lipid concentrations and the incidence of CHD under low-dose simvastatin treatment in subjects with a history of CHD. We adopted a surveillance model for the study because patients already had a history of coronary disease and thus placebo control was not possible for ethical and feasibility reasons.

Simvastatin reduces the serum TC and LDL-C concentrations and increases high-density lipoprotein cholesterol (HDL-C) concentration in patients with hypercholesterolemia, and without a history of CHD, and a relationship exists between the serum lipid concentrations and the relative risk of coronary events[14] In this report, we examine the relationship between serum lipid concentrations and the incidence of coronary events under low-dose simvastatin treatment in patients with previous CHD.

Methods

Study Design

The design of the J-LIT study has been described previously;[15] but briefly this study involved 6,500 general practitioners throughout Japan and enrolled 52,421 patients; men aged 35–70 years and postmenopausal women aged under 70 years, with a TC concentration ≥220 mg/dl. Of those enrolled, patients with documented CHD[16] (ICD codes I 20 to I 25 and a history of coronary intervention) at the time of enrollment were selected for the secondary prevention cohort study. Exclusion criteria included a recent acute myocardial infarction (MI) or stroke within the past month, uncontrolled diabetes mellitus, serious concomitant hepatic or renal disease, secondary hypercholesterolemia, malignancy or any illness with a poor prognosis. Patients were selected from throughout Japan and received open-labeled simvastatin 5–10 mg/day for 6 years. The lipid concentration, adverse events and CHD-related events were monitored. Another lipid-lowering agent was permitted if the serum TC concentration did not respond adequately to simvastatin alone. The primary endpoints were coronary events, including acute MI[17] and sudden cardiac death. The secondary endpoints were other cardiac events such as deterioration of angina pectoris indicated by hospitalization or the requirement for coronary intervention. All CHD events during the study period were assessed by the Endpoint Classification Committee. Each patient was informed of the study purpose, as well as drug efficacy and the need for long-term treatment.

Statistical Analysis

For baseline patient characteristics, the study group was divided into 5 subgroups based on the average serum TC concentration during the treatment. The relationship between baseline characteristics and average TC concentration during treatment was analyzed with a trend test. For risk of coronary events, patients were stratified according to average lipid concentrations (TC, LDL-C, triglyceride (TG) and HDL-C) during the treatment period and according to the ratio of LDL-C/HDL-C during that time. Relative risks with a 95% confidence interval (CI) for the primary and secondary endpoints were calculated using the Cox proportional-hazard model[18] with adjustment for baseline characteristics (gender, age, hypertension, diabetes mellitus, smoking habit, and a history of MI). We excluded 74 patients because data on their smoking habit was not available. Continuous data are expressed as average ± SD. For all statistical analysis, $p<0.05$ was considered significant. All statistical calculations were performed using SAS software (V.6.12, SAS Institute Inc, Cary, NC, USA).

Results

Follow-up

Of the 52,421 patients enrolled, 5,127 were screened for the secondary prevention cohort study[15] In the present study, data collected from 4,673 patients were analyzed and data from 454 patients were excluded for the following reasons: lack of follow-up data (93), violation of inclusion/exclusion criteria (5), unwillingness to participate (1), and incomplete covariance (355). The average length of follow-up was 5.3 years per subject and after 6 years there were 3,348 patients remained. In 6 years, 203 patients died.

Table 1 Baseline Characteristics in the Subgroups of Patients Classified by Serum Total Cholesterol (TC) Concentration During Simvastatin Treatment

	TC (mg/dl)						
	<180	180–199	200–219	220–239	≥240	p value	Total
n	491	1,095	1,351	946	716		4,599
Male gender (%)	60.9	46.5	40.3	36.7	33.9	*	42.2
Age (years)	60.9±6.6	60.7±6.9	60.2±6.9	59.8±7.0	58.9±7.5	*	60.1±7.0
Obesity (%)	38.4	35.8	33.9	38.9	41.9	*	37.1
Hypertension (%)	49.3	48.7	46.0	47.4	47.3		47.5
Diabetes mellitus (%)	22.4	18.4	15.9	16.3	22.9		18.4
Cerebrovascular disease (%)	4.5	3.3	3.3	3.9	4.5		3.7
Renal disease (%)	3.9	3.6	1.6	1.7	2.7	*	2.5
Hepatic disease (%)	7.5	7.1	6.7	7.5	8.7		7.3
History of MI (%)	39.3	26.7	21.8	21.2	19.8	*	24.4
ECG abnormality (%)	69.7	71.5	69.7	70.1	73.3		70.8
Family history of CHD (%)	11.2	9.4	9.2	10.1	12.2		10.1
Smoking habit (%)	23.2	16.7	15.9	14.7	18.0	*	17.0
Alcohol consumption (%)	39.0	33.4	30.6	30.6	28.7	*	31.9
TC (mg/dl)	249±22	255±23	261±24	271±31	290±38	*	265±30
LDL-C (mg/dl)	168±23	170±26	176±28	183±33	202±40	*	179±32
TG (mg/dl)	194±183	186±118	183±116	204±151	216±180	*	194±144
HDL-C (mg/dl)	47.1±13.3	50.3±14.4	51.6±15.1	51.3±15.2	51.6±15.6	*	50.7±14.9

*Obesity, body mass index ≥25 kg/m²; MI, myocardial infarction; CHD, coronary heart disease; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglycerides. p value for trend test, *<0.05.*

The relationships between baseline patient characteristics and average serum TC concentration were analyzed with a trend test when patients were grouped according to their average serum TC concentration during treatment. The percentage of male patients, age, incidence of renal disease, and percentage of smokers and drinkers at baseline decreased proportionally as the average serum TC concentration increased. On the other hand, the percentage of obesity increased as the average serum TC concentration increased (Table 1).

Changes in Serum Lipid Levels With Simvastatin

Serum concentrations of TC, LDL-C and TG decreased significantly from baseline (265, 179 and 194 mg/dl, respectively) to 213 (19.6%), 130 (27.3%), and 167 (13.9%) mg/dl, respectively, after 6 months of treatment. Those concentrations were well-controlled for 6 years and were reduced to 211, 125, and 154 mg/dl, respectively, at the end of the study (Fig 1). The mean serum HDL-C concentration increased from 50.7 mg/dl (pretreatment) to 51.8 mg/dl after 6 months of treatment, and gradually increased to 56.1 mg/dl after 6 years of treatment. Over the course of the study, the average reductions in serum TC, LDL-C, and TG concentrations were 19.8±10.5%, 28.6±15.6%, and 15.9±40.1%, respectively, and the average increase in the serum HDL-C concentration was 4.7±25.0%.

Relationship Between the Risk of Coronary Events and Lipid Concentrations During Treatment

During the 6 years of treatment, 110 patients developed coronary events (primary endpoint), and the rate of incidence was 4.45 events per 1,000 patients-year (Table 2):

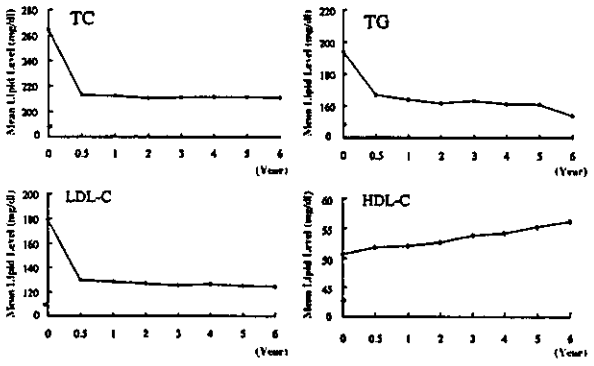

Fig 1. Sequential changes in serum lipid levels in hypercholesterolemic patients with a history of CHD who were maintained on low-dose simvastatin.

non-fatal MI (67 patients), fatal MI (38 patients), and sudden cardiac death (5 patients). Deterioration of angina pectoris (secondary endpoint) occurred in 95 patients. Overall, coronary heart disease occurred in 205 patients and the rate was 8.29 per 1,000 patients-year.

The risk of coronary events was a function of the average LDL-C concentration and inversely related to that of HDL-C during treatment (Table 3). No correlation existed between the relative risk of coronary events and the TC concentration in patients with TC concentration <240 mg/dl. However, the relative risk increased in patients whose average TC concentration was ≥240 mg/dl. The average TG concentration did not correlate with the risk of coronary events. Patients with an HDL-C concentration <40 mg/dl had a higher risk of coronary events compared with those who had a HDL-C concentration from 40 to 49 mg/dl. The risk of coronary events was lower in patients with HDL-C concentration ≥60 mg/dl than in patients with a concentration of 40–49 mg/dl. Each 10 mg/dl decrease in LDL-C and each 10 mg/dl increase in HDL-C lowered the relative risk of coronary events by 8.0% (95% confidence interval 3.8–12.0) and 28.3% (95% CI 13.9–40.3), respectively.

Relationship Between the Risk of Coronary Events and Baseline Patient Characteristics

Of the baseline characteristics, male patients had a higher risk for coronary events, with a relative risk of 2.61, compared with female patients (Fig 2). Age and obesity (body mass index ≥25 kg/m^2) did not affect the incidence of coronary events. Diabetes mellitus and a history of MI (relative risk, 2.76) increased the incidence of coronary events as well as smoking (relative risk, 1.41; p=0.133). Alcohol consumption decreased the risk and possibly protected patients. Hypertension did not affect the risk of coronary events and although renal disease tended to increase the risk, it was not statistically significant.

Discussion

The present study monitored 5,127 patients with hypercholesterolemia and a previous history of CHD for 6 years to examine the relation between serum lipid concentrations and the recurrence of coronary events. Patients were maintained on low-dose simvastatin (5–10 mg/day) under ordinary clinical care. The accumulated treatment term was approximately 24,747 patients-year. After 6 months of treatment, the serum concentrations of TC and LDL-C were lower than the baseline values, and the HDL-C concentration was higher. HDL-C concentration continued to increase during the study period. This pattern of changes in lipid concentrations during treatment was similar in that

Table 2 Incidence of Coronary Heart Disease (CHD) in Patients With Hypercholesterolemia and a History of CHD During the 6-Year Low-Dose Simvastatin Treatment Study

	No. of patients	*Incidence rate (/1,000 patients-year)*
Primary end point (coronary events)	*110*	*4.45*
MI (nonfatal)	*67*	*2.71*
MI (fatal)	*38*	*1.54*
Cardiac sudden death	*5*	*0.20*
Secondary end point	*95*	*3.84*
Angina pectoris (definite)	*95*	*3.84*
Total	*205*	*8.29*

MI, myocardial infarction.

Table 3 Risk of Coronary Events and Serum Lipids Concentration During the 6-Year Low-Dose Simvastatin Treatment Study in Patients With Hypercholesterolemia

	Study population	*No. of events*	*Relative risk*	*95% CI*	*p value*
TC (mg/dl)					
<180	*491*	*11*	*0.68*	*(0.34–1.38)*	*0.29*
180–199	*1,095*	*27*	*1.00*		
200–219	*1,351*	*26*	*0.91*	*(0.53–1.56)*	*0.72*
220–239	*946*	*20*	*1.07*	*(0.60–1.92)*	*0.81*
≥240	*716*	*21*	*1.65*	*(0.92–2.94)*	*0.09*
LDL-C (mg/dl)					
<100	*643*	*9*	*0.70*	*(0.32–1.54)*	*0.38*
100–119	*1,237*	*21*	*1.00*		
120–139	*1,362*	*34*	*1.61*	*(0.94–2.78)*	*0.08*
140–159	*789*	*21*	*1.95*	*(1.06–3.58)*	*<0.05*
≥160	*534*	*17*	*2.27*	*(1.19–4.32)*	*<0.05*
TG (mg/dl)					
<100	*854*	*21*	*1.36*	*(0.78–2.37)*	*0.28*
100–149	*1,611*	*31*	*1.00*		
150–249	*1,628*	*41*	*1.16*	*(0.73–1.86)*	*0.53*
≥250	*504*	*12*	*1.03*	*(0.53–2.03)*	*0.93*
HDL-C (mg/dl)					
<40	*669*	*32*	*1.60*	*(0.99–2.58)*	*0.06*
40–49	*1,417*	*36*	*1.00*		
50–59	*1,261*	*24*	*0.87*	*(0.52–1.47)*	*0.61*
≥60	*1,252*	*13*	*0.58*	*(0.31–1.11)*	*0.09*
LDL-C/HDL-C					
<2.0	*1,241*	*11*	*0.75*	*(0.34–1.63)*	*0.47*
2.0–2.4	*1,120*	*15*	*1.00*		
2.5–2.9	*898*	*26*	*2.18*	*(1.15–4.11)*	*<0.05*
3.0–3.4	*655*	*22*	*2.40*	*(1.24–4.62)*	*<0.01*
≥3.5	*651*	*28*	*3.05*	*(1.63–5.72)*	*<0.001*

CI, confidence intervals; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol.

observed in patients without a previous history of CHD![4]

During the 6 years of treatment, 110 patients developed CHD and the incidence of recurrent CHD was 4.45 events per 1,000 patients-years. We found in our primary prevention cohort study that the serum TC and LDL-C concentrations positively correlated and that of serum HDL-C inversely correlated with the risk of CHD in patients without a history of CHD![4] In the primary prevention cohort study, 209 patients developed coronary events, including fatal MI (51 patients), non-fatal MI (147 patients) and sudden cardiac death (11 patients). The incidence of coronary events in patients with a history of CHD was 5-fold higher than in patients without the history; the incidence was 4.45 per 1,000 patients-year for those with a history of CHD and 0.91 in patients without CHD.

Early studies established that an elevated TC concentration was an independent risk factor for CHD and death![1–4] However, in the present study, patients with a TC concentration ≥240 mg/dl developed CHD more often than patients with TC <240 mg/dl. The relationship between the TC concentration and the risk of coronary events was less clear wheres there was a strong relationship between the risk of coronary events and the LDL-C or HDL-C concentration. The serum LDL-C concentration positively correlated and serum HDL-C inversely correlated with the incidence of coronary events. The observation in TC concentration may be the result of opposite effects on coronary events influenced by the 2 lipoprotein-cholesterol components of TC because it has been established that LDL-C is a risk factor and HDL-C is, inversely, a negative risk factor for CHD?[2,19,20] In our previous primary prevention cohort study, TG concentration was a risk factor for coronary events, although the association was not strong. In the present study, serum TG concentration was not a risk factor for coronary events. The LDL-C/HDL-C ratio proportionally correlated with the incidence of coronary events, which was consistent with data from the primary prevention cohort study![4] The present results suggest that monitoring TC, LDL-C and HDL-C concentrations is crucial in the prevention of CHD progression. We also analyzed the risk of coronary events in 5 subgroups divided by equal number of subjects (about 920 patients in each group) along the average concentration of serum lipids during the study. The

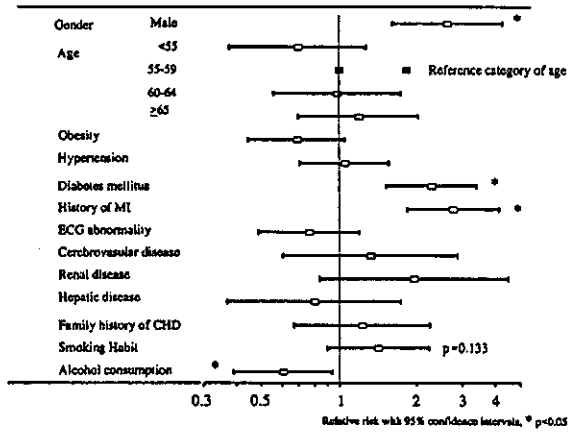

Fig 2. Correlation between the risk of coronary events and baseline characteristics of patients with a history of CHD treated by simvastatin therapy. Bars express the relative risk with 95% confidence intervals. *p<0.05. ECG, electrocardiogram; MI, myocardial infarction; Obesity, body mass index ≥25 kg/m^2.

occurrence of coronary events in relation to lipid concentration in this analysis was confirmed to be similar with the results obtained when subgroups were divided by a constant interval of serum lipid concentrations.

Other risk factors for coronary events included male patients, history of MI, diabetes mellitus, and smoking. Hypertension was not a risk factor in this study, probably because of stricter management of patient blood pressure. To elucidate further, analysis of the relationship between coronary events and patient blood pressure during treatment would be necessary. For patients who have any of these risk factors, especially diabetes mellitus, normalizing the lipid concentrations is important?[1-26]

Because the J-LIT study was conducted under the usual clinical conditions in a target population of patients throughout Japan, our findings can be reasonably extrapolated to the general Japanese population. We conclude from the data that serum choresterol concentrations relate to the incidence of coronary events in hypercholesterolemic patients under low-dose simvastatin treatment. A reasonable treatment strategy to prevent coronary events in Japanese hypercholesterolemic patients with prior CHD under low-dose statin might be regulating the serum lipid concentration to at least less than 120 mg/dl for LDL-C and more than 40 mg/dl for HDL-C.

Acknowledgments

We are grateful to Dr Heizo Tanaka, National Institute of Health and Nutrition, for expert statistical analysis of the data. This study was supported in part by a grant from the Banyu Pharmaceutical Co, Ltd, Tokyo, Japan.

References

1. National Cholesterol Education Program. Second report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II). *Circulation* 1994; **89:** 1333–1445.
2. Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels: The Framingham Study. *JAMA* 1986; **256:** 2835–2838.
3. Stamler J, Wentworth D, Neaton JD. Is the relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356,222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). *JAMA* 1986; **256:** 2823–2828.
4. Keys A, Menotti A, Aravanis C, Blackburn H, Djordevic BS, Buzina R, et al. The Seven Countries Study: 2,289 deaths in 15 years. *Prev Med* 1984; **2:** 141–154.
5. Pedersen TR, Wilhelmsen L, Faergeman O, Strandberg TE, Thorgeirsson G, Troedsson L, et al. and Scandinavian Simvastatin Survival Study Group: Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: The Scandinavian Simvastatin Survival Study (4S). *Lancet* 1994; **344:** 1383–1389.
6. Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, et al. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels: Cholesterol and Recurrent Events Trial investigators. *N Engl J Med* 1996; **335:** 1001–1009.
7. Tonkin A, Aylward P, Colquhoun D, Glasziou P, Harris P, Hunt D, et al. and the Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) study group. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels: The Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *N Engl J Med* 1998; **339:** 1349–1357.
8. Sugimoto K, Chikamori T, Kitaoka H, Furuno T, Seo H, Takata J, et al. Efficacy of cholesterol lowering therapy on the prognosis of Japanese patients with coronary heart disease. *J Noninvasive Cardiol* 1999; **3:** 86–90.
9. Todd PA, Goa KL. Simvastatin: A review of its pharmacological properties and therapeutic potential in hypercholesterolemia. *Drugs* 1990; **40:** 584–607.
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. Okayama A, Ueshima H, Marmot MG, Nakamura M, Kita Y, Yamakawa M. Changes in total serum cholesterol and other risk factors for cardiovascular disease in Japan 1980–1989. *Int J Epidemiol* 1993; **22:** 1038–1047.
13. Investigating Committee on Guidelines for Diagnosis and Treatment of Hyperlipidemias, Japan Atherosclerosis Society. Guidelines for diagnosis and treatment of hyperlipidemias in adults. *Jpn J Atheroscler* 1997; **25:** 1–34.
14. Matsuzaki M, Kita T, Mabuchi H, Matsuzawa Y, Nakaya, N, Oikawa S, et al. and the J-LIT Study Group. Large scale cohort study on the relationship between serum cholesterol concentration and coronary events under low-dose simvastatin therapy in Japanese patients with hypercholesterolemia: Primary Prevention Study of the Japan Lipid Intervention Trial (J-LIT). *Circ J* 2002; 66 (accepted for publication).
15. Matsuzawa Y, Itakura H, Kita T, Mabuchi H, Matsuzaki M, Nakaya N, et al. and the J-LIT Study Group: Design and baseline characteristics of a cohort study in Japanese patients with hypercholesterolemia: The Japan Lipid Intervention Trial (J-LIT). *Curr Ther Res* 2000; **61:** 219–243.
16. International Statistical Classification of Diseases and Related Health Problems, Vol. 1, 10th Revision. Geneva: World Health Organization, 1992; 471–513.
17. WHO MONICA Project. MONICA Manual, revised edn. Geneva: Cardiovascular Disease Unit, WHO, 1990.
18. Cox DR. Regression methods of life tables (with discussion). *JR Stat Soc* 1972; **B34:** 187–220.
19. Miwa K, Nakagawa K. Risk factors that discriminate 'high-risk' from 'low-risk' Japanese patients with coronary artery disease. *Jpn Circ J* 2000; **64:** 825–830.
20. Saku K, Zhang B, Liu R, Shirai K, Arakawa K. Associations among serum lipoprotein(a) levels, apolipoprotein(a) phenotypes, and myocardial infarction in patients with extremely low and high levels of serum lipoprotein(a). *Jpn Circ J* 1999; **63:** 659–665.
21. Jarrett RJ, McCartney P, Keen H. The Bedford survey: Ten year mortality rates in newly diagnosed diabetics, borderline diabetics and normoglycaemic controls and risk indices for coronary heart disease in borderline diabetics. *Diabetologia* 1982; **22:** 79–84.
22. Butler WJ, Ostrander LD Jr, Carman WJ, Lamphiear DE. Mortality from coronary heart disease in the Tecumseh study: Long-term effect of diabetes mellitus, glucose tolerance and other risk factors. *Am J Epidemiol* 1985; **121:** 541–547.
23. Borch-Johnsen K, Kreiner S. Proteinuria: Value as predictor of cardiovascular mortality in insulin dependent diabetes mellitus. *BMJ* 1987; **294:** 1651–1654.
24. Krolewski AS, Kosinski EJ, Warram JH, Leland OS, Busick KJ, Asmal AC, et al. Magnitude and determinants of coronary artery disease in juvenile-onset, insulin-dependent diabetes mellitus. *Am J Cardiol* 1987; **59:** 750–755.
25. Jarrett RJ, Shipley MJ. Type 2 (non-insulin-dependent) diabetes mellitus and cardiovascular disease: Putative association via common antecedents: Further evidence from the Whitehall Study. *Diabetologia* 1988; **31:** 737–740.
26. Morrish NJ, Stevens LK, Head J, Fuller JH, Jarrett RJ, Keen H. A prospective study of mortality among middle-aged diabetic patients (the London Cohort of the WHO Multinational Study of Vascular Disease in Diabetics). II: Associated risk factors. *Diabetologia* 1990; **33:** 542–548.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 11, Issue of March 14, pp. 9327–9331, 2003
Printed in U.S.A.

Vortex-mediated Mechanical Stress Induces Integrin-dependent Cell Adhesion Mediated by Inositol 1,4,5-Trisphosphate-sensitive Ca^{2+} Release in THP-1 Cells*

Received for publication, December 4, 2002, and in revised form, January 7, 2003
Published, JBC Papers in Press, January 7, 2003, DOI 10.1074/jbc.M212316200

Noboru Ashida‡, Hajime Takechi‡, Toru Kita§, and Hidenori Arai‡¶

From the Department of ‡Geriatric and §Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, 606-8507, Japan

In the downstream regions of stenotic vessels, cells are subjected to a vortex motion under low shear forces, and atherosclerotic plaques tend to be localized. It has been reported that such a change of shear force on endothelial cells has an atherogenic effect by inducing the expression of adhesion molecules. However, the effect of vortex-induced mechanical stress on leukocytes has not been investigated. In this study, to elucidate whether vortex flow can affect the cell adhesive property, we have examined the effect of vortex-mediated mechanical stress on integrin activation in THP-1 cells, a monocytic cell line, and its signaling mechanisms. When cells are subjected to vortex flow at 400–2,000 rpm, integrin-dependent cell adhesion to vascular cell adhesion molecule-1 or fibronectin increased in a speed- and time-dependent manner. Next, to examine the role of Ca^{2+} in this integrin activation, various pharmacological inhibitors involved in Ca^{2+} signaling were tested to inhibit the cell adhesion. Pretreatment of cells with BAPTA-AM, thapsigargin +$NiCl_2$, or U-73122 (a phospholipase C inhibitor) inhibited cell adhesion induced by vortex-mediated mechanical stress. We also found that W7 (a calmodulin inhibitor) blocked the cell adhesion. However, pretreatment of cells with $GdCl_3$, $NiCl_2$, or ryanodine did not affect the cell adhesion. These data indicate that vortex-mediated mechanical stress induces integrin activation through calmodulin and inositol 1,4,5-trisphosphate-mediated Ca^{2+} releases from intracellular Ca^{2+} stores in THP-1 cells.

The nature of blood flow patterns and shear forces within blood vessels may be very variable depending upon vessel size, shape, branching, and partial obstructions (1). Biomechanical forces induced within the cardiovascular system affect gene expression in cells of blood vessel walls (2, 3) and functions of the cells in the vessel wall and in the fluid phase (4–8). Changes of shear forces occur in bifurcated or stenotic regions where atherosclerotic regions are prone to develop.

According to the multistep theory in cell transmigration, monocytes roll on the endothelial cells, interact with selectins, adhere to the endothelial cells by firm adhesion to ICAM-1[1] and vascular cell adhesion molecule-1 (VCAM-1), and then migrate into the subendothelium (9). Rolling of monocytes on endothelial cells is dependent on the binding of E-selectin and sialyl Lewis X, and adhesion to the endothelium is dependent on the interaction between integrins on monocytes and adhesion molecules on the endothelial cells, such as VCAM-1 and ICAM-1. Integrins consist of several subtypes, and each subtype is specific for each ligand. For example, $\alpha4\beta1$ integrin, VLA-4, binds to VCAM-1, and $\beta2$ integrins bind to ICAM-1. Fibronectin, one of the extracellular matrix proteins, is also known to bind to $\beta1$ integrins, mainly to $\alpha5\beta1$ integrin. Thus activation of adhesion molecules in endothelial cells and leukocytes is important for the cell migration process.

In this study, we hypothesized that leukocyte adhesion might be increased at bifurcations and in the downstream of the restricted vessels. In normal laminar flow, it has been reported that human leukocytes respond to fluid shear stress by retracting pseudopods and down-regulation of integrins (10, 11), which is a requirement for normal passage of circulating leukocytes through the microcirculation. In the downstream of the region where the vessel lumen is partially occluded, however, a backward vortex can be observed where cells in the fluid phase are subjected to a vortex motion under low shear forces (1, 12–14). Although such a change of shear force on endothelial cells can regulate the expression of adhesion molecules resulting in the progression of atherosclerosis (15, 16), the effect of vortex-mediated mechanical stress on leukocytes has not yet been determined. If vortex-induced mechanical stress can induce cell adhesion in leukocytes, leukocytes would be more prone to attach to the endothelial lining in the turbulent flow because the residence time of leukocytes in the regions with nonlaminar flow is longer than in those with laminar flow (12, 13).

A variety of signaling systems are induced by a mechanosensor in endothelial cells. As a mechanosensor, stretch-activated channels have been reported to regulate Ca^{2+} influx induced by flow stress in cells such as endothelial cells or smooth muscle cells (17). There is much evidence that stretch increases intracellular Ca^{2+} levels (4, 17). Thus the importance of Ca^{2+} signaling in endothelial mechanotransduction has been established. However, the role of Ca^{2+} in cell response to the mechanical stress in leukocytes has not been examined so far. Therefore, the aim of this study was to examine the effect of

* This study was supported by Grants-in-aid 13307034 and 14570657 and Center of Excellence Grant 12CE2006 from the Japanese Ministry of Education, Science, Sports, and Culture (12CE2006) and a research grant for health sciences from the Japanese Ministry of Health and Welfare. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Geriatric Medicine, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan. Tel.: 81-75-751-3463; Fax: 81-75-751-3463; E-mail: harai@kuhp.kyoto-u.ac.jp.

[1] The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MES, 4-morpholineethanesulfonic acid; VLA-4, very late antigen-4; PLC, phospholipase C.

mechanical stress on integrin-dependent cell adhesion in human monocytic THP-1 cells and to elucidate the role of Ca^{2+} signaling involved in this process.

EXPERIMENTAL PROCEDURES

Reagents—RPMI medium was obtained from Nissui Pharmaceuticals Co. Ltd. (Tokyo, Japan). Fetal calf serum was purchased from Grand Cayman (British West Indies). L-glutamine and penicillin/streptomycin were obtained from Bio Whittaker (Walkersville, MD). Recombinant human soluble VCAM-1 and ICAM-1 were from Genzyme/Techne (Minneapolis, MN). Fibronectin, thapsigargin, W-7, ryanodine, U-73122, bovine serum albumin, RGDS peptides, and RGES peptides were from Sigma. Anti-human α4 (VLA-4) antibody was from Upstate Biotechnology (Lake Placid, NY). $GdCl_3 \cdot 6H_2O$ and $NiCl_2 \cdot 6H_2O$ were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BAPTA-AM was from Dojindo (Kumamoto, Japan).

Cell Lines—The monocytic cell line THP-1 was a generous gift from Dr. K. Nishida (Daiichi Pharmaceuticals Co. Ltd., Tokyo) and was cultured in RPMI supplemented with L-glutamine and penicillin/streptomycin plus 10% fetal calf serum in an atmosphere of 95% air and 5% CO_2 at 37 °C.

Cell Adhesion Assay—Cell adhesion assays were carried out essentially as described (18). Briefly, polystyrene 96-well flat-bottomed microtiter plates (Costar 3595, Corning Inc., Corning, NY) were coated with 50 μl of soluble VCAM-1 (2.5 μg/ml), soluble ICAM-1 (2.5 μg/ml), or fibronectin (10 μg/ml) for 1 h at room temperature. After incubation, wells were blocked by incubation with 200 μl of 10 mg/ml heat-denatured bovine serum albumin for 30 min at room temperature. Control wells were filled with 10 mg/ml heat-denatured bovine serum albumin. One hundred μl of THP-1 cells suspended at a concentration of 10^6/ml in 10% fetal calf serum-RPMI were incubated for the indicated times in a CO_2 incubator at 37 °C after exposure to vortex flow by vortex machine (MS1 minishaker from IKA Works, Wilmington, NC). After incubation, nonadherent cells were removed by centrifugation (top side down) at 48 × *g* for 5 min. The plates were then centrifuged inversely at 80 × *g* for 5 min. Attached cells were fixed with 5% glutaraldehyde for 30 min at room temperature. Cells were washed three times with water, and 100 μl of 0.1% crystal violet in 200 mM MES (pH 6.0) was added to each well and incubated at room temperature for 20 min. Excess dye was removed by washing with water three times, and the bound dye was solubilized with 100 μl of 10% acetic acid. The absorbance of each well at 595 nm was then measured using a multiscan enzyme-linked immunosorbent assay reader (SPECTRA classic, Tecan, Maennedorf, Austria). Each sample was assayed in triplicate. The absorbance was linear to the cell number up to OD of 1.9 (data not shown). For example, 0.05 of OD. represents adhesion of about 2,000 cells, and 0.5 of OD represents adhesion of about 25,000 cells.

RESULTS

Vortex-mediated Mechanical Stress Increased Adhesion of THP-1 Cells to VCAM-1 and Fibronectin—To determine the regulation of integrin avidity or affinity by mechanical stress mediated by vortex flow, we studied adhesion of THP-1 cells to purified adhesion molecules. Cell adhesion to soluble VCAM-1, soluble ICAM-1, and fibronectin was determined after cells were exposed to vortex flow for 5 s at 1,500 rpm to mimic vortices that may occur in the cardiovascular system (12, 13, 19). Vortex-mediated mechanical stress increased adhesion of THP-1 cells to VCAM-1 and fibronectin by approximately five-fold but not to ICAM-1 (Fig. 1).

Vortex-mediated cell adhesion to VCAM-1 and fibronectin increased in a speed-dependent manner (Fig. 2). To show that this cell adhesion is dependent on α4β1 and α5β1 integrins, we preincubated the cells with anti-α4 antibody and RGDS peptides. Preincubation of the cells with anti-α4 antibody inhibited vortex-mediated cell adhesion to VCAM-1 by about 80%, but not with control IgG (Fig. 3*A*). Preincubation with RGDS, but not with REDS peptides, inhibited vortex-mediated cell adhesion to fibronectin (Fig. 3*B*). We also studied the change of β1 integrin expression on THP-1 cells induced by vortex-mediated mechanical stress, but we could not find any change of the expression by flow cytometry (data not shown). These data indicate that cell adhesion in our assay depends on the interaction between integrins and their ligands and that vortex-mediated mechanical stress increased the avidity or affinity of both α4β1 and α5β1 integrins in THP-1 cells.

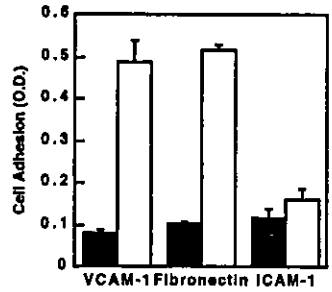

FIG. 1. **Vortex flow stimulates cell adhesion to VCAM-1 and fibronectin, but not ICAM-1, in THP-1 cells.** THP-1 cells were subjected to adhesion assays on ICAM-1, VCAM-1, or fibronectin for 5 min (*VCAM-1, fibronectin*) or 10 min (*ICAM-1*) with (*open bar*) or without (*filled bar*) vortexing at 1,500 rpm for 5 s. Data represent the mean ± S.D. of triplicate measurements from three independent experiments.

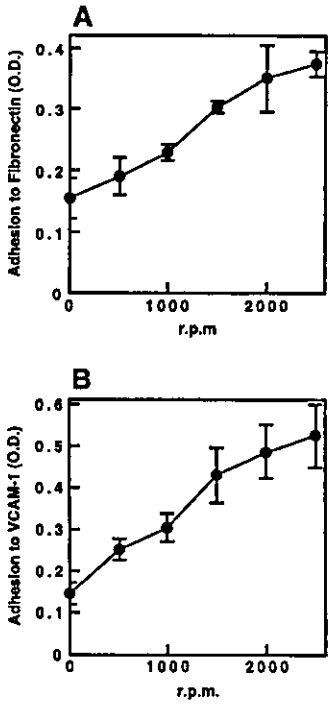

FIG. 2. **Speed-dependence of vortex-induced adhesion of THP-1 cells to VCAM-1 and fibronectin.** THP-1 cells were subjected to adhesion assays on VCAM-1 or fibronectin for 5 min after vortexing at the indicated speeds for 5 s. Data represent the mean ± S.D. of triplicate measurements from three independent experiments.

Transient Integrin Activation after Vortex-mediated Mechanical Stress—Next, we studied the time-dependent effect of vortex flow on cell adhesion to VCAM-1 and fibronectin. We found that vortex-mediated mechanical stress increased cell adhesion to both VCAM-1 and fibronectin quite rapidly, reaching a peak at 2–5 s of stimulation, indicating that such a brief vortex stimulation is enough to activate β1 integrin (Fig. 4). To examine reversibility of this integrin activation, cells were vortexed at 1,500 rpm for 5 s and left static for the indicated minutes. Cell adhesion to VCAM-1 or fibronectin was then determined. After the cells were left static for only 4 min, the cell adhesion induced by vortex flow was rapidly reduced to ~50% (Fig. 5), showing that this integrin activation induced by vortex-mediated mechanical stress is quite transient and reversible.

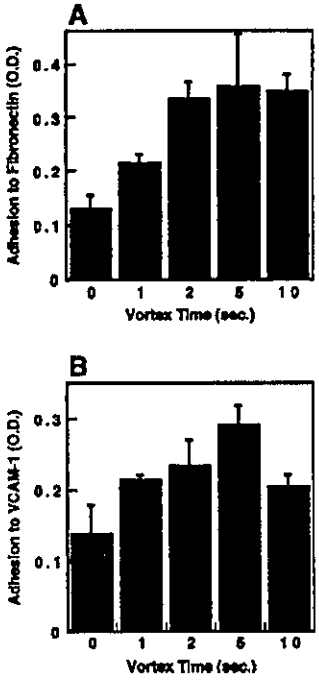

FIG. 3. **Time-dependent increase of vortex-induced adhesion of THP-1 cells to VCAM-1 and fibronectin.** THP-1 cells were subjected to adhesion assays on VCAM-1 or fibronectin for 5 min after vortexing at 1,500 rpm for the indicated seconds. Data represent the mean ± S.D. of triplicate measurements from three independent experiments.

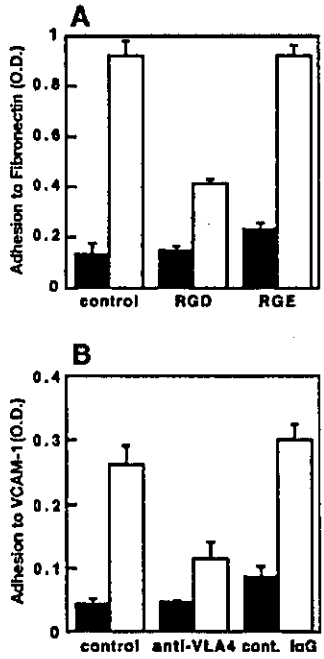

FIG. 4. **Cell adhesion depends on β1 integrin.** THP-1 cells were preincubated with 10 μg/ml anti-α4 antibody or control IgG (for VCAM-1) and 2 mM RGDS or RGES peptide (for fibronectin) for 1 h in an atmosphere of 95% air and 5% CO_2 at 37 °C. After the incubation, cells were subjected to adhesion assays on VCAM-1 or fibronectin with (*open bar*) or without (*filled bar*) vortexing at 1,500 rpm for 5 s. Data represent the mean ± S.D. of triplicate measurements from three independent experiments.

Integrin Activation Induced by Vortex-mediated Mechanical Stress Depends on IP_3-sensitive Ca^{2+} Release from Intracellular Stores—Calcium signals are reported to be important for

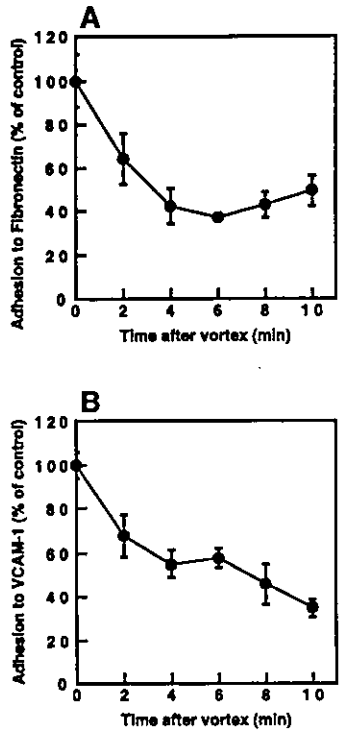

FIG. 5. **Vortex-induced integrin activation is transient and reversible.** THP-1 cells were subjected to adhesion assays on VCAM-1 or fibronectin for 5 min after left static for the indicated minutes after vortexing at 1,500 rpm for 5 s. Data represent the mean ± S.D. of triplicate measurements from three experiments. The value at the baseline was expressed as 100%.

various cell responses such as integrin activation leading to cell adhesion (20). To determine whether Ca^{2+} is involved in integrin activation induced by vortex-mediated mechanical stress, we next pretreated the cells with BAPTA-AM, an intracellular Ca^{2+}chelator. Pretreatment of the cells with BAPTA-AM inhibited vortex-mediated cell adhesion to fibronectin (Fig. 6*A*) and VCAM-1 (data not shown), indicating that intracellular Ca^{2+} is necessary for this integrin activation. To determine whether a stretch-activated Ca^{2+} channel, a well known sensing system for mechanical stress (17), or Ca^{2+} influx from the extracellular space is involved in integrin activation induced by vortex-mediated mechanical stress, we next pretreated the cells with $GdCl_3 \cdot 6H_2O$, a specific stretch-activated channel inhibitor, or $NiCl_2 \cdot 6H_2O$, a nonspecific Ca^{2+} influx inhibitor. Pretreatment of cells with these inhibitors did not affect vortex-mediated cell adhesion to fibronectin (Fig. 6*B*) or VCAM-1 (data not shown), indicating that this integrin activation does not depend on stretch-activated channels or Ca^{2+} influx from outside of the cells. These data indicate that Ca^{2+} release from intracellular Ca^{2+} stores such as endoplasmic reticulum may play a key role for this phenomenon.

Ca^{2+} is released from the intracellular Ca^{2+} stores via two known channels, one sensitive to inositol 1,4,5-trisphosphate (IP_3) and the other sensitive to ryanodine. Therefore, to determine the mechanism of Ca^{2+} release from intracellular Ca^{2+} stores, we pretreated the cells with thapsigargin, an inhibitor of Ca^{2+}-ATPase that inhibits IP_3-dependent Ca^{2+} release from intracellular stores (21, 22). Because thapsigargin itself induces sustained elevation of intracellular calcium mediated by capacitative Ca^{2+} influx (23, 24), we added $NiCl_2$ to block this Ca^{2+} influx. Pretreatment of THP-1 cells with thapsigargin and $NiCl_2$ inhibited vortex-mediated mechanical stress-induced cell adhesion to fibronectin (Fig. 6*B*) and VCAM-1 (data

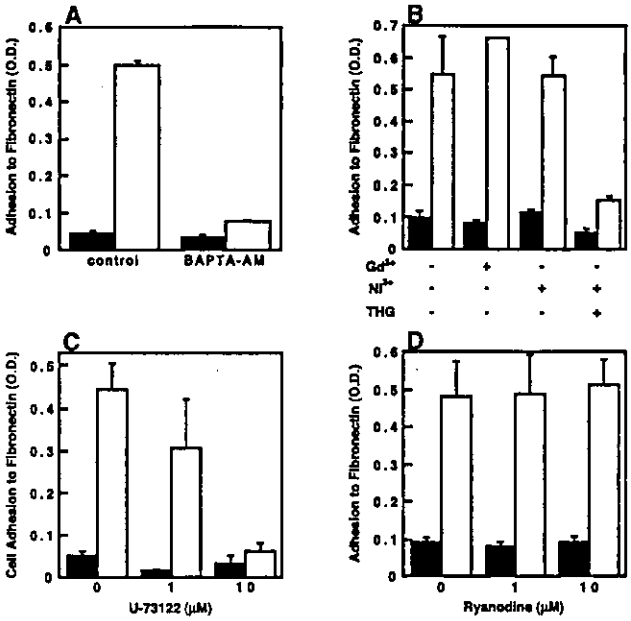

FIG. 6. **Integrin activation induced by vortex-mediated mechanical stress depends on IP_3-sensitive Ca^{2+} release from intracellular stores.** *A*, THP-1 cells were preincubated with 50 μM BAPTA-AM for 1 h in an atmosphere of 95% air and 5% CO_2 at 37 °C. After incubation, cells were subjected to adhesion assays on fibronectin for 5 min with (*open bar*) or without (*filled bar*) vortexing at 1,500 rpm for 5 s. Data represent the mean ± S.D. of triplicate measurements from three independent experiments. *B*, THP-1 cells were preincubated with 50 μM $GdCl_3 \cdot 6H_2O$ for 1 h or 1 mM $NiCl_2 \cdot 6H_2O$ for 1 h followed by treatment with or without 1 μM thapsigargin (*THG*) for 3 h in an atmosphere of 95% air and 5% CO_2 at 37 °C. After incubation, cells were subjected to adhesion assays on fibronectin for 5 min with (*open bar*) or without (*filled bar*) vortexing at 1,500 rpm for 5 s. Data represent the mean ± S.D. of triplicate measurements from three independent experiments. *C* and *D*, THP-1 cells were preincubated with the indicated concentrations of U-73122 (*C*) or ryanodine (*D*) for 1 h in an atmosphere of 95% air and 5% CO_2 at 37 °C. After incubation, cells were subjected to adhesion assays on VCAM-1 for 5 min with (*open bar*) or without (*filled bar*) vortexing at 1,500 rpm for 5 s. Data represent the mean ± S.D. of triplicate measurements from three independent experiments.

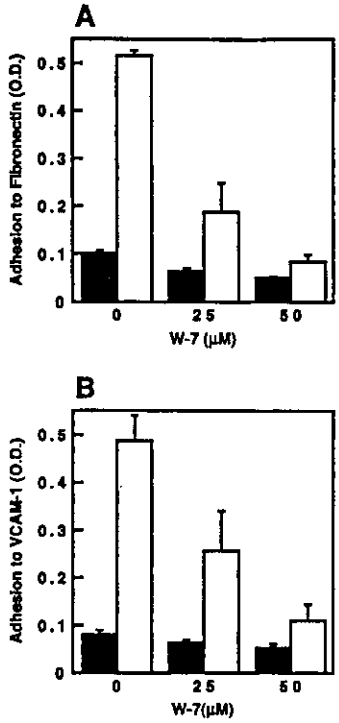

FIG. 7. **Calmodulin inhibitor inhibits vortex-induced adhesion to VCAM-1 and fibronectin in a dose-dependent manner.** THP-1 cells were preincubated with W-7 (calmodulin inhibitor) at indicated concentrations for 2 h in an atmosphere of 95% air and 5% CO_2 at 37 °C. After incubation, cells were subjected to adhesion assays on VCAM-1 or fibronectin for 5 min with (*open bar*) or without (*filled bar*) vortexing at 1,500 rpm for 5 s. Data represent the mean ± S.D. of triplicate measurements from three independent experiments.

not shown). We also pretreated the cells with U-73122, a specific PLC inhibitor, because mechanical stimulation of a single cell can activate PLC to elevate IP_3 (25). Pretreatment of the cells with U-73122 inhibited vortex-mediated cell adhesion to fibronectin and VCAM-1 (data not shown) in a dose-dependent manner (Fig. 6*C*).

To examine the role of ryanodine-sensitive Ca^{2+} release from intracellular Ca^{2+} stores, we next pretreated the cells with ryanodine, which can inhibit ryanodine-sensitive Ca^{2+} release (26). Pretreatment of THP-1 cells with ryanodine up to 10 μM did not affect vortex-mediated cell adhesion to fibronectin (Fig. 6*D*). These data indicate that IP_3-dependent Ca^{2+} release from intracellular Ca^{2+} stores plays a key role in this phenomenon.

Calmodulin Is Also Necessary for Integrin Activation Induced by Vortex-mediated Mechanical Stress—We also examined the potential role of Ca^{2+}-calmodulin in integrin activation induced by vortex-mediated mechanical stress. To determine the involvement of calmodulin in integrin activation induced by vortex-mediated mechanical stress, we pretreated the cells with W-7, a calmodulin inhibitor, before vortexing the cells. Pretreatment of cells with W-7 inhibited vortex-mediated cell adhesion to VCAM-1 and fibronectin in a dose-dependent manner (Fig. 7), indicating that calmodulin is also involved in integrin activation induced by vortex-mediated mechanical stress.

DISCUSSION

In this study we have examined the effect of vortex-mediated mechanical stress on integrin-dependent cell adhesion in human monocytic THP-1 cells and have clearly shown that a brief period of vortex-mediated mechanical stress activated β1 integrin, resulting in cell adhesion to VCAM-1 and fibronectin in a transient and reversible manner. We have also shown that IP_3-dependent Ca^{2+} release from intracellular Ca^{2+} stores and calmodulin are involved in this integrin activation. This mechanism might explain why atherosclerosis is prone to progress in bifurcated or stenotic regions, and this may be a novel aspect of atherosclerosis and inflammation.

Most of the studies on mechanotransduction in the cardiovascular field have been done in endothelial cells and smooth muscle cells. The endothelial cells are normally subjected to mechanical stimuli from shear stress and from strain associated with stretch of the vessel wall. These stimuli can be detected by a mechanosensor that initiates a variety of signal transduction cascades (17, 27). For example, in response to the change in shear stress the endothelium can change the gene expression of various cytokines and adhesion molecules (15, 16, 28) that would be related to the promotion of atherosclerosis, thrombosis, and inflammation. Few studies, however, have been conducted to elucidate the changes in the adhesive property of leukocytes in the vortex flow, which might be also related to the induction of atherosclerosis. Fukuda *et al.* (11) have reported that human leukocytes respond to fluid shear stress by retracting pseudopods and down-regulate the integrin expression under the laminar flow condition, which would help leukocytes to run in the vessel wall. However, in the tortuous cardiovascular system, such as branching of the vessels and downstream of partially occluded vessels, leukocytes and plate-

lets can be subjected to differing shear forces under nonlaminar flow patterns (1, 12, 13). In this study, therefore, we exposed cells to vortex flow in order to mimic vortices that may occur in the cardiovascular system. In the study of platelet aggregation, a stirring bar has been used to expose platelets to vortex flow (19). Because it is important to expose whole cells to vortex flow instantaneously to mimic the *in vivo* situation, vortexing the cells in a vortex machine would be more reasonable to stimulate the cells *in vitro*. Establishing an *in vivo* model would be more important to show the relevance of this data to *in vivo* situations.

In previous studies, the endothelial intracellular Ca^{2+} concentration in response to mechanical stress is biphasic, consisting of an initial transient rise that depends on Ca^{2+} release from IP_3-sensitive stores, followed by a sustained elevation mediated by Ca^{2+} influx (22, 29, 30). However, in this report we have shown that Ca^{2+} influx from the extracellular space is not necessary for integrin activation induced by vortex-mediated mechanical stress on THP-1 cells. Our data also clearly indicate that IP_3-dependent Ca^{2+} release from intracellular Ca^{2+} stores plays a key role in this mechanism. Although the reason why only Ca^{2+} release from intracellular stores is required for vortex-mediated integrin activation remains unclear, it might be because of the shortness of vortex stimulation and integrin activation.

Calmodulin is a Ca^{2+} binding protein and is reported to be important for various cell responses, such as integrin activation leading to T cell adhesion (20) and aggregation (31). Our study clearly demonstrates that calmodulin also plays an essential role in regulating integrin activation induced by vortex-mediated mechanical stress as shown in various cell responses (32, 33). However, at present it is not clear how Ca^{2+} release from intracellular stores can be linked to the activation of calmodulin and integrin activation in THP-1 cells. Further studies, therefore, are required to clarify this mechanism.

In this study we have not been able to identify the sensing mechanism for vortex-induced mechanical stress in THP-1 cells. There is a possibility that a mechanosensor itself is not involved in this process. The forces applied at the cell surface might be transmitted to other locations via cytoskeleton. This kind of mechanotransduction is shown in the area of mechanical stretch (34). Therefore, an explanation of the sensing mechanism would be required to understand this process. Further understanding of how leukocyte adhesion functions in the tortuous cardiovascular system would enhance our knowledge of the nuances of the atherosclerotic and inflammatory process and should facilitate the development of drugs to regulate the process.

In summary, we have provided clear evidence that vortex-mediated mechanical stress on THP-1 cells quickly induces Ca^{2+}- and calmodulin-dependent integrin activation, and IP3-dependent Ca^{2+} release from intracellular Ca^{2+} stores is involved in its mechanism. These findings might enlighten another aspect of increased atherosclerosis at stenotic or bifurcated regions.

Acknowledgment—We thank Hitomi Sagawa for excellent technical assistance.

REFERENCES

1. Turitto, V. T., and Hall, C. L. (1998) *Thromb. Res.* **92,** (suppl.) 25–31
2. Malek, A. M., Gibbons, G. H., Dzau, V. J., and Izumo, S. (1993) *J. Clin. Invest.* **92,** 2013–2021
3. Resnick, N., Yahav, H., Khachigian, L. M., Collins, T., Anderson, K. R., Dewey, F. C., and Gimbrone, M. A., Jr. (1997) *Adv. Exp. Med. Biol.* **430,** 155–164
4. Mo, M., Eskin, S. G., and Schilling, W. P. (1991) *Am. J. Physiol.* **260,** H1698–1707
5. Sriramarao, P., Languino, L. R., and Altieri, D. C. (1996) *Blood* **88,** 3416–3423
6. Kroll, M. H., Hellums, J. D., McIntire, L. V., Schafer, A. I., and Moake, J. L. (1996) *Blood* **88,** 1525–1541
7. Miyazaki, Y., Nomura, S., Miyake, T., Kagawa, H., Kitada, C., Taniguchi, H., Komiyama, Y., Fujimura, Y., Ikeda, Y., and Fukuhara, S. (1996) *Blood* **88,** 3456–3464
8. Weiss, H. J., Hawiger, J., Ruggeri, Z. M., Turitto, V. T., Thiagarajan, P., and Hoffmann, T. (1989) *J. Clin. Invest.* **83,** 288–297
9. Butcher, E. C. (1991) *Cell* **67,** 1033–1036
10. Moazzam, F., DeLano, F. A., Zweifach, B. W., and Schmid-Schonbein, G. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5338–5343
11. Fukuda, S., Yasu, T., Predescu, D. N., and Schmid-Schonbein, G. W. (2000) *Circ. Res.* **86,** E13–18
12. Ku, D. N., Glagov, S., Moore, J. E., Jr., and Zarins, C. K. (1989) *J. Vasc. Surg.* **9,** 309–316
13. Cao, J., and Rittgers, S. E. (1998) *Ann. Biomed. Eng.* **26,** 190–199
14. Palmen, D. E., van de Vosse, F. N., Janssen, J. D., and van Dongen, M. E. (1994) *J. Biomech.* **27,** 581–590
15. Mohan, S., Mohan, N., Valente, A. J., and Sprague, E. A. (1999) *Am. J. Physiol.* **276,** C1100–1107
16. Nagel, T., Resnick, N., Atkinson, W. J., Dewey, C. F., Jr., and Gimbrone, M. A., Jr. (1994) *J. Clin. Invest.* **94,** 885–891
17. Brakemeier, S., Eichler, I., Hopp, H., Kohler, R., and Hoyer, J. (2002) *Cardiovasc. Res.* **53,** 209–218
18. Ashida, N., Arai, H., Yamasaki, M., and Kita, T. (2001) *J. Biol. Chem.* **276,** 16555–16560
19. Soslau, G., Schechner, A. J., Alcasid, P. J., and Class, R. (2000) *Thromb. Res.* **97,** 15–27
20. van Kooyk, Y., Weder, P., Heije, K., de Waal Malefijt, R., and Figdor, C. G. (1993) *Cell Adhes. Commun.* **1,** 21–32
21. Yellowley, C. E., Jacobs, C. R., and Donahue, H. J. (1999) *J. Cell. Physiol.* **180,** 402–408
22. Oike, M., Droogmans, G., and Nilius, B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2940–2944
23. Granfeldt, D., Samuelsson, M., and Karlsson, A. (2002) *J. Leukoc. Biol.* **71,** 611–617
24. Kuroiwa-Matsumoto, M., Hirano, K., Ahmed, A., Kawasaki, J., Nishimura, J., and Kanaide, H. (2000) *Br. J. Pharmacol.* **131,** 115–123
25. Sanderson, M. J., Charles, A. C., Boitano, S., and Dirksen, E. R. (1994) *Mol. Cell. Endocrinol.* **98,** 173–187
26. Meissner, G. (1986) *J. Biol. Chem.* **261,** 6300–6306
27. Lansman, J. B., Hallam, T. J., and Rink, T. J. (1987) *Nature* **325,** 811–813
28. Tsuboi, H., Ando, J., Korenaga, R., Takada, Y., and Kamiya, A. (1995) *Biochem. Biophys. Res. Commun.* **206,** 988–996
29. Geiger, R. V., Berk, B. C., Alexander, R. W., and Nerem, R. M. (1992) *Am. J. Physiol.* **262,** C1411–1417
30. Helmlinger, G., Berk, B. C., and Nerem, R. M. (1996) *J. Vasc. Res.* **33,** 360–369
31. Andrews, R. K., Suzuki-Inoue, K., Shen, Y., Tulasne, D., Watson, S. P., and Berndt, M. C. (2002) *Blood* **99,** 4219–4221
32. Exton, J. H. (1985) *Am. J. Physiol.* **248,** E633–647
33. Johns, A., Leijten, P., Yamamoto, H., Hwang, K., and van Breemen, C. (1987) *Am. J. Cardiol.* **59,** 18A-23A
34. Ingber, D. E. (1997) *Annu. Rev. Physiol.* **59,** 575–599

Circ J 2002; **66**: 1087–1095

Large Scale Cohort Study of the Relationship Between Serum Cholesterol Concentration and Coronary Events With Low-Dose Simvastatin Therapy in Japanese Patients With Hypercholesterolemia

—— Primary Prevention Cohort Study of the Japan Lipid Intervention Trial (J-LIT) ——

Masunori Matsuzaki, MD; Toru Kita, MD*; Hiroshi Mabuchi, MD**; Yuji Matsuzawa, MD†; Noriaki Nakaya, MD††; Shinichi Oikawa, MD‡; Yasushi Saito, MD‡‡; Jun Sasaki, MD§; Kazuaki Shimamoto, MD§§; Hiroshige Itakura, MD¶, ¶¶ and the J-LIT Study Group

Hyperlipidemia is a well-established risk factor for primary coronary heart disease (CHD). Although simvastatin is known to lower serum lipid concentrations, the protective effect of such lipid-lowering therapy against primary CHD has not been established in Japanese patients with hypercholesterolemia. The Japan Lipid Intervention Trial was a 6-year, nationwide cohort study of 47,294 patients treated with open-labeled simvastatin (5–10 mg/day) and monitored by physicians under standard clinical conditions. The aim of the study was to determine the relationship between the occurrence of CHD and the serum lipid concentrations during low-dose simvastatin treatment. Simvastatin reduced serum concentrations of total cholesterol (TC), low-density lipoprotein- cholesterol (LDL-C) and triglyceride (TG), by 18.4%, 26.8% and 16.1% on average, respectively, during the treatment period. The risk of coronary events was higher when the average TC concentration was ≥240 mg/dl and the average LDL-C concentration was ≥160 mg/dl. The incidence of coronary events increased in the patients with TG concentration ≥300 mg/dl compared with patients with TG concentration <150 mg/dl. The high-density lipoprotein cholesterol (HDL-C) inversely correlated with the risk of coronary events. The J-curve association was observed between average TC or LDL-C concentrations and total mortality. Malignancy was the most prevalent cause of death. The health of patients should be monitored closely when there is a remarkable decrease in TC and LDL-C concentrations with low-dose statin. A reasonable strategy to prevent coronary events in Japanese hypercholesterolemic patients without prior CHD under low-dose statin treatment might be regulating the serum lipid concentrations to at least <240 mg/dl for TC, <160 mg/dl for LDL-C, <300 mg/dl for TG, and >40 mg/dl for HDL-C. (*Circ J* 2002; **66**: 1087–1095)

Key Words: Cholesterol-lowering medication; Coronary heart disease; Hyperlipidemia; Longitudinal study; Risk factors; Simvastatin; Total mortality

Hypercholesterolemia is a known and significant risk factor for the development of coronary heart disease (CHD) and death[1] Epidemiologic studies from Western countries, such as the Framingham Study[2] have established that a high concentration of serum cholesterol confers a high risk of CHD. The incidence of CHD in the Japanese population is relatively lower than that reported in the Western countries[3–5] Cholesterol-lowering therapy with resins and fibrates has been shown to reduce the risk of CHD[6–8] Recent primary and secondary prevention studies have indicated that statins also reduce the incidence of CHD[9–13] The mechanism of statin action is selective inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes a rate-limiting step of cholesterol synthesis[14] and as a consequence, reduces the serum total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) concentrations[15]

Statins are now the most widely prescribed drugs worldwide and are established as the first-line treatment for hyperlipidemia. Although cholesterol-lowering therapy is also prescribed widely for Japanese patients with hypercholesterolemia, the relationship between serum lipid concentrations and the incidence of CHD under low-dose statin treatment has not been completely elucidated. The

(Received May 27, 2002; revised manuscript received August 8, 2002; accepted August 23, 2002)

*Yamaguchi University Graduate School of Medicine, Ube, *Kyoto University Graduate School of Medicine, Kyoto, **Kanazawa University Graduate School of Medicine, Kanazawa, †Osaka University Graduate School of Medicine, Suita, ††Fussa general hospital, Fussa, ‡Nippon Medical School, Tokyo, ‡‡Chiba University Graduate School of Medicine, Chiba, §International University Graduate School of Health and Welfare, Fukuoka, §§Sapporo Medical University School of Medicine, Sapporo and ¶Ibaraki Christian University, Hitachi, Japan and ¶¶Chairman of Central Committee.*

Mailing address: Masunori Matsuzaki, MD, Department of Cardiovascular Medicine, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-kogushi, Ube, Yamaguchi 755-8550, Japan. E-mail: masunori@yamaguchi-u.ac.jp

Table 1 Baseline Characteristics of the Population in the Primary Prevention Cohort Study

					TC during treatment (mg/dl)	
	<160	160–179	180–199	200–219	220–239	240–259
n	461	2,065	7,233	12,494	10,671	5,380
Male gender (%)	61.6	43.5	33.8	30.7	29.8	27.8
Age (years)	57.4±8.8	59.0±8.0	58.9±7.6	58.2±7.7	57.6±7.8	56.7±7.9
Obesity (%)	37.0	36.5	33.2	33.0	33.1	34.4
Hypertension (%)	52.5	57.3	53.8	48.3	42.5	39.1
Diabetes mellitus (%)	20.8	20.2	17.2	14.7	13.4	13.6
Cerebrovascular disease (%)	6.3	5.6	4.3	3.0	2.3	2.0
Renal disease (%)	4.8	2.6	2.4	1.9	1.7	1.9
Hepatic disease (%)	16.5	11.1	8.0	7.7	7.1	8.0
ECG abnormality (%)	18.0	17.8	14.7	12.9	11.7	11.8
Family history of CHD (%)	5.0	3.8	4.5	4.4	4.5	5.2
Smoking habit (%)	31.2	21.2	16.7	15.2	15.6	16.3
Alcohol consumption (%)	44.9	32.9	28.7	28.0	28.7	27.8
TC (mg/dl)	253±40	252±24	256±22	264±34	272±28	282±30
LDL-C (mg/dl)	165±34	167±26	171±26	177±28	185±30	193±33
TG (mg/dl)	263±270	211±188	185±140	183±132	192±159	205±174
HDL-C (mg/dl)	45.6±13.5	48.8±13.8	52.1±14.6	53.2±14.8	53.7±15.1	54.0±15.5

TC, total cholesterol; Obesity, body mass index ≥25 kg/m²; CHD, coronary heart disease; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglycerides.
p value for trend test <0.05.

Japan Lipid Intervention Trial (J-LIT) is the first nationwide study conducted to determine the relationship between serum lipid concentration and the development of CHD under low-dose simvastatin treatment. In order to evaluate this relationship, we used a surveillance study under common medical practice.

We have already reported that the serum lipid concentrations in Japanese patients with hypercholesterolemia are well-controlled by low-dose simvastatin (initial dose, 5–10 mg/day),[16] and in the present study, we examined how serum cholesterol concentrations relate to the incidence of CHD and overall mortality in a large number of these patients who did not have a history of CHD.

Methods

Subjects

The J-LIT study enrolled 52,421 patients with a serum TC concentration ≥220 mg/dl; men aged 35–70 years and postmenopausal women under 70 years of age who were selected from throughout Japan. Patients who had been treated with a lipid lowering agent, were screened for eligibility after a washout period of at least 4 weeks; the washout period was at least 12 weeks for patients previously treated with probucol. Exclusion criteria included a recent acute myocardial infarction (MI) or stroke (≤1 month), uncontrolled diabetes mellitus, serious concomitant hepatic or renal disease, secondary hypercholesterolemia, malignancy or any other illness with a poor prognosis. Patients without documented CHD (ICD[17] codes: I20 to I25) or a history of any coronary intervention at the time of enrollment were assigned to the primary prevention cohort.

Study Design

The design of the J-LIT study has been described previously,[16] but in brief it involved 6,500 general practitioners throughout Japan. During the screening period, body weight and blood pressure were determined and fasting serum lipid profiles were measured twice at monthly intervals at the local recruiting sites. Indicators of hepatic and renal function were assessed, and an electrocardiogram (ECG) was recorded. Patients were treated with open-labeled simvastatin at a dose of 5–10 mg/day and all patients, including those who discontinued simvastatin for any reason, were monitored for 6 years. Their lipid concentrations, adverse events, and CHD-related events were recorded. Cholesterol concentrations were determined locally in study institutions. The inter-hospital differences were assessed twice in 1996 and 1999 using standards distributed to sampled institutions throughout the country, and no inter-hospital differences in measurement of cholesterol were found. Dietary changes and exercise therapy for hyperlipidemia were recommended to the patients by the investigators. Additional lipid-lowering agents were allowed only when the serum TC concentration did not respond adequately to simvastatin alone. No restrictions were placed on the administration of medical treatment for complications. The LDL-C concentration in patients with a serum triglyceride (TG) concentration ≤400 mg/dl was calculated using the Friedewald formula[18] Body weight, blood pressure, and the serum lipid concentrations were measured every 6 months after enrollment and patients were asked about drug compliance, number of cigarettes smoked, alcohol consumption, and amount of exercise. Every 12 months, hepatic and renal functions were monitored and an ECG was recorded.

The primary end-points of the study were major coronary events, such as acute MI or sudden cardiac death. The secondary end-points were the occurrence of other cardiovascular events, such as the onset of angina pectoris, and death from any cause. All CHD-related events and deaths that occurred during the study period were reviewed and determined by the Endpoint Classification Committee. The adverse drug reactions (ADRs) were evaluated by the Adverse Event Subcommittee. Each patient was informed of the study purpose, as well as drug efficacy and the need for long-term treatment. Written informed consent was not obtained from the patients because a commercially available simvastatin preparation was used for the open-labeled study.

Statistical Analysis

All data, including those obtained after the termination of

260–279 2,110	≥280 1,387	p value	Total 41,801
30.2	32.4	*	31.6
56.1±8.0	54.6±8.2	*	57.8±7.8
35.2	36.2		33.7
35.6	33.0	*	45.9
16.0	17.5	*	15.2
2.4	1.7	*	3.0
2.4	3.0		2.1
8.0	8.9	*	8.0
10.9	11.8	*	12.9
7.1	7.9	*	4.8
17.8	20.0	*	16.5
29.5	30.9	*	28.9
294±39	322±57	*	270±34
203±38	233±59	*	182±33
222±254	256±317		195±169
53.7±15.8	52.0±17.2	*	52.9±15.1

simvastatin therapy were analyzed. For baseline characteristics, the patients were divided into 8 subgroups based on their average serum TC concentration during the treatment. The average lipid concentrations were calculated using the data obtained throughout the study period. The data for lipid concentrations acquired after the onset of disease other than a primary or secondary end-point were excluded. For analysis of baseline patient age and lipid profiles, continuous variables within and between subgroup were assessed using analysis of variance by trend test. For analysis of baseline characteristics determined by categorical outcomes, differences between groups were compared using the Mantel-Haenszel test. Patients were classified into 3–8 subgroups based on the average lipid concentrations during treatment. TC, TG, LDL-C, and high-density lipoprotein cholesterol (HDL-C) concentrations and the ratio of LDL-C/HDL-C were classified into discrete intervals of 20, 150, 20, 10 mg/dl and 0.5, respectively. Reference categories were set for the subgroups, according to the guidelines[19] of normal ranges with an upper limit of 220 mg/dl for TC, 150 mg/dl for TG, 140 mg/dl for LDL-C and with the lower limit of 40 mg/dl for HDL-C. The reference category for the ratio of LDL-C/HDL-C was set on the subgroup with 2.0–2.4. We calculated the relative risks, with 95% confidence intervals (CI) for each end-point of each subgroup relative to the reference category, using the Cox proportional-hazards model[20] with adjustment for gender and age at baseline (as a continuous variable), hypertension, diabetes mellitus, and smoking habit. We excluded 559 patients from this analysis because information about their smoking habits was not available. In addition, the effects on each baseline characteristic at each end-point were assessed, except for the effect of age, which was not adjusted because it was treated as a continuous variable. Data are expressed as the average±SD. For all statistical analyses, $p<0.05$ was considered to be significant. All statistical calculations were performed using the SAS software (V.6.12, SAS Institute Inc, Cary, NC, USA).

Results

Follow-up

A total of 47,294 of the 52,421 patients enrolled in the J-LIT study, were eligible for the primary prevention cohort, and the remaining 5,127 patients who had a history of CHD were enrolled in the secondary prevention cohort, and the clinical characteristics of which are reported elsewhere[16] In the present study, data collected from 42,360 patients were analyzed and data from 4,934 patients were excluded for the following reasons: lack of follow-up data (932 patients), violation of inclusion/exclusion criteria (63 patients), unwillingness to participate (6 patients), and incomplete covariance (3,933 patients). Of the study patients, 31,766 were followed up by the investigators through to the end of the 6th year (average length of follow up, 5.39 years per subject). For the analysis of the baseline characteristics, patients were stratified according to their serum TC concentrations during treatment with every 20 mg/dl. In the analysis with the trend test, a trend in the relationship between average serum total cholesterol concentration during treatment and the baseline characteristics of patients was observed. The percentage of male patients, age, incidence of hypertension, diabetes mellitus, cerebrovascular disease, hepatic disease and abnormal ECG, percentage of patients with a family history of CHD, smoking and drinking increased as average serum total cholesterol concentration during treatment decreased (Table 1). The incidence of obesity and renal disease was similar in all groups. The decrease in average serum TC concentration during treatment was proportional to the patients' baseline concentrations of TC, LDL-C and HDL-C.

Table 2 Adverse Drug Reaction During the 6-Year Simvastatin Therapy

	No. of patients	Incidence rate (%)
Hepatic	450	0.97
Musculoskeletal	388	0.84
Digestive	256	0.55
Body as a whole; general	178	0.38
Skin	166	0.36
Kidney	82	0.18
Mental and nervous system	80	0.17
Blood	56	0.12
Laboratory test abnormal	59	0.13
Miscellaneous	19	0.04

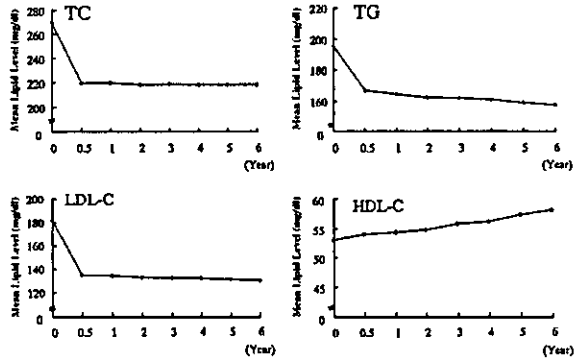

Fig 1. Sequential changes in serum lipid concentrations in patients maintained on low-dose simvastatin.

Safety

Simvastatin was well tolerated: ADRs were reported in 1,478 patients (2,194 events) for an overall ADR frequency of 3.2% over 6 years (Table 2). The most frequently ob-

Table 3 Incidence of Coronary Heart Disease in Patients With Hypercholesterolemia Receiving Low-Dose Simvastatin for 6-Years

	No. of patients		Incidence rate (/1,000 patients-year)	
Primary end point (coronary events)	*209*		*0.91*	
MI (nonfatal)		*147*		*0.64*
MI (fatal)		*51*		*0.22*
Cardiac sudden death		*11*		*0.05*
Secondary end point	*146*		*0.64*	
Angina pectoris (definite)		*146*		*0.64*
Total	*355*		*1.55*	

MI, myocardial infarction.

Table 4 Risk of Coronary Events and Lipids Concentration During the 6-Year Treatment Study of Low-Dose Simvastatin

	Study population	No. of events	Relative risk	95% confidence intervals	p value
TC (mg/dl)					
<180	*2,526*	*14*	*1.29*	*(0.70–2.38)*	*0.241*
180–199	*7,233*	*36*	*1.38*	*(0.88–2.16)*	*0.16*
200–219	*12,494*	*40*	*1.00*		
220–239	*10,671*	*45*	*1.47*	*(0.96–2.25)*	*0.08*
240–259	*5,380*	*37*	*2.63*	*(1.68–4.12)*	*<0.001*
≥260	*3,497*	*35*	*4.03*	*(2.55–6.38)*	*<0.001*
LDL-C (mg/dl)					
<100	*4,025*	*14*	*0.74*	*(0.40–1.35)*	*0.32*
100–119	*9,376*	*38*	*1.03*	*(0.67–1.59)*	*0.89*
120–139	*12,622*	*44*	*1.00*		
140–159	*9,089*	*41*	*1.45*	*(0.95–2.22)*	*0.09*
160–179	*3,931*	*29*	*2.59*	*(1.62–4.15)*	*<0.001*
≥180	*2,367*	*34*	*5.71*	*(3.64–8.97)*	*<0.001*
TG (mg/dl)					
<150	*23,140*	*88*	*1.00*		
150–299	*16,060*	*91*	*1.26*	*(0.93–1.69)*	*0.13*
≥300	*2,577*	*28*	*2.16*	*(1.38–3.37)*	*<0.001*
HDL-C (mg/dl)					
<40	*4,161*	*47*	*1.45*	*(1.01–2.07)*	*<0.05*
40–49	*11,897*	*85*	*1.00*		
50–59	*12,522*	*51*	*0.63*	*(0.44–0.89)*	*<0.01*
≥60	*13,221*	*24*	*0.30*	*(0.19–0.48)*	*<0.001*
LDL-C/HDL-C					
<2.0	*10,808*	*23*	*0.67*	*(0.39–1.14)*	*0.14*
2.0–2.4	*10,197*	*32*	*1.00*		
2.5–2.9	*8,949*	*33*	*1.21*	*(0.74–1.96)*	*0.45*
3.0–3.4	*5,730*	*44*	*2.54*	*(1.61–4.00)*	*<0.001*
≥3.5	*5,726*	*68*	*4.02*	*(2.63–6.13)*	*<0.001*

TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol.

served ADR was hepatic dysfunction and 760 events occurred in 450 subjects for an incidence of 0.97%. The incidence of musculoskeletal and digestive ADRs were 0.84 and 0.55%, respectively. Rhabdomyolysis was not observed in any patients for the entire 6 years of this study.

Changes in Serum Lipid Concentrations With Simvastatin

The average serum concentrations of TC, TG, and LDL-C decreased from their average baseline concentrations of 270 to 221, 196 to 167, and 182 to 132 mg/dl, respectively, after 6 months of treatment, and these concentrations were well maintained during the 6 years (Fig 1). The average lipid concentrations during the treatment for TC, TG and LDL-C were 220 mg/dl, 164 mg/dl and 134 mg/dl, respectively. The average serum HDL-C concentration increased from a baseline of 52.9 mg/dl to 54.0 mg/dl after 6 months of treatment, and continued to further increase to 58.1 mg/dl at the 6th year. The average percent changes in the TC, LDL-C, TG and HDL-C concentrations during the treatment period were −18.4±10.3%, −26.8±15.0%, −16.1±42.5%, and +4.5±29.8%, respectively.

Relationship Between the Risk of Coronary Events and Average Lipid Concentrations During Treatment

Coronary events occurred in 209 patients during the course of the study with a rate of incidence of 0.91 events per 1,000 patients-year (Table 3). Fatal MI occurred in 51 patients, non-fatal MI in 147 patients, and sudden cardiac death in 11 patients. Unequivocal angina pectoris (secondary end-point) developed in 146 patients.

The average serum concentrations of TC and LDL-C were closely related to the risk of coronary events for 6 years (Table 4). The risk of coronary events was higher in patients whose TC concentration was ≥240 mg/dl, compared with those whose TC concentrations were between 200 and 219 mg/dl (the reference category). Likewise, in

patients with a LDL-C concentration ≥160 mg/dl, the risk of coronary events was higher than in those with a concentration between 120 and 139 mg/dl (the reference category). A group of patients with an average TG concentration ≥300 mg/dl had a higher incidence of coronary events than the group with an average TG concentration <150 mg/dl. In contrast, the average serum HDL-C concentration was inversely related to the risk of coronary events. The incidence of coronary events was lower in patients with an average HDL-C concentration ≥50 mg/dl and higher in those with an average HDL-C concentration <40 mg/dl compared with patients whose HDL-C concentration was between 40 and 49 mg/dl. The incidence of coronary events and the average lipid concentrations during the treatment were found to be strongly related because each 10 mg/dl decrease in the TC, LDL-C and TG concentrations and each 10 mg/dl increase in the HDL-C concentration reduced the risk of coronary events by 11.3%, 15.8%, 1.2%, and 37.5%, respectively. In comparison, the incidence of coronary events was less correlated with the baseline concentrations of TC, LDL-C and HDL-C because a 10 mg/dl decrease in baseline serum TC, LDL-C, and TG concentrations and 10 mg/dl increase in baseline serum HDL-C concentration reduced the risk of coronary events by a mere 1.5%, 7.3%, 0.1%, and 21.6%, respectively.

Relationship Between the Risk of Coronary Events and Baseline Patient Characteristics (Fig 2)

The risk of coronary events was analyzed using multiple regression. Male patients had a higher risk, with a relative risk of 2.29 compared with female patients. Age correlated with the incidence of coronary events: coronary events occurred more often in patients with aged 60 years or more than in patients younger than 60 years old. Hypertension, diabetes mellitus, ECG abnormalities, cerebrovascular disease, a family history of CHD and a smoking habit were also risk factors for coronary events. In contrast, patients' alcohol consumption reduced the risk for coronary events; the average consumption was approximately 38 g/day per patient (measured as absolute alcohol).

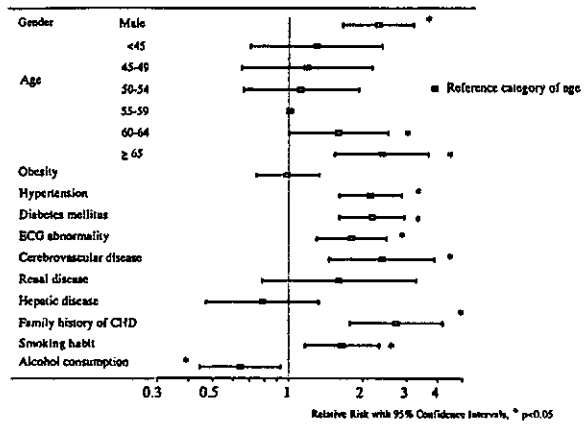

Fig 2. Relationship between the relative risk of coronary events and baseline characteristics of patients maintained on low-dose simvastatin. Bars express the relative risk with a 95% confidence interval. Obesity, body mass index ≥25 kg/m².

Relationship Between the Relative Risk of Overall Mortality and Lipid Concentrations

During the study period, 844 patients died (3.69 deaths

Table 5 Relative Risk of Death and Serum Lipid Concentrations During Treatment of Hypercholesterolemia With Low-Dose Simvastatin

	Study population	*No. of events*	*Relative risk*	*95% confidence intervals*	*p value*
TC (mg/dl)					
<160	*461*	*28*	*2.76*	*(1.86–4.10)*	*<0.001*
160–179	*2,065*	*77*	*1.72*	*(1.33–2.23)*	*<0.001*
180–199	*7,233*	*161*	*1.13*	*(0.92–1.38)*	*0.25*
200–219	*12,494*	*222*	*1.00*		
220–239	*10,671*	*178*	*1.03*	*(0.84–1.25)*	*0.79*
240–259	*5,380*	*79*	*1.01*	*(0.78–1.30)*	*0.95*
260–279	*2,110*	*49*	*1.68*	*(1.23–2.28)*	*<0.01*
≥280	*1,387*	*43*	*2.58*	*(1.85–3.58)*	*<0.001*
LDL-C (mg/dl)					
<80	*839*	*30*	*1.72*	*(1.17–2.53)*	*<0.01*
80–99	*3,186*	*76*	*1.16*	*(0.90–1.51)*	*0.26*
100–119	*9,376*	*211*	*1.20*	*(0.99–1.44)*	*0.07*
120–139	*12,622*	*219*	*1.00*		
140–159	*9,089*	*154*	*1.07*	*(0.87–1.31)*	*0.54*
160–179	*3,931*	*75*	*1.32*	*(1.02–1.72)*	*<0.05*
180–199	*1,403*	*25*	*1.37*	*(0.90–2.07)*	*0.14*
≥200	*964*	*33*	*2.92*	*(2.03–4.22)*	*<0.001*
TG (mg/dl)					
<150	*23,140*	*425*	*1.00*		
150–299	*16,060*	*353*	*1.13*	*(0.98–1.31)*	*0.09*
≥300	*2,577*	*58*	*1.29*	*(0.97–1.70)*	*0.08*
HDL-C (mg/dl)					
<40	*4,161*	*135*	*1.30*	*(1.06–1.60)*	*<0.05*
40–49	*11,897*	*286*	*1.00*		
50–59	*12,521*	*217*	*0.75*	*(0.63–0.90)*	*<0.01*
60–69	*7,536*	*94*	*0.55*	*(0.44–0.70)*	*<0.001*
≥70	*5,686*	*105*	*0.84*	*(0.67–1.05)*	*0.13*

TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol.

Table 6 Causes of Death and Total Cholesterol (TC) Concentration of Patients Treated With Low-Dose Simvastatin for 6-Year

	TC (mg/dl)	Study population	No. of deaths	Relative risk	95% confidence intervals	p value
Cardiac	Subtotal	41,801	83			
	<160	461	4	6.23	(1.99–19.52)	<0.01
	160–179	2,065	5	1.83	(0.64–5.23)	0.26
	180–199	7,233	15	1.82	(0.85–3.89)	0.12
	200–219	12,494	12	1.00		
	220–239	10,671	16	1.80	(0.85–3.81)	0.12
	240–259	5,380	16	4.04	(1.91–8.56)	<0.001
	260–279	2,110	6	3.96	(1.48–10.58)	<0.01
	>280	1,387	9	10.67	(4.45–25.54)	<0.001
Cerebrovascular /other vascular	Subtotal	41,801	188			
	<160	461	4	1.48	(0.54–4.11)	0.45
	160–179	2,065	16	1.34	(0.77–2.33)	0.31
	180–199	7,233	35	0.92	(0.60–1.40)	0.70
	200–219	12,494	58	1.00		
	220–239	10,671	36	0.81	(0.53–1.22)	0.31
	240–259	5,380	21	1.04	(0.63–1.71)	0.89
	260–279	2,110	8	1.03	(0.49–2.17)	0.93
	>280	1,387	10	2.25	(1.14–4.41)	<0.05
Malignancy	Subtotal	41,801	313			
	<160	461	12	3.16	(1.72–5.81)	<0.001
	160–179	2,065	31	1.85	(1.22–2.80)	<0.01
	180–199	7,233	61	1.13	(0.81–1.57)	0.47
	200–219	12,494	86	1.00		
	220–239	10,671	77	1.13	(0.83–1.54)	0.43
	240–259	5,380	22	0.72	(0.45–1.15)	0.17
	260–279	2,110	16	1.42	(0.83–2.42)	0.20
	>280	1,387	8	1.24	(0.60–2.57)	0.56
Accident/suicide	Subtotal	41,801	72			
	<160	461	3	2.87	(0.86–9.64)	0.09
	160–179	2,065	3	0.67	(0.20–2.22)	0.51
	180–199	7,233	12	0.82	(0.41–1.64)	0.57
	200–219	12,494	24	1.00		
	220–239	10,671	14	0.72	(0.37–1.39)	0.32
	240–259	5,380	8	0.88	(0.39–1.96)	0.75
	260–279	2,110	3	0.87	(0.26–2.90)	0.82
	>280	1,387	5	2.40	(0.91–6.36)	0.08
Others	Subtotal	41,801	181			
	<160	461	5	2.67	(1.05–6.80)	<0.05
	160–179	2,065	22	2.59	(1.54–4.36)	<0.001
	180–199	7,233	38	1.40	(0.90–2.17)	0.14
	200–219	12,494	42	1.00		
	220–239	10,671	35	1.07	(0.68–1.68)	0.77
	240–259	5,380	12	0.81	(0.43–1.54)	0.52
	260–279	2,110	16	2.92	(1.64–5.21)	<0.001
	>280	1,387	11	3.61	(1.85–7.04)	<0.001
Total deaths	Subtotal	41,801	837			

per 1,000 patients-year). The J-curve was observed between average TC concentration and total mortality (Table 5): the relative risk of death was higher in patients with a TC concentration <180 mg/dl or ≥260 mg/dl compared with the other groups. A similar pattern was observed between average LDL-C concentration and total mortality. Significantly lower total mortality was observed in patients with an average HDL-C concentration between 50 and 69 mg/dl, whereas there was higher mortality in patients with a HDL-C concentration <40 mg/dl compared with those whose average HDL-C concentration was between 40 and 49 mg/dl. There was no significant relationship between average TG concentration and total mortality. Of the 41,801 patients evaluated, 461 (1.1%) had an average TC concentration <160 mg/dl (40.2% reduction) during the treatment. Among the highly responsive population of patients to low-dose simvastatin therapy, 28 patients died at an average of 3.30±1.59 years after starting the treatment and of them, 12 died from malignancy (4 cases of gastric cancer, the highest, and 2 cases of lung cancer). Malignancy was the most common cause of death in most TC subgroups, followed by cerebrovascular diseases 1 other vascular diseases (Table 6). Death from cardiac disease occurred in 83 of 41,801 patients, including 51 who died from MI, 11 from sudden cardiac death, and 21 from other cardiac diseases. There was no obvious correlation between the relative risk of accident/suicide and serum TC concentration.

Relationship Between the Relative Risk of Death and Baseline Patient Characteristics

The relative risk of death was analyzed using multiple regression (Fig 3). Male patients had higher risk of death compared with female patients, and the incidence of death increased with age. Obesity did not correlate with the risk of death. Hypertension, diabetes mellitus, ECG abnormalities, cerebrovascular disease, and renal and hepatic diseases

also were risk factors for death. A smoking habit tended to increase the risk of death, but not significantly. Alcohol consumption reduced the risk of death.

Discussion

The J-LIT, a long-term prospective cohort study on the use of simvastatin, is the first epidemiological study in Japan to demonstrate a relationship between serum lipid concentrations and the incidence of primary onset of CHD or total mortality in Japanese patients with hypercholesterolemia and low-dose statin administration.

The overall frequency of ADRs during the simvastatin treatment was 3.2% over 6 years, which suggests that simvastatin is safe and well-tolerated.

After 6 months of treatment, the serum concentrations of TC and LDL-C had changed to 18.4% and 26.8% below baseline, respectively, and these concentrations were maintained throughout the 6 years of the study, however the concentration of HDL-C continued to increase during the treatment period.

The incidence of coronary events in Japanese patients without prior CHD in this study was 0.91 events per 1,000 patients-year, much lower than in Western countries[21–22] Since the relative risk of coronary events in men was 2.3 times higher than in women, and two-thirds of the patients enrolled in the J-LIT study were women, this low male/female ratio may have contributed to the overall low incidence of coronary events.

In the J-LIT study, patients with average TC concentration ≥240 mg/dl developed coronary events more frequently than those with a concentration <240 mg/dl during simvastatin (5–10 mg/day) treatment. The incidence of MI significantly increased when the TC concentration rose above 220 mg/dl in the Framingham Study[2] and a correlation between serum cholesterol and the risk of MI was also reported in an Okinawan population[23] The reason for having more coronary events at a TC concentration ≥240 mg/dl in the J-LIT study, not ≥220 mg/dl as in the Framingham study, could be simvastatin's anti-atherosclerotic effect, which is presumed to be the result of its pleiotropic actions on coronary vessels. It has been reported that simvastatin inhibits smooth muscle cell migration[24] and inflammatory reactions[25] and improves the responsiveness of endothelium cells to the factors influencing blood vessels[26]

It is well documented that an elevated LDL-C concentration is an independent risk factor for CHD and death! When the J-LIT subgroups were divided on the basis of a constant interval of serum lipid concentration, the average serum concentration of LDL-C closely correlated with the risk of coronary events, whereas the concentration of HDL-C was inversely correlated. Coronary events did not occur in any of the 871 patients (4,633 patient-years) whose baseline HDL-C concentration was ≥90 mg/dl, and an increase in the HDL-C concentration as a result of statin treatment may prevent CHD[27,28] We observed an average reduction of LDL-C concentration of 48 mg/dl below baseline during 6 years of simvastatin treatment, and an increase in HDL-C concentration by 5.2 mg/dl. Using these combined changes in lipid concentrations and previously calculated rate of reduction in coronary events of 15.8% per 10 mg/dl reduction in LDL-C and 37.5% per 10 mg/dl increase in HDL-C, 66% reduction in coronary events during the treatment was predicted.

TG concentration was not a strong risk factor for coronary events in the present study and others have reported that the relationship between the TG concentration and the incidence of MI is greatly affected by the serum TC and HDL-C concentrations of the patient[29,30] In a recent epidemiological study, a close relationship between the concentration of serum TG and coronary events was reported in Japanese patients[31] thus further examination is needed to clarify the relationship.

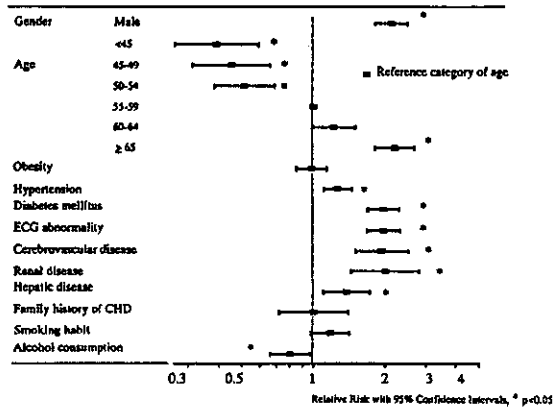

Fig 3. Relationship between the relative risk of death and baseline characteristics of patients maintained on low-dose simvastatin. Bars express the relative risk with a 95% confidence interval. Obesity, body mass index ≥25 kg/m².

We also analyzed the risk of coronary events in 8 subgroups divided by equal number of subjects (approx. 5,200 patients in each group), in addition to the average concentrations of serum lipids during the study. The occurrence of coronary events in relation to the lipid concentrations in this analysis was similar with the result from the subgroups divided by constant interval of serum lipid concentrations. The correlation between the incidence of coronary events and the baseline concentrations of TC, LDL-C or HDL-C was weak in the present study. In another clinical intervention trial, a 6-month simvastatin regimen prevented the occurrence of MI, which was more closely correlated with average TC concentration during treatment rather than the baseline lipid concentrations[8] and our present result is consistent with that finding.

The risk factors for coronary events, other than the average serum lipid concentrations, were male gender, age, hypertension, diabetes mellitus, ECG abnormality, cerebrovascular disease, a family history of CHD and smoking. Numerous epidemiologic studies have documented that hypertension, diabetes mellitus and smoking are risk factors for MI[32–44] To minimize the risk of coronary events, eliminating those risk factors as well as normalizing the lipid concentrations is important, especially for patients with hypertension or diabetes mellitus. Alcohol consumption was a negative risk factor for coronary events and that finding was noted in other several epidemiologic studies[45–49]

In a minority of patients with exceptional lowering of the TC concentration (<160 mg/dl) by low-dose simvastatin therapy, the relative risk of death was similar to the group of patients in which the treatment had little effect on patient TC concentration ≥280 mg/dl. The J-curve was observed between TC or LDL-C concentrations and total mortality: the relative risk of death was higher in patients with a TC concentration <180 mg/dl or ≥260 mg/dl compared with the other patient group. The patients with an exceptionally low

TC concentration, the so-called 'hyper-responders' to simvastatin, had a higher relative risk of death from malignancy than in the other patient groups. Almost all patients in this group showed a marked decrease in TC concentrations at 6 months after starting the treatment and maintained the same concentration throughout the study period, therefore the exceptionally low TC concentration may have been caused by underlying diseases that were responsible for the patient's death. It is still unclear why those hyperresponsive patients responded to simvastatin so remarkably, but the effect should be noted. The 1990 National Heart, Lung, and Blood Institute Conference on Low Blood Cholesterol reported a U-shaped association between serum cholesterol concentration and death based on data from cohort studies[50] and it was concluded that the cause of the higher death rates in those with a TC concentration <160 mg/dl was probably a mixed bag of mechanisms that had yet to be clarified. The PROCAM[51] and MRFIT[52] studies also observed an association between low TC concentration and malignancy. The data of the J-LIT study were obtained from observations during low dose simvastatin (5 mg/day) treatment in which the average serum TC concentration of patients was 270 mg/dl at baseline and decreased to 220 mg/dl during the treatment. On the other hand, the PROCAM and MRFIT were epidemiological studies and therefore we cannot directly compare our results with those studies. Further analysis is necessary to elucidate why the hyper-responders had an increased risk of death; their baseline characteristics will be described and discussed in detail in the future. Nevertheless, the health of patients who show a remarkable decrease in TC or LDL-C concentration with low-dose statin therapy should be monitored closely.

The present study demonstrates a relationship between serum lipid concentrations and the incidence of coronary events in Japanese patients with hypercholesterolemia under low-dose simvastatin treatment. A reasonable strategy to prevent coronary events in Japanese hypercholesterolemic patients without prior CHD under low-dose statin treatment might be to regulate the serum lipid concentrations to at least <240 mg/dl for TC, <160 mg/dl for LDL-C and <300 mg/dl for TG and >40 mg/dl for HDL-C. The relationship between the relative risk of death and the concentrations of the different lipids requires further study.

Study Limitation

Although we have named this study 'The Japan Lipid Intervention Trial', in reality it was a cohort and observational study rather than an intervention study.

Acknowledgments

Authors are grateful to Dr Heizo Tanaka, National Institute of Health and Nutrition for expert statistical analysis of the data. This study was supported in part by a grant from Banyu Pharmaceutical Co, Ltd, Tokyo, Japan.

References

1. National Cholesterol Education Program. Second report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II). *Circulation* 1994; **89:** 1333–1445.
2. Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels: The Framingham Study. *JAMA* 1986; **256:** 2835–2838.
3. Keys A, Menotti A, Aravanis C, Blackburn H, Djordevic BS, Buzina R, et al. The Seven Countries Study: 2,289 deaths in 15 years. *Prev Med* 1984; **13:** 141–154.
4. Sekikawa A, Satoh T, Hayakawa T, Ueshima H, Kuller LH. Coronary heart disease mortality among men aged 35–44 years by prefecture in Japan in 1995–1999 compared with that among white men aged 35–44 by state in the United States in 1995–1998: Vital statistics data in recent birth cohort. *Jpn Circ J* 2001; **65:** 887–892.
5. 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.
6. The Lipid Research Clinics Coronary Primary Prevention Trial. Results I: Reduction in incidence of coronary heart disease. *JAMA* 1984; **251:** 351–364.
7. Frick MH, Elo O, Haapa K, Heinonen OP, Heinsalmi P, Helo P, et al. Helsinki Heart Study: Primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia: Safety of treatment, changes in risk factors, and incidence of coronary heart disease. *N Engl J Med* 1987; **317:** 1237–1245.
8. A co-operative trial in the primary prevention of ischaemic heart disease using clofibrate: Report from the Committee of Principal Investigators. *Br Heart J* 1978; **40:** 1069–1118.
9. Pedersen TR, Wilhelmsen L, Faergeman O, Strandberg TE, Thorgeirsson G, Troedsson L, et al. and Scandinavian Simvastatin Survival Study Group: Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: The Scandinavian Simvastatin Survival Study (4S). *Lancet* 1994; **344:** 1383–1389.
10. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia: West of Scotland Coronary Prevention Study Group. *N Engl J Med* 1995; **333:** 1301–1307.
11. Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, et al. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels: Cholesterol and Recurrent Events Trial investigators. *N Engl J Med* 1996; **335:** 1001–1009.
12. Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA, et al. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: Results of AFCAPS /TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. *JAMA* 1998; **279:** 1615–1622.
13. Tonkin A, Aylward P, Colquhoun D, Glasziou P, Harris P, Hunt D, et al. and the Long-term Intervention with Pravastatin in Ischemic Disease (LIPID) study group. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels: The Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *N Engl J Med* 1998; **339:** 1349–1357.
14. Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, et al. Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc Natl Acad Sci USA* 1980; **77:** 3957–3961.
15. Todd PA, Goa KL. Simvastatin: A review of its pharmacological properties and therapeutic potential in hypercholesterolaemia. *Drugs* 1990; **40:** 583–607.
16. Matsuzawa Y, Itakura H, Kita T, Mabuchi H, Matsuzaki M, Nakaya N, et al and the J-LIT Study Group. Design and baseline characteristics of a cohort study in Japanese patients with hypercholesterolemia: The Japan Lipid Intervention Trial (J-LIT). *Curr Ther Res* 2000; **61:** 219–243.
17. International Statistical Classification of Diseases and Related Health Problems, Vol. 1, 10th Revision. Geneva: World Health Organization, 1992; 471–513.
18. Friedwald WT, Levy RI, Fredrickson DS. Estimation of the level of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972; **18:** 499–502.
19. Investigating Committee on Guidelines for Diagnosis and Treatment of Hyperlipidemias, Japan Atherosclerosis Society. Guidelines for diagnosis and treatment of hyperlipidemia in adults. *Jpn J Atheroscler* 1997; **25:** 1–34.
20. Cox DR. Regression models and life tables (with discussion). *J R Stat Soc* 1972; **B34:** 187–220.
21. Tunstall-Pedoe H, Kuulasmaa K, Mahonen M, Tolonen H, Ruokokoski E, Amouyel P. Contribution of trends in survival and coronary-event rates to changes in coronary heart disease mortality: 10-year results from 37 WHO MONICA project populations: Monitoring trends and determinants in cardiovascular disease. *Lancet* 1999; **353:** 1547–1547.
22. Menotti A, Blackburn H, Seccareccia F, Kromhout D, Nissinen A, Aravanis C, et al. The relation of chronic diseases to all-cause mortality risk: The Seven Countries Study. *Ann Med* 1997; **29:** 135–141.

23. 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.
24. Hidaka Y, Eda T, Yonemoto M, Kamei T. Inhibition of cultured vascular smooth muscle cell migration by simvastatin (MK-733). *Atherosclerosis* 1992; **95:** 87–94.
25. Strandberg TE, Vanhanen H, Tikkanen MJ. Effect of statins on C-reactive protein in patients with coronary artery disease. *Lancet* 1999; **353:** 118–119.
26. O'Driscoll G, Green D, Taylor RR. Simvastatin, an HMG-coenzyme A reductase inhibitor, improves endothelial function within 1 month. *Circulation* 1997; **95:** 1126–1131.
27. Boden WE. High-density lipoprotein cholesterol as an independent risk factor in cardiovascular disease: Assessing the data from Framingham to the Veterans Affairs High-Density Lipoprotein Intervention Trial. *Am J Cardiol* 2000; **86:** 19L–22L.
28. Saku K, Zhang B, Liu R, Shirai K, Arakawa K. Associations among serum lipoprotein(a) levels, apolipoprotein(a) phenotypes, and myocardial infarction in patients with extremely low and high levels of serum lipoprotein(a). *Jpn Circ J* 1999; **63:** 659–665.
29. Austin MA. Plasma triglyceride as a risk factor for coronary heart disease: The epidemiologic evidence and beyond. *Am J Epidemiol* 1989; **129:** 249–259.
30. Assmann G, Schulte H, Funke H, von Eckardstein A. The emergence of triglycerides as a significant independent risk factor in coronary artery disease. *Eur Heart J* 1998; **19:** M8–M14.
31. Iso H, Naito Y, Sato S, Kitamura A, Okamura T, Sankai T, et al. Serum triglycerides and risk of coronary heart disease among Japanese men and women. *Am J Epidemiol* 2001; **153:** 490–499.
32. The Sixth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Arch Intern Med* 1997; **157:** 2413–2446.
33. Jarrett RJ, McCartney P, Keen H. The Bedford survey: Ten year mortality rates in newly diagnosed diabetics, borderline diabetics and normoglycaemic controls and risk indices for coronary heart disease in borderline diabetics. *Diabetologia* 1982; **22:** 79–84.
34. Butler WJ, Ostrander LD Jr, Carman WJ, Lamphiear DE. Mortality from coronary heart disease in the Tecumseh study: Long-term effect of diabetes mellitus, glucose tolerance and other risk factors. *Am J Epidemiol* 1985; **121:** 541–547.
35. Borch-Johnsen K, Kreiner S. Proteinuria: Value as predictor of cardiovascular mortality in insulin dependent diabetes mellitus. *BMJ* 1987; **294:** 1651–1654.
36. Krolewski AS, Kosinski EJ, Warram JH, Leland OS, Busick EJ, Asmal AC, et al. Magnitude and determinants of coronary artery disease in juvenile-onset, insulin-dependent diabetes mellitus. *Am J Cardiol* 1987; **59:** 750–755.
37. Jarrett RJ, Shipley MJ. Type 2 (non-insulin-dependent) diabetes mellitus and cardiovascular disease: Putative association via common antecedents: Further evidence from the Whitehall Study. *Diabetologia* 1988; **31:** 737–740.
38. Morrish NJ, Stevens LK, Head J, Fuller JH, Jarrett RJ, Keen H. A prospective study of mortality among middle-aged diabetic patients (the London Cohort of the WHO Multinational Study of Vascular Disease in Diabetics). II: Associated risk factors. *Diabetologia* 1990; **33:** 542–548.
39. US Department of Health and Human Services. Cardiovascular disease: The health consequences of smoking. DHHS Publication No. (PHS) 84-5024. Washington DC: US Department of Health and Human Services, Public Health Service, Centers for Disease Control, Center for Chronic Disease Prevention and Health Promotion, Office on smoking and Health, 1983.
40. US Department of Health and Human Services. Reducing the health consequences of smoking, 25 years of progress: A report of the Surgeon General. DHHS Publication No. (CDC) 89-8411. Washington DC: US Department of Health and Human Services, Public Health Service, Centers for Disease Control, Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 1989.
41. Willett WC, Green A, Stampfer MJ, Speizer FE, Colditz GA, Rosner B, et al. Relative and absolute excess risks of coronary heart disease among women who smoke cigarettes. *N Engl J Med* 1987; **317:** 1303–1309.
42. Kannel WB, Neaton JD, Wentworth D, Thomas HE, Stamler J, Hulley SB, et al. Overall and coronary heart disease mortality rates in relation to major risk factors in 325,348 men screened for the MRFIT (Multiple Risk Factor Intervention Trial). *Am Heart J* 1986; **112:** 825–836.
43. LaCroix AZ, Lang J, Scherr P, Wallace RB, Cornoni-Huntley J, Berkman L, et al. Smoking and mortality among older men and women in three communities. *N Engl J Med* 1991; **324:** 619–625.
44. Jonas MA, Oates JA, Ockene JK, Hennekens CH. Statement on smoking and cardiovascular disease for health care professionals: American Heart Association. *Circulation* 1992; **86:** 1664–1669.
45. Stampfer MJ, Colditz GA, Willett WC, Speizer FE, Hennekens CH. A prospective study of moderate alcohol consumption and the risk of coronary disease and stroke in women. *N Engl J Med* 1988; **319:** 267–273.
46. Rimm EB, Giovannucci EL, Willett WC, Colditz GA, Ascherio A, Rosner B, et al. Prospective study of alcohol consumption and risk of coronary disease in men. *Lancet* 1991; **338:** 464–468.
47. Kitamura A, Iso H, Sankai T, Naito Y, Sato S, Kiyama M, et al. Alcohol intake and premature coronary heart disease in urban Japanese men. *Am J Epidemiol* 1998; **147:** 59–65.
48. Solomon CG, Hu FB, Stampfer MJ, Colditz GA, Speizer FE, Rimm EB, et al. Moderate alcohol consumption and risk of coronary heart disease among women with type 2 diabetes mellitus. *Circulation* 2000; **102:** 494–499.
49. Ajani UA, Gaziano JM, Lotufo PA, Liu S, Hennekens CH, Buring JE, et al. Alcohol consumption and risk of coronary heart disease by diabetes status. *Circulation* 2000; **102:** 500–505.
50. Hulley SB, Walsh JM, Newman TB. Health policy on blood cholesterol: Time to change directions. *Circulation* 1992; **86:** 1026–1029.
51. Cullen P, Schulte H, Assmann G. The Munster Heart Study (PROCAM): Total mortality in middle-aged men is increased at low total and LDL cholesterol concentrations in smokers but not in non-smokers. *Circulation* 1997; **96:** 2128–2136.
52. Sherwin RW, Wentworth DN, Cutler JA, Hulley SB, Kuller LH, Stamler J. Serum cholesterol levels and cancer mortality in 361,662 men screened for the Multiple Risk Factor Intervention Trial. *JAMA* 1987; **257:** 943–948.

GATA-6 Is Involved in PPARγ-Mediated Activation of Differentiated Phenotype in Human Vascular Smooth Muscle Cells

Mitsuru Abe, Koji Hasegawa, Hiromichi Wada, Tatsuya Morimoto, Tetsuhiko Yanazume, Teruhisa Kawamura, Maretoshi Hirai, Yutaka Furukawa, Toru Kita

Objective—Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear receptor superfamily involved in the growth and differentiation of many cell types. Although the activation of PPARγ in human vascular smooth muscle cells (VSMCs) inhibits the growth of these cells, the precise mechanism of this effect is unknown. PPARγ-mediated growth inhibition of VSMCs is associated with the induction of the differentiated phenotype. A zinc finger transcription factor, GATA-6, has been implicated in the maintenance of the differentiated phenotype in VSMCs.

Methods and Results—The administration of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a naturally occurring PPARγ ligand, and troglitazone, a thiazolidinedione derivative, induced the expression of smooth muscle myosin heavy chain and smooth muscle α-actin, highly specific markers for differentiated VSMCs. Stimulation of proliferative VSMCs with PPARγ ligands also increased the activity of the transfected wild-type smooth muscle myosin heavy chain promoter but not that of the mutant promoter, in which a GATA-6 binding site was mutated. Compatible with the role of GATA-6, both 15d-PGJ_2 and troglitazone upregulated the DNA binding activity of GATA-6 in proliferative VSMCs.

Conclusions—The activation of PPARγ-dependent pathways induces the differentiated phenotype in proliferative VSMCs, and this induction is mediated, in part, through a GATA-6–dependent transcriptional mechanism. (*Arterioscler Thromb Vasc Biol.* 2003;23:404-410.)

Key Words: peroxisome proliferator-activated receptor-γ ■ GATA-6 ■ vascular smooth muscle cells ■ phenotypic modulation

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily. PPARγ, the most intensively studied isoform, has been implicated in such diverse pathways as lipid and glucose homeostasis, control of cellular proliferation, and differentiation.[1,2] The activation of PPARγ with a ligand, such as a class of antidiabetic, insulin-sensitizing agents known as thiazolidinediones,[3] or eicosanoid derivatives, including 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2),[4] results in its heterodimerization with the retinoid X receptor and the binding of their complex to the PPAR response element (PPRE) of target genes.[5] Since its discovery, PPARγ has been shown to be expressed in monocytes/macrophages, the heart, vascular smooth muscle cells (VSMCs), endothelial cells, and atherosclerotic lesions.[6,7] Recently, it has been shown that thiazolidinediones inhibit the development of atherosclerosis in LDL receptor–deficient mice[8] and apolipoprotein E–knockout mice[9] and of restenosis in rat models of balloon angioplasty.[10] In vitro, the thiazolidinediones and 15d-PGJ_2 inhibit the proliferation of VSMCs through a PPARγ-dependent pathway.[7,10] However, the precise mechanisms of this growth inhibition are unknown at present.

In contrast to normal medial VSMCs, the proliferating VSMCs within thickened arterial intimas observed in atherosclerosis express low levels of proteins that are characteristic of differentiated VSMCs.[11,12] These proteins include smooth muscle isoforms of contractile proteins, such as smooth muscle myosin heavy chain (SM-MHC) and smooth muscle α-actin (SM-α-actin).[11] Although the mechanisms involved in specifying the proliferative/synthetic or differentiated/contractile VSMC phenotype are largely unknown, the recent observations suggest the role of a zinc finger transcriptional protein, GATA-6, as an important regulator of VSMC phenotype.[13–15] We have recently demonstrated that GATA-6 binds a conserved GATA-like motif within the rat SM-MHC promoter and activates this promoter in a sequence-specific manner. In addition, this motif is required for the upregulated expression of the SM-MHC gene during differentiation of VSMCs.[16] For all of these reasons, we are interested in investigating the role of GATA-6 in PPARγ-mediated growth inhibition/differentiation of VSMCs. To achieve this, we

Received July 8, 2002; revision accepted January 5, 2003.

From the Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

Correspondence to Koji Hasegawa, MD, PhD, Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan. E-mail koj@kuhp.kyoto-u.ac.jp

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000059405.51042.A0

examined whether PPARγ ligands such as 15d-PGJ_2 and troglitazone activate the transcription of a smooth muscle–specific contractile protein, and if so, whether GATA-6 is involved in this process.

Methods

Plasmid Constructs

A plasmid pSM-MHC chloramphenicol acetyltransferase CAT, consisting of the bacterial CAT driven by 1346 bp of the rat SM-MHC gene promoter,[17] was a generous gift from Dr Gray K. Owens (University of Virginia, Charlottesville). Plasmid constructs pwtSM-MHCluc and pmutSM-MHCluc contain firefly luciferase reporter cDNA driven by the wild-type 836-bp rat SM-MHC promoter or by the corresponding promoter in which the GATA-6 binding site is mutated, respectively.[16] pRSVCAT and pRSVluc contain the reporter gene driven by Rous sarcoma virus long-terminal repeat sequences.[18] The expression plasmid encoding human GATA-6 (phGATA-6) and that encoding human PPARγ (phPPARγ) were kindly donated by Dr Kenneth Walsh (Boston University, Boston, Mass)[13] and Dr Krishna Chatterjee (University of Cambridge, Cambridge, UK),[19] respectively. pPPREluc, containing 3 copies of the PPRE (GTCGACAGGGGACCAGGACAAAGGTCACG-TTCGGGAGTCGAC) in direct orientation upstream of the basal reporter construct tk-luc, was provided by Dr Kazuhiko Umesono (Kyoto University, Kyoto, Japan).[5] Plasmids were purified by anion exchange chromatography (Qiagen), quantified by the measurement of OD_{260}, and examined on agarose gels stained with ethidium bromide before use.

Cell Culture

Human aortic VSMCs were obtained from Kurabo Industries Ltd and cultured with 5% fetal bovine serum in the presence of growth factors as previously described.[16] To induce the differentiated phenotype, VSMCs were cultured in low-serum media (1% fetal bovine serum) in the absence of growth factors.

Measurement of DNA Synthesis and Assay for Cellular Viability

The incorporation of the thymidine analogue, 5-bromo-2′-deoxyuridine (BrdU), was measured to determine the effects of 15d-PGJ_2 or troglitazone on DNA synthesis. VSMCs (passage 6) were plated in a 96-well microplate and cultured in low-serum media (1% fetal bovine serum) in the absence of growth factors for 48 hours to induce the differentiated phenotype. Thereafter, these cells were stimulated with growth factors and 5% fetal bovine serum to reenter the cell cycle in the presence or absence of 15d-PGJ_2 or troglitazone for 18 hours. The cells kept as the differentiated phenotype were used as a control. BrdU was then added, and the incubation was continued for an additional 6 hours. Subsequent BrdU incorporation assays were carried out according to the protocols supplied by the manufacturer (Biotrak, Amersham Pharmacia Biotech).

A colorimetric assay based on the mitochondrial activity of living cells to cleave the tetrazolium ring and reduce the tetrazolium salt, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Wako Pure Chemicals Industries, Osaka), to formazan was used to quantify cell viability as described previously.[20]

Immunocytochemistry and Western Blotting

Immunocytochemical staining was performed by the use of an anti–SM-MHC monoclonal antibody (Sigma Chemicals) indirect immunoperoxidase method as described previously.[18] For Western blotting, we used monoclonal antibodies (SM-MHC and β-actin obtained from Sigma Chemicals; SM-α-actin from Dako Laboratories). Immunoblots were quantified by densitometry by using NIH Image 1.62 as described previously.[18]

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from VSMCs as described previously.[16] Double-stranded wild-type oligonucleotide (wt SM-MHC GATA) from rat SM-MHC upstream sequences (−827/−795 relative to the transcription start site) and mutant oligonucleotide (mut SM-MHC GATA) of corresponding length, in which the GATA-6 binding site was mutated, were prepared as previously described.[16] An oligonucleotide containing consensus PPRE (5′-CAAAAC-TAGGTCAAAGGTCA-3′),[5] mutant PPRE (5′-CAAAACTAG-CACAAAGCACA-3′), or the consensus Sp1 site was purchased from Santa Cruz Biotechnology. Electrophoretic mobility shift assays (EMSAs) were carried out as described previously.[18]

Transfection and Luciferase/CAT Assays

VSMCs (passages 6) were transfected with 2 μg of DNA in a 60-mm plate by using lipofectAMINE PLUS (Life Technologies, Inc) as previously described.[16] The lysates from these cells were subjected to assays for luciferase and CAT activities as described previously.[18]

Statistical Analysis

Data are presented as mean±SE. Statistical comparisons were performed by unpaired 2-tailed Student's t tests or ANOVA with Scheffe's test where appropriate, with $P<0.05$ indicating significance.

Results

15d-PGJ_2 and Troglitazone Inhibit DNA Synthesis in Proliferative VSMCs

To examine the effect of PPARγ ligands such as 15d-PGJ_2 and thiazolidinediones on human VSMC proliferation, we measured BrdU, a thymidine analogue, incorporation in the presence of various concentrations of 15d-PGJ_2 or troglitazone after stimulation with growth factors and 5% fetal bovine serum. As shown in Figure 1A, administering 10^{-9} to 10^{-7} mol/L of 15d-PGJ_2 (Cayman Chemical Co) into proliferative VSMCs resulted in mild but not significant inhibition of BrdU incorporation. The inhibition was significant at concentrations of 10^{-6}, 5×10^{-6}, and 10^{-5} mol/L. As shown in Figure 1C, administering 10^{-9} to 10^{-6} mol/L of troglitazone (a gift from Sankyo Pharmaceutical Co, Tokyo, Japan) into proliferative VSMCs did not significantly inhibit BrdU incorporation, although it appeared to have some inhibitory effects. Significant inhibition was observed at the concentrations of 5×10^{-6} and 10^{-5} mol/L.

To rule out the possibility that 15d-PGJ_2 and troglitazone induced VSMC death, cell viability was assessed with the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The data in Figures 1B and 1D show that 10^{-5} mol/L of 15d-PGJ_2 or troglitazone induced cell death. However, 15d-PGJ_2 or troglitazone in the range of 10^{-9} to 5×10^{-6} mol/L had no effect on cell viability, as indicated by equivalent mitochondrial activity in untreated and treated VSMCs of proliferative phenotype.

15d-PGJ_2 and Troglitazone Induce the Expression of SM-MHC and SM-α-Actin in Proliferative VSMCs

To explore whether PPARγ ligands induce the differentiated phenotype, we examined the effects of 15d-PGJ_2 or troglitazone on the expression of SM-MHC and SM-α-actin, highly specific markers for differentiated VSMCs. Proliferative VSMCs were incubated with 15d-PGJ_2 (10^{-9} to 10^{-6} mol/L)