism for the glomerular changes [25]. RT-PCR revealed that SREBP-1c-overexpressing glomeruli also had markedly higher expression of components of NADPH oxidase system, including P47 phox and p67 phox, compared to control mice (Fig. 2). PU.1, an upstream transcription factor of these genes, was also strongly activated. As a result, eNOS expression was repressed.

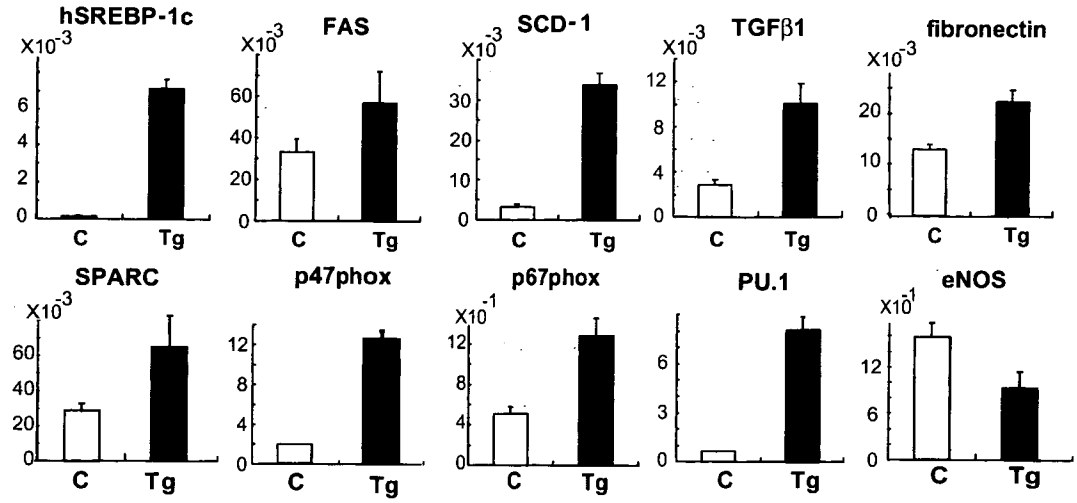

Fig. 2. Activation of glomerular NADPH oxidase in PEPCK-SREBP-1cTg mice. mRNA amounts of SREBP-1c target, growth factors, and NADPH oxidase subunit genes in isolated glomeruli from SREBP-1c transgenic and control mice were estimated by quantitative RT-PCR. 36B4 mRNA level was used as an internal control for each gene expression. Relative expression ratio to control is shown. Data are means ± SD ($n = 3$).

STZ-induced diabetes and activation of glomerular SREBP-1c had similar and connected effects on profibrotic and oxidative stress

PEPCK TgSREBP-1c mice were further treated with streptozocin (STZ) to super-impose diabetic changes on SREBP-1c-induced nephropathy. Two months after STZ-induced diabetes, urinary excretion was increased in both the STZ-treated control group and non-treated Tg groups to a similar level. STZ-treated SREBP-1c transgenic mice exhibited a trend toward a further, but not additive increase in proteinuria (Fig. 3A). Endogenous glomerular SREBP-1c expression in the kidney was elevated slightly in the STZ-treated control mice and robustly in the untreated SREBP-1c transgenic mice (Fig. 3B). Thus, total (transgene and endogenous) SREBP-1c expression was further enhanced in glomeruli of STZ-treated Tg mice. Type 4 collagen, TGFβ1, and fibronectin tended to be increased individually by STZ treatment and SREBP-1c overexpression (Fig. 3B). Combination of STZ treatment and SREBP-1c overexpression did not show additive effects. These data indicate that diabetic nephropathy and SREBP-1c-induced renal dysfunction are not independent processes. Oxidative stress has been postulated to be the mediator of the diabetic nephropathy [3,4,26,27]. The oxidative stress marker 8-OHdG, assessed by both urinary excretion and renal production, showed trends of increasing in STZ-diabetic mice and in the transgenic mice (Fig. 3C and D). The values were significantly elevated in STZ-treated PEPCK-TgSREBP-1c kidneys. Taken together, overexpression of glomerular SREBP-1c caused mesangial-proliferative glomerulonephropathy with activation of NADPH oxidase, mimicking diabetic glomerulonephropathy.

Amelioration of diabetic nephropathy in STZ-treated SREBP-1 knockout mice

To ascertain the role of SREBP-1c in diabetic nephropathy, SREBP-1 knockout mice were treated with STZ. Absence of SREBP-1c completely abolished the increase in urinary albumin excretion induced by diabetes in the STZ-treated mice, indicating the crucial role of SREBP-1c in diabetic nephropathy (Fig. 4A). The protection against nephropathy in kidneys from SREBP-1 null mice was associated with amelioration of diabetes-induced oxidative stress as measured by both urinary and kidney 8-OHdG (Fig. 4C and D). Meanwhile, TG content in the kidney was not different between groups (Fig. 4B).

Effects of SREBP-1c on oxidative and profibrotic genes in mesangial cells

To estimate the effects of SREBP-1c on glomerulus as observed in SREBP-1c transgenic mice in vitro, MES13 mesangial cells were subjected to modification of SREBP-1c expression. Adenoviral overexpression of nuclear SREBP-1c activated TGFβ1 whereas adenoviral dominant-negative SREBP-1c dose-dependently suppressed TGFβ1 expression (Fig. 4E). Gain and loss of function in SREBP-1c by these adenoviral vectors in the cultured cells were confirmed by up- and down-regulation of FAS expression, respectively. Expression of p47phox was dose-dependently activated by adenoviral SREBP-1c (Fig. 4F). These data support the notion that the gene expression pattern observed in glomeruli of PEPCK-TgSREBP-1c mice and STZ-induced diabetic mice was caused by SREBP-1c expression in mesangial cells.

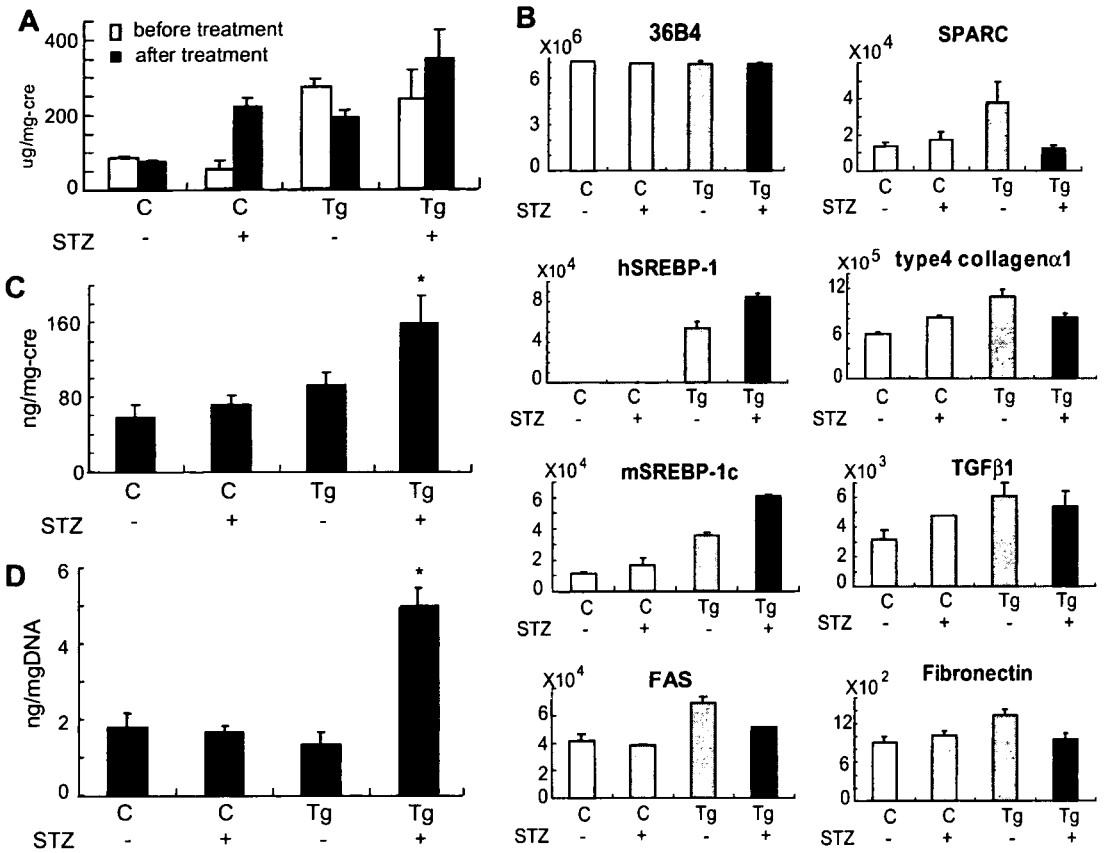

Fig. 3. Effects of STZ-induced diabetes on proteinuria, glomerular gene expression, and oxidative stress in PEPCK-TgSREBP-1c mice. Changes of urinary albumin levels (A), quantitative real-time PCR analysis of glomeruli (B), 8-OHdG levels in urine (C) and in mitochondria DNA from kidney (D) in STZ-treated (diabetic) and -untreated (non-diabetic) control and SREBP-1c transgenic mice. Amounts of molecular copies with 36B4 mRNA as an internal control are shown (means ± SE) ($n = 3$). Data for (A, C, and D) are means ± SE ($n = 8$). $^*P < 0.05$ vs. control mice.

Discussion

Our current study clearly demonstrates that SREBP-1c is involved in diabetic glomerulo-nephropathy. Transgenic overexpression of nuclear SREBP-1c caused mesangial proliferation, accumulation of PAS-, and PAM-positive extracellular matrix, leading to increased proteinuria. Blood glucose, glycohemoglobin, and lipids were not increased, and thus cannot explain the renal damage. Therefore, it is conceivable that renal changes were due to glomerular expression of SREBP-1c. The data from SREBP-1 KO and MES13 cell supported this conclusion.

Involvement of SREBP-1 in nephropathy has been shown in PEPCK-Tg-SREBP-1a mice and several obesity-induced insulin resistant mice such as db/db, ob/ob, and DIO mice [9–13]. These previous reports implicate that activation of renal SREBP-1a and/or SREBP-1c caused renal dysfunction through marked lipid accumulation in glomeruli. In contrast, our study focused on involvement of SREBP-1c specifically in diabetic nephropathy caused by hyperglycemia. Our current data implicate that hyperglycemia could induce glomerular SREBP-1c and contribute to nephropathy. PEPCK-TgSREBP-1c mice are good model for this. Considering that renal TG content was significantly reduced in this model, activation of SREBP-1c per se was responsible for the glomerulopathy through a mechanism other than lipid accumulation. The transcriptional activity of SREBP-1c is much weaker than that of SREBP-1a, explaining the difference in lipogenic gene activation between the two models. Intriguingly, oxidative stress processes were significantly activated by this level of SREBP-1c. The molecular mechanism, although discussed later, is currently unknown. It could be related to high oxygen consumption leading glomeruli to be vulnerable to oxidative stress.

It has been shown that activation of renal TGFβ1 is observed in various glomerular diseases as a final common pathway to renal fibrosis. Our data indicate that SREBP-1c activation is consistently associated with patho-histological fibrosis and TGFβ1 activation both in vivo and in vitro, accompanying induction of fibrosis markers such as collagen type IV, fibronectin, and SPARC. However, our preliminary data did not support that TGFβ1 is a direct gene target of SREBP-1c. A human TGFβ1 promoter construct

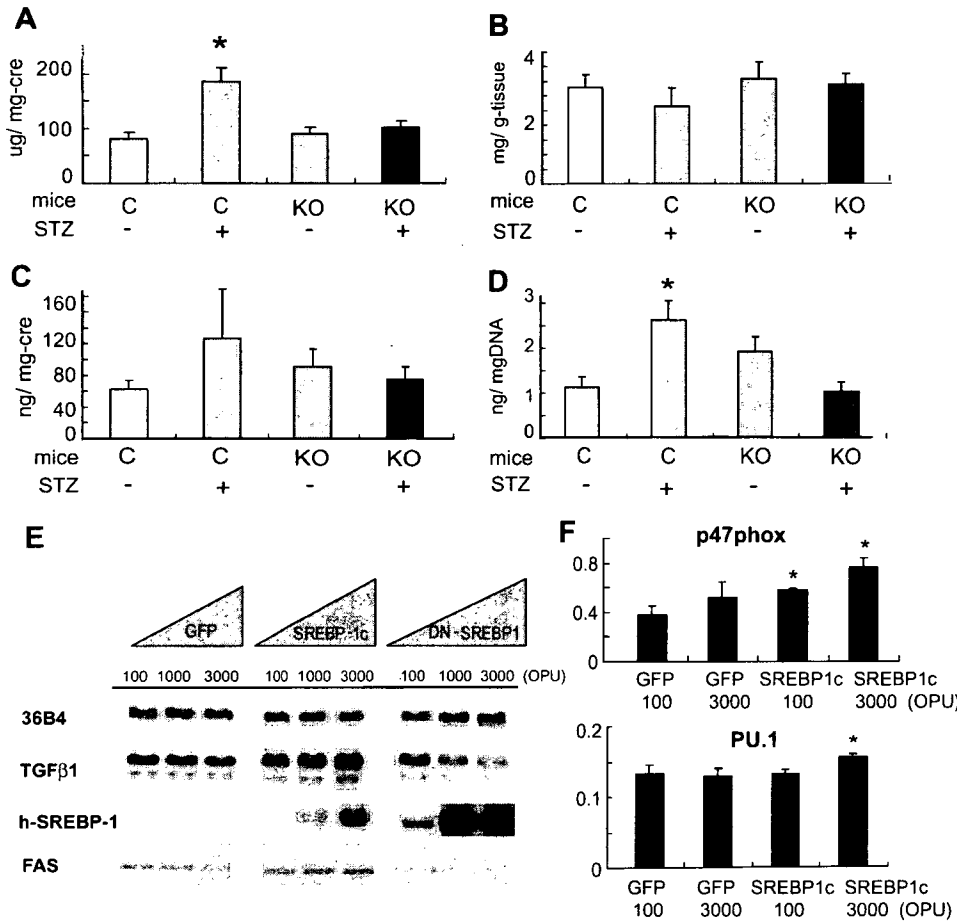

Fig. 4. Amelioration of diabetic nephropathy in STZ-teated SREBP-1-null mice (A–D) and Effect of SREBP-1c on TGFβ1 (E), p47phox and PU.1 (F) in mesangial cells. Changes of urinary albumin levels (A), TG contents of kidneys (B), Urinary 8-OHdG levels in urine (C) and mitochondria DNA from kidneys (D) in STZ-treated (diabetic) and -untreated (non-diabetic) wild control and SREBP-1KO mice ($n = 8$). Mice were sacrificed 6weeks after the induction of diabetes. Data are means ± SE. $^{*}P < 0.01$ vs. untreated control mice, untreated SREBP-1KO mice and SREBP-1KO mice treated with STZ. $^{*}P < 0.05$ vs. untreated control mice and SREBP-1KO mice treated with STZ. (E,F) Mesangial cell line, MES13 cells were infected with adenovirus containing cDNA for either GFP, SREBP-1c, or dominant-negative form SREBP-1 (DN-SREBP1). Expression of indicated genes was estimated by Northern blotting (E) and real-time PCR (F). 36B4 mRNA level was used as an internal control. Relative expression ratio to 36B4 is shown. Data are means ± SE ($n = 3$). $^{*}P < 0.05$ vs. GFP100OPU ($n = 3$).

containing the upstream regulatory elements, as previously reported [28], was not activated by co-transfection of the SREBP-1 expression plasmid in the luciferase reporter assays. However, we cannot rule out that activation of TGFβ1 could indirectly result from SREBP-1c-mediated oxidative stress in glomeruli.

The elevation of 8-OHdG levels in urine and kidney suggests that cellular stress, especially oxidative stress, may mediate the pathogenesis of SREBP-1c-mediated renal changes. It has also been reported that induction of reactive oxygen species is involved in diabetic nephropathy. The protective roles of SREBP-1 disruption against proteinuria, oxidative stress markers, and profibrotic gene expression in gloemrulus strongly support that SREBP-1c is deeply involved in this pathological process. We previously reported that overnutrition caused SREBP-1c activation and led to insulin resistance in the liver and insulin secretion defect in pancreatic β-cells through repression of IRS-2, PDX-1, and activation of granuphilin, respectively [14,15,29]. Here, in glomerulus, activation of SREBP-1c could affect expression of some unknown gene(s) enhancing oxidative stress in a process independent of lipid accumulation. PU-1/NADPH oxidase is the most likely SREBP-1c candidate target. Thus, we propose a novel hypothesis linking SREBP-1c to diabetic nephropathy in which hyperglycemia activates mesangial SREBP-1c, ROS, and mesangial proliferation and glomerular fibrosis leading to diabetic nephropathy. Any step of this sequence could be a therapeutic target for diabetic nephropathy. Further studies are needed to clarify the precise molecular mechanisms by which SREBP-1c is activated, and activates the NADPH oxidase-mediated oxidative stress pathway.

Acknowledgments

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References

[1] M. Ravid, D. Brosh, D. Ravid-Safran, Z. Levy, R. Rachmani, Main risk factors for nephropathy in type 2 diabetes mellitus are plasma cholesterol levels, mean blood pressure, and hyperglycemia, Arch. Intern. Med. 158 (1998) 998–1004.

[2] X.Z. Ruan, Z. Varghese, S.H. Powis, J.F. Moorhead, Human mesangial cells express inducible macrophage scavenger receptor, Kidney Int. 56 (1999) 440–451.

[3] M.L. Onozato, A. Tojo, A. Goto, T. Fujita, C.S. Wilcox, Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: effects of ACEI and ARB, Kidney Int. 61 (2002) 186–194.

[4] P.A. Craven, F.R. DeRubertis, V.E. Kagan, M. Melhem, R.K. Studer, Effects of supplementation with vitamin C or E on albuminuria, glomerular TGF-beta, and glomerular size in diabetes, J. Am. Soc. Nephrol. 8 (1997) 1405–1414.

[5] S.A. Jones, J.T. Hancock, O.T. Jones, A. Neubauer, N. Topley, The expression of NADPH oxidase components in human glomerular mesangial cells: detection of protein and mRNA for p47phox, p67phox, and p22phox, J. Am. Soc. Nephrol. 5 (1995) 1483–1491.

[6] S.A. Jones, V.B. O'Donnell, J.D. Wood, J.P. Broughton, E.J. Hughes, O.T. Jones, Expression of phagocyte NADPH oxidase components in human endothelial cells, Am. J. Physiol. 271 (1996) H1626–H1634.

[7] S. Greiber, T. Munzel, S. Kastner, B. Muller, P. Schollmeyer, H. Pavenstadt, NAD(P)H oxidase activity in cultured human podocytes: effects of adenosine triphosphate, Kidney Int. 53 (1998) 654–663.

[8] Y.A. Suh, R.S. Arnold, B. Lassegue, J. Shi, X. Xu, D. Sorescu, A.B. Chung, K.K. Griendling, J.D. Lambeth, Cell transformation by the superoxide-generating oxidase Mox1, Nature 401 (1999) 79–82.

[9] T. Jiang, S.E. Liebman, M.S. Lucia, J. Li, M. Levi, Role of altered renal lipid metabolism and the sterol regulatory element binding proteins in the pathogenesis of age-related renal disease, Kidney Int. 68 (2005) 2608–2620.

[10] T. Jiang, Z. Wang, G. Proctor, S. Moskowitz, S.E. Liebman, T. Rogers, M.S. Lucia, J. Li, M. Levi, Diet-induced obesity in C57BL/6J mice causes increased renal lipid accumulation and glomerulosclerosis via a sterol regulatory element-binding protein-1c-dependent pathway, J. Biol. Chem. 280 (2005) 32317–32325.

[11] G. Proctor, T. Jiang, M. Iwahashi, Z. Wang, J. Li, M. Levi, Regulation of renal fatty acid and cholesterol metabolism, inflammation, and fibrosis in Akita and OVE26 mice with type 1 diabetes, Diabetes 55 (2006) 2502–2509.

[12] L. Sun, N. Halaihel, W. Zhang, T. Rogers, M. Levi, Role of sterol regulatory element-binding protein 1 in regulation of renal lipid metabolism and glomerulosclerosis in diabetes mellitus, J. Biol. Chem. 277 (2002) 18919–18927.

[13] Z. Wang, T. Jiang, J. Li, G. Proctor, J.L. McManaman, S. Lucia, S. Chua, M. Levi, Regulation of renal lipid metabolism, lipid accumulation, and glomerulosclerosis in FVBdb/db mice with type 2 diabetes, Diabetes 54 (2005) 2328–2335.

[14] T. Ide, H. Shimano, N. Yahagi, T. Matsuzaka, M. Nakakuki, T. Yamamoto, Y. Nakagawa, A. Takahashi, H. Suzuki, H. Sone, H. Toyoshima, A. Fukamizu, N. Yamada, SREBPs suppress IRS-2-mediated insulin signalling in the liver, Nat. Cell Biol. 6 (2004) 351–357.

[15] A. Takahashi, K. Motomura, T. Kato, T. Yoshikawa, Y. Nakagawa, N. Yahagi, H. Sone, H. Suzuki, H. Toyoshima, N. Yamada, H. Shimano, Transgenic mice overexpressing nuclear SREBP-1c in pancreatic beta-cells, Diabetes 54 (2005) 492–499.

[16] H. Shimano, J.D. Horton, I. Shimomura, R.E. Hammer, M.S. Brown, J.L. Goldstein, Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells, J. Clin. Invest. 99 (1997) 846–854.

[17] H. Shimano, I. Shimomura, R.E. Hammer, J. Herz, J.L. Goldstein, M.S. Brown, J.D. Horton, Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a targeted disruption of the SREBP-1 gene, J. Clin. Invest. 100 (1997) 2115–2124.

[18] P.A. Harper, J.M. Robinson, R.L. Hoover, T.C. Wright, M.J. Karnovsky, Improved methods for culturing rat glomerular cells, Kidney Int. 26 (1984) 875–880.

[19] M. Kakimoto, T. Inoguchi, T. Sonta, H.Y. Yu, M. Imamura, T. Etoh, T. Hashimoto, H. Nawata, Accumulation of 8-hydroxy-2′-deoxyguanosine and mitochondrial DNA deletion in kidney of diabetic rats, Diabetes 51 (2002) 1588–1595.

[20] M. Fujimoto, Y. Maezawa, K. Yokote, K. Joh, K. Kobayashi, H. Kawamura, M. Nishimura, A.B. Roberts, Y. Saito, S. Mori, Mice lacking Smad3 are protected against streptozotocin-induced diabetic glomerulopathy, Biochem. Biophys. Res. Commun. 305 (2003) 1002–1007.

[21] T. Kobayashi, S. Uehara, T. Ikeda, H. Itadani, H. Kotani, Vitamin D3 up-regulated protein-1 regulates collagen expression in mesangial cells, Kidney Int. 64 (2003) 1632–1642.

[22] S. Furukawa, T. Fujita, M. Shimabukuro, M. Iwaki, Y. Yamada, Y. Nakajima, O. Nakayama, M. Makishima, M. Matsuda, I. Shimomura, Increased oxidative stress in obesity and its impact on metabolic syndrome, J. Clin. Invest. 114 (2004) 1752–1761.

[23] H. Ding, M. Hashem, C. Triggle, Increased oxidative stress in the streptozotocin-induced diabetic apoE-deficient mouse: changes in expression of NADPH oxidase subunits and eNOS, Eur. J. Pharmacol. 561 (2007) 121–128.

[24] H. Oda, W.F. Keane, Lipids in progression of renal disease, Kidney Int. Suppl. 62 (1997) S36–S38.

[25] M. Kitada, D. Koya, T. Sugimoto, M. Isono, S. Araki, A. Kashiwagi, M. Haneda, Translocation of glomerular p47phox and p67phox by protein kinase C-beta activation is required for oxidative stress in diabetic nephropathy, Diabetes 52 (2003) 2603–2614.

[26] P. Dandona, K. Thusu, S. Cook, B. Snyder, J. Makowski, D. Armstrong, T. Nicotera, Oxidative damage to DNA in diabetes mellitus, Lancet 347 (1996) 444–445.

[27] J.W. Baynes, S.R. Thorpe, Role of oxidative stress in diabetic complications: a new perspective on an old paradigm, Diabetes 48 (1999) 1–9.

[28] I. Kinjyo, H. Inoue, S. Hamano, S. Fukuyama, T. Yoshimura, K. Koga, H. Takaki, K. Himeno, G. Takaesu, T. Kobayashi, A. Yoshimura, Loss of SOCS3 in T helper cells resulted in reduced immune responses and hyperproduction of interleukin 10 and transforming growth factor-beta 1, J. Exp. Med. 203 (2006) 1021–1031.

[29] T. Kato, H. Shimano, T. Yamamoto, T. Yokoo, Y. Endo, M. Ishikawa, T. Matsuzaka, Y. Nakagawa, S. Kumadaki, N. Yahagi, A. Takahashi, H. Sone, H. Suzuki, H. Toyoshima, A.H. Hasty, S. Takahashi, H. Gomi, T. Izumi, N. Yamada, Granuphilin is activated by SREBP-1c and involved in impaired insulin secretion in diabetic mice, Cell Metab. 4 (2006) 143–154.

A Secreted Soluble Form of LR11, Specifically Expressed in Intimal Smooth Muscle Cells, Accelerates Formation of Lipid-Laden Macrophages

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Objective—Macrophages play a key role in lipid-rich unstable plaque formation and interact with intimal smooth muscle cells (SMCs) in early and progressive stages of atherosclerosis. LR11 (also called sorLA), a member of low-density lipoprotein receptor family, is highly and specifically expressed in intimal SMCs, and causes urokinase-type plasminogen activator receptor-mediated degradation of extracellular matrices. Here we investigated whether the secreted soluble form of LR11 (solLR11) enhances adhesion, migration, and lipid accumulation in macrophages using animal models and cultured systems.

Methods and Results—Immunohistochemistry showed solLR11 expression in thickened intima of balloon-denuded rat artery. Macrophage infiltration into the cuff-injured artery was markedly reduced in LR11-deficient mice. In vitro functional assays using THP-1–derived macrophages showed that solLR11 (1 μg/mL) significantly increased acetylated low-density lipoprotein uptake by THP-1 cells and cell surface levels of scavenger receptor SR-A 1.7- and 2.8-fold, respectively. SolLR11 dose-dependently increased the migration activity of THP-1 macrophages and adhesion to extracellular matrices 2.0- and 2.1-fold, respectively, at 1 μg/mL. These effects of solLR11 were almost completely inhibited by a neutralizing anti-urokinase–type plasminogen activator receptor antibody.

Conclusion—SolLR11, secreted from intimal SMCs, regulates adhesion, migration, and lipid accumulation in macrophages through activation of urokinase-type plasminogen activator receptor. The formation of lipid-laden macrophages in atherosclerotic plaques possibly is regulated by SolLR11 of intimal SMCs. (***Arterioscler Thromb Vasc Biol.* 2007;27:1050-1056.**)

Key Words: atherosclerosis ■ foam cells ■ macrophages ■ scavenger receptors ■ smooth muscle cells

The early recruitment of monocytes to the arterial neointima, their subsequent differentiation to macrophages, and lipid accumulation are key events in the pathogenesis of atherosclerosis.[1,2] Coincidentally, smooth muscle cells (SMCs) migrate and accumulate in the developing neointimal lesion, where intimal SMCs secrete extracellular matrices, such as elastin, collagen and proteoglycans, inflammatory cytokines, and several proteases.[3,4]

Recent functional studies using genetically modified animals or cells have revealed that certain receptors belonging to the family of low-density lipoprotein (LDL) receptor relatives (LRs) are important regulators of migration, proliferation, and secretory functions of SMCs.[5–10] We have demonstrated that LR11 is abundantly and specifically expressed in intimal SMCs during intimal thickening in a variety of experimental models of atherogenesis, and that its expression is elevated in early stages of neointimal formation.[11–13] LR11 enhances the migration of SMCs by increasing cell-surface urokinase-type plasminogen activator (uPA) receptor (uPAR) levels. LR11 is secreted in soluble form from isolated cultured SMCs, especially in the logarithmic growth phase, and tumor necrosis factor-α converting enzyme is responsible for the shedding of the large ectodomain of LR11.[14,15] This secreted soluble form of LR11 has biological activity toward SMC migration, different from that of the membrane-bound form.[11,16] This finding strongly suggested a solLR11-mediated interaction of intimal SMC and other players, particularly macrophages, in the intima. However, the role of intimal SMCs in the process of lipid accumulation in macrophages has not been well characterized.

The uPAR on monocytes/macrophages is implicated in the pathological infiltration of monocytes into the intima and in the process of foam cell formation.[17,18] Cell-surface expression of uPAR is significantly elevated in monocytes of subjects with acute myocardial infarction and contributes to enhanced cell adhesion in vitro.[17] In apoE$^{-/-}$ mice, overex-

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pression of human uPAR in macrophages enhances cell adhesion to the aortic wall,[18] and targeted overexpression of uPA, a ligand of uPAR, in macrophages accelerates atherosclerosis with increased foam cell formation.[19]

Thus, solLR11 might be expected to modify the macrophage foam cell formation through the activation of uPAR-mediated extracellular matrix degradation. Here we demonstrate the presence of solLR11 in hyperplastic intima, and show that solLR11 deficiency drastically reduces the infiltration of lipid-laden macrophages into the intima of $LR11^{-/-}$ mice on a high-fat diet using a cuff-injury model. Cell culture experiments showed that recombinant solLR11 increases the migration and adhesion of macrophages to extracellular matrix and SMCs through enhanced expression of adhesion molecules, as well as lipid accumulation through scavenger receptors. These results support a novel function of intimal SMCs in the regulation of macrophage-foam cell formation in the process of atherosclerosis.

Materials and Methods

Antibodies and Cells

Preparation and properties of the monoclonal and polyclonal antibodies against human and mouse LR11, 5-4-30-19-2 and pm11, respectively, were described previously.[11] Monoclonal antibodies against SR-A (KT022) was obtained from Wako (Tokyo, Japan). Polyclonal or monoclonal antibodies against uPAR (AF807), VLA-4 (BBA37) and P-selectin glycoprotein ligand (PSGL)-1 (MAB996) and recombinant platelet-derived growth factor (PDGF)-BB (520-BB) were from R&D systems (Minneapolis, Minn). Monoclonal antibody against Mac-3 was from BD Pharmingen (San Diego, Calif). Primary cultures of SMCs were prepared from the isolated medial layer of rat aortas as described.[20] COS7 cells were from ATCC (CRL-1651; Manassas, Va). THP-1 cells were obtained from ATCC (TIB-202) and maintained in RPMI 1640 containing 10% fetal bovine serum. THP-1 cells were differentiated to macrophages (THP-1 macrophages) by treatment with 200 nM of phorbol 12-myristate, 13-acetate (PMA; Promega, Madison, Wis) for 24 hours at 37°C in the presence or absence of purified solLR11 at 1 μg/mL (unless indicated otherwise) and/or of the indicated antibodies.

Animal Experiments

All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Ethics Board. Male Wistar rats (Charles River Laboratories, Chiba, Japan), weighing 400 to 450 grams, were anesthetized, and the left common carotid artery was denuded by ballooning as described.[21] The left carotid arteries were isolated at 7 or 14 days after injury and used for histochemical staining, immunohistochemistry and Western blot. Female $LR11^{-/-}$ and $LR11^{+/+}$ mice, aged ≈40 weeks fed a high-fat diet (Research Diets, Inc; 60 kcal% fat supplied from lard and soybean oil, 20 kcal% carbohydrate from sucrose and maltodextrin, and 20 kcal% protein from casein) from 3 days before surgery, were anesthetized, and the left femoral artery was sheathed with a polyethylene cuff made of PE90 tubing as described,[11] then maintained on high-fat diet. The left femoral arteries were isolated at 7 days after cuff placement and used for histochemical staining and immunohistochemistry.

Generation of Knockout Mouse

$LR11^{-/-}$ mice were generated as described (Jiang et al, submitted). Briefly, an LR11 targeting vector was constructed with short (3.3 kb) and long (4.4 kb) arms of homology and a Neo cassette (3.9 kb) to target the first exon of mouse LR11. Cultured embryonic stem cells were transfected with the LR11 targeting vector, homologous recombinant clones were selected with G418, and confirmed by Southern blotting. Germline-transmitted chimeras obtained were crossbred with C57BL6/J females, and resulting heterozygous offspring were interbred. Wild-type, heterozygous, and homozygous mutant mice were born in Mendelian ratios. All mice born were maintained under standard animal house conditions with a 12-hour light/dark cycle and were fed ad libitum with regular chow diet.

Immunohistochemistry and Western Blot

Serial paraffin-embedded sections (5 μm) were used for immunohistostaining as described.[12] Briefly, sections were pretreated with 3% H_2O_2 to inactivate endogenous peroxidase. Slides were then stained with anti-LR11 (pm11, 1:50) or anti-Mac3 (1:25) for 1 hour at 25°C in the presence of 0.1% bovine serum albumin. Vectastain ABC-AP kit (Vector Laboratories) was used with biotin-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies (Wako) according to the manufacturer's instructions. Slides were counterstained with hematoxylin-eosin and elastica van Gieson. Western blot analysis was performed as described previously[22] using anti-LR11 (pm11, 1:500), anti-VLA-4 (1:250), anti-SR-A (1:250) and anti-uPAR (1:250).

Construction, Expression, and Purification of SolLR11

Materials and Methods for this study are fully described in the online data supplement section (please see http://atvb.ahajournals.org). Briefly, we first constructed an expression plasmid for the soluble form of LR11 lacking 104 C-terminal amino acids containing the transmembrane region. COS7 cells were transfected with the expression construct and solLR11 was purified using Ni^{2+}-chelating chromatography. The biological activity of purified solLR11 was confirmed by a SMC migration assay.[13]

Adhesion and Migration

Cell adhesion was determined in 96-well plates as described.[23] Wells were coated with 5 μg/mL collagen or fibronectin for 2 hours at 37°C. THP-1 macrophages were fluorescently labeled by loading with Calcein-AM dye for 1 hour at 5×10^6 cells/mL in RPMI containing 1% fetal bovine serum. Calcein-loaded cells were then added to the extracellular matrix coated plates at 2.5×10^5 cells/well, and incubated for 30 minutes at 37°C. Nonadherent cells were removed by gently washing with phosphate-buffered saline, and adherent cells were analyzed by measuring fluorescence using a fluorescence microplate reader, SPECTRAmax GEMINI XS (Molecular Devices, Menlo Park, Calif). Cell migration was measured in a 96-well micro-Boyden chamber with collagen type I-coated filters as described.[13] The lower chamber contained RPMI 1640 with 5 ng/mL PDGF-BB, and THP-1 macrophages were added to the upper chamber and incubated for 4 hours at 37°C. Migrated cells were quantitated using a fluorescence microplate reader.

Acetyl-LDL Uptake

THP-1 macrophages were seeded on 96-well culture plates and incubated with the indicated concentrations of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated LDL (DiI-AcLDL) for 4 hours at 37°C. Then, unincorporated DiI-AcLDL was removed by washing with phosphate-buffered saline. DiI-AcLDL uptake was measured using a fluorescence microplate reader.

Statistics

The results are shown as mean±SD for each index. Comparison of data were performed using the Student t test or Williams test; $P<0.05$ was considered significant.

Results

LR11, Expressed in Intimal SMCs, Is Secreted as a Soluble Form in the Intima of Balloon-Denuded Artery

A soluble form of LR11 is secreted from cultured SMCs and induces the migration activity of SMCs together with the

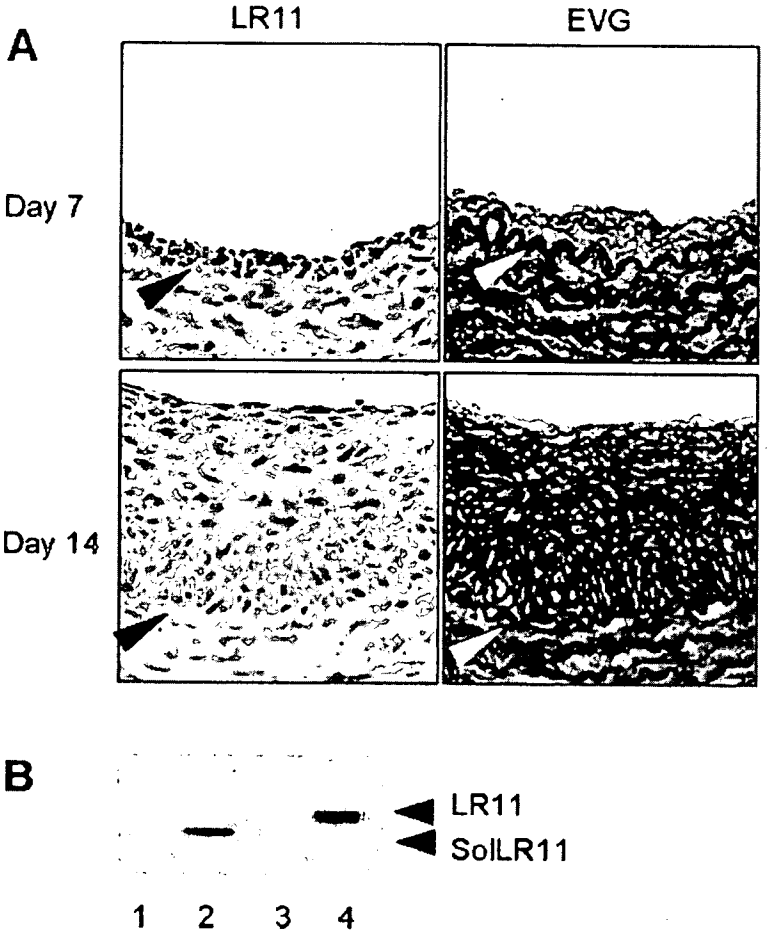

Figure 1. SolLR11 expression in intimal SMCs in balloon-denuded rat artery. A, Sections of balloon-denuded carotid artery were subjected to histological analysis using elastica van Gieson staining (EVG), and to immunohistochemistry with anti-LR11 antibody (pm11) at day 7 (top) and day 14 (bottom) after injury. Arrowheads indicate the internal elastic layers. B, Intima from day 14 balloon-denuded carotid artery was homogenized and analyzed by Western blotting with anti-LR11 antibody (pm11). Ln 1: mock/COS7; lane 2: solLR11/COS7; lane 3: medial layer extract; lane 4: intimal layer extract. Arrowheads indicate the full-length and truncated soluble LR11, respectively.

membrane-anchored form.[11] To investigate the pathophysiological relevance of solLR11 in the process of neointimal formation, the expression of soluble and membrane-anchored LR11 proteins were analyzed in the rat balloon injury model. Immunohistochemistry and Western blot showed that LR11 is highly and specifically expressed in intimal SMCs, and that its expression is higher at day 7 after injury than at day 14 (Figure 1A). This is in agreement with the finding that LR11 is specifically expressed in the proliferating phase of SMCs in culture.[11] Using the samples of thickend intima obtained at day 14, secreted solLR11 with reduced molecular size compared with that of membrane-bound LR11, was detected in intimal homogenates, as expected from the results in cultured SMCs (Figure 1B).

Macrophage Infiltration and Lipid Accumulation in Intima of Cuff-Injured Artery Is Inhibited in LR11 Knockout Mice

Blocking LR11's function by neutralizing antibody significantly reduced neointimal thickening in cuff-injured femoral artery in mice.[11] We have recently established LR11 knockout mice, in which the coronary arterial structure appears histopathologically normal (Jiang et al, submitted). To clarify the role of solLR11 in neointimal formation, we applied cuff injury in femoral artery in the $LR11^{-/-}$ mice on a high-fat diet. Infiltration of Mac3-positive macrophages and lipid accumulation in macrophages were detected at 7 days after cuff placement, and elastin-rich neointimal thickening was observed at day 28 in wild-type mice on a hig- fat diet (Figure 2). The intimal thickness at day 28 after cuff injury was significantly reduced in the $LR11^{-/-}$ mice compared with the mice on normal chow diet (Jiang et al, submitted). Surprisingly, infiltration of Mac3-positive and lipid-laden macrophages was significantly decreased in the SMC-rich early neointima. These data suggest that LR11 is involved in lipid accumulation and macrophage infiltration into the intima at an early stage of injury-induced neointimal formation.

Expression, Purification, and Biological Activity of Recombinant SolLR11

To investigate the mechanism of decrease in intimal lipid-laden macrophages after cuff injury, we analyzed the effect of solLR11 on macrophages using the established cell line, THP-1. Recombinant solLR11 was expressed using a COS7 expression system and purified by single step Ni^{2+}-chelating chromatography (supplemental Figure I, available online at http://atvb.ahajournals.org). The addition of purified recombinant solLR11 at 1, 10, and 100 μg/mL strongly increased the PDGF-induced migration activity of SMCs when compared with SMCs transfected with vector alone or vector containing full-length LR11 (supplemental Figure I). The enhancement of SMC's migrating activities by LR11s were completely blocked by anti-LR11 antibody.