arteriosclerosis using PWV measurement, this measurement will become an extremely useful means for the prevention of cardiovascular diseases in the elderly. It has been reported that both the frequency of calcification of the aorta, as seen on CT images, and carotid intima-media thickness, as measured on echoaortographic images, increase as PWV increases.[11] It has also been reported that cfPWV is significantly higher in patients with hypertension and/or diabetes mellitus.[2,3,12] Blacher *et al.* found by comparison of values of cfPWV in subjects with and without atherosclerosis that PWV in subjects with atherosclerosis was significantly higher than that in subjects without atherosclerosis, and they reported that the risk of cardiovascular disease, even in subjects without atherosclerosis, increased with increases in cfPWV.[13]

In a previous study, we examined baPWV and impaired glucose tolerance in middle-aged and elderly male subjects of a general population, who participated in the medical examination in the same towns in 2000, and we showed that baPWV is a useful indicator of the progression of arteriosclerosis and that baPWV starts to increase not from the DM stage but from the borderline blood sugar stage.[3] The results of that study also suggested that the blood sugar level is likely to promote the progression of arteriosclerosis in a manner dependent on their values.

In the present investigation, we measured SBP, DBP, BMI, FBS, TC, TG, HDL and baPWV in elderly male subjects of a general population. The values of BMI, TC, TG and HDL in those subjects are comparable to mean values for age-matched Japanese, and there were no significant intergroup differences (Table 1). Therefore, it may be possible to directly assess the relationships of baPWV with blood pressure and blood sugar levels without the effects of other risk factors. In the present study, subjects with ABI ≤ 0.9, indicating arteriosclerosis obliterans,[5] were excluded. Subjects with a past history of arteriosclerotic diseases such as angina pectoris, myocardial infarction and cerebral infarction were also excluded. The subjects in the present study can therefore be considered to be relatively healthy.

In the elderly subjects in the present investigation, as was also previously reported for middle-aged subjects, baPWV increased as blood pressure and blood sugar level increased, and positive correlations were found between blood pressure and baPWV and between blood sugar level and baPWV (Fig. 1). In the NT group, baPWV did not increase significantly with an increase in the level of blood sugar. We have already examined the relationship between baPWV and impaired glucose tolerance in middle-aged people and found that baPWV was already starting to increase at the borderline blood sugar stage.[3] Although further study is needed to determine the reason for the difference between baPWV in middle-aged people and that in elderly people, the results of the present study suggest that the combination of hypertension and impaired glucose tolerance in elderly people promotes the progression of arteriosclerosis. It is also thought that maintenance of normal blood pressure until an elderly age delays the progression of arteriosclerosis by impaired glucose tolerance.

A comparison of the NT and HT groups showed that baPWV increased significantly regardless of glucose tolerance impairment, and baPWV in the HT group increased as the level of blood sugar increased. A tendency for baPWV to be high was found even at the IFG stage, and baPWV was significantly higher in the DM group than in the NGT group ($P = 0.002$). The results suggest that baPWV is elevated in people with HT even at the stage of IFG and that, conversely, arteriosclerosis strongly progresses in people with IFG if complicated by hypertension. By a multiple regression analysis using baPWV as a dependent variable, age, SBP and FBS level were selected as independent variables. It is conceivable that SBP and FBS level independently contribute to the elevation of baPWV. The above-described results suggest that strict control of blood pressure and blood sugar levels in the elderly might be necessary for the prevention of arteriosclerosis.

It is well known that PWV increases as age increases. This is thought to reflect the development of arteriosclerosis due to aging. However, to date, it has been difficult to detect differences in the degree of progression of arteriosclerosis by the presence or absence of risk factors. There have been very few studies in which the relationship between risk factors and arteriosclerosis in elderly subjects was investigated using change in PWV as an end point. Mackey *et al.* recently reported results of a 4-year follow-up study on the relationship between arteriosclerosis and risk factors in elderly subjects in whom cfPWV had been measured.[14] They used measurements of cfPWV to estimate the degree of arteriosclerosis in 356 male and female subjects aged 70–96 years, and they reported that the degree of arteriosclerosis was positively correlated with systolic blood pressure, age, fasting blood sugar level and blood sugar level 2 h after a meal, insulin level 2 h after a meal, triglyceride, girth of the abdomen and heart rate. After adjustment for age and blood pressure, heart rate in men and heart rate as well as girth of the abdomen in women remained positively correlated with degree of arteriosclerosis. Their results suggest that the degree of arteriosclerosis in the elderly is related to states of risk factors, particularly heart rate and insulin resistance, during the past several years. Although our investigation was a cross-sectional study on baPWV and current risk factors, the finding of a significant correlation between elevation of blood sugar level and baPWV suggests that there is a link between insulin resistance and baPWV. Our results are considered to be compatible with the results reported by Mackey *et al.*

In our previous cohort study, we investigated the relationships between FBS and plasma glucose concentrations 2 h after load (2h-PG) in 1991 and 1992 and baPWV in 2001 and 2002.[15] Significant positive correlations were found between 2h-PG in 1991 and 1992 and baPWV in 2001 and 2002. Multiple regression analysis was performed on baPWV with regard to sex, BMI, TC and 2h-PG, and then 2h-PG was selected as an independent variable. In that cohort study, we suggested that 2h-PG plays a key role in the increase in baPWV after 10 years. The DECODE study group recently reported results of a 7-year follow-up study on the relationships of FBS and 2h-PG with risk of death.[16] They suggested that 2h-PG as well as FBS identified individuals at increased risk of death. In our cohort study, baPWV was higher in both the IFG group and the IGT group than in the NGT group. It was not clarified whether baPWV was affected more by an increase in FBS or by an increase in 2h-PG. In our cohort study, baPWV, FBS and 2h-PG were not measured at the same time. Further investigation is therefore need.

Rachel *et al.* recently reported that excess body weight is associated with higher aortic stiffness in whites and African Americans.[17] They used measurements of cfPWV in 186 young adults (20–40 years old) and 177 older adults (41–70 years old), and the subjects were divided into three groups on the basis of BMI: a normal weight group (BMI < 25 kg/m^2), an overweight group ($25 \leq$ BMI ≤ 30 kg/m^2) and an obese group (BMI > 30 kg/m^2). They found a significant positive correlation between BMI and cfPWV. In the obese group, cfPWV was higher than that in the normal weight group. Although cfPWV in the overweight group was not higher than in the normal weight group in 20–30 year old subjects, it increased in parallel with increases in age and finally reached the same level as that in the obese group in 41–59 year old subjects. Their results suggest that BMI is the strongest independent predictor of cfPWV. Nakanishi *et al.* recently reported that BMI affects cfPWV after 9 years in 2186 Japanese males.[18] In our previous cohort study, we also found that BMI affects baPWV after 10 years in 530 Japanese subjects. In the present cross-sectional study, there was no correlation between BMI and baPWV, and BMI was not selected as an independent variable in multiple regression analysis performed on baPWV. The discrepancy between the results for white and African Americans and those for Japanese might be due to differences in races. This was because very few Japanese have a BMI greater then 30 kg/m^2. A further cross-sectional study is needed to determine whether visceral fat accumulation or waist to hip ratio affects baPWV in Japanese people. In our present cross-sectional study, BMI did not affect baPWV. On the other hand, in the previous longitudinal study, it was suggested that BMI has an influence on PWV after about 10 years in Japanese. Further study is needed to determine the time at which a high BMI starts to affect PWV in Japanese people.

Hypertension and diabetes mellitus are risk factors for arteriosclerosis, and their combination exerts a synergistic effect on the progression of arteriosclerosis and on the development of arteriosclerotic disease. The results of the present study suggest that strict control of blood pressure and blood sugar level might be necessary in order to prevent the development of arteriosclerotic diseases even in the elderly if mild impairment of glucose tolerance is combined with hypertension.

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Editor's comment

This article was selected by the Japan Geriatric Society for its outstanding contribution to geriatrics.

Original Article

Olmesartan Ameliorates Insulin Sensitivity by Modulating Tumor Necrosis Factor-α and Cyclic AMP in Skeletal Muscle

Koichi YAMAGUCHI, Nobuyuki URA, Hideyuki MURAKAMI, Nobuhiko TOGASHI, Masaya HYAKUKOKU, Katsuhiro HIGASHIURA, and Kazuaki SHIMAMOTO

We have reported that tumor necrosis factor (TNF)-α in skeletal muscle is one of the determinants of insulin resistance and that the renin-angiotensin system may be related to the regulation of TNF-α in skeletal muscle. Recent studies have suggested the involvement of cyclic adenosine monophosphate (cAMP) in the regulation of TNF-α in vascular smooth muscle cells or monocytes. The aim of this study was to determine the relationship between cAMP and TNF-α in skeletal muscle in connection with the renin-angiotensin system. Six-week-old male Sprague-Dawley rats were fed either normal rat chow or fructose-rich chow for 6 weeks. For the last 2 weeks of a 6-week period, the rats were treated with a vehicle or with an angiotensin II type 1 receptor antagonist (olmesartan medoxomil, 0.1 mg/kg/day). TNF-α levels in the soleus muscle were significantly higher and cAMP levels in the soleus muscle were significantly lower in fructose-fed rats than in control rats. Olmesartan increased cAMP and reduced TNF-α simultaneously in fructose-fed rats. There was a significant negative correlation between levels of cAMP and TNF-α. Moreover, a cAMP analogue reduced TNF-α levels in the soleus muscle. These results indicate that the increase in TNF-α *via* suppression of cAMP may affect the induction of insulin resistance. In addition, the facts that olmesartan increased cAMP and decreased TNF-α suggest that a part of the TNF-α regulation by angiotensin II might consist of modulation of cAMP through Gi protein activation in skeletal muscle. (*Hypertens Res* 2005; 28: 773–778)

***Key Words*: insulin resistance, cyclic adenosine monophosphate, tumor necrosis factor-α, angiotensin II**

Introduction

Recently, insulin resistance (IR) and hyperinsulinemia have been shown to be common findings in patients with essential hypertension, diabetes mellitus, or hyperlipidemia (*1*). These impairments of glucose metabolism are thought to be associated with each other and to play a role in the development of arteriosclerosis and cardiovascular diseases (*2*, *3*). IR has been reported in several animal models of hypertension, including spontaneously hypertensive rats (*4*) and fructose-fed rats (FFR). In FFR, it has been shown that a diet of fructose induces a decrease in insulin sensitivity and an increase in blood pressure (BP) (*5*). Skeletal muscle is thought to be a major regulator of systemic insulin sensitivity. Euglycemic hyperinsulinemic glucose clamp studies have demonstrated that skeletal muscle accounts for over 80% of glucose disposal under hyperinsulinemic conditions in humans. Lesions of atherosclerosis occur mainly in large and medium-sized elastic and muscular arteries and can lead to ischemia of the heart, brain or extremities, resulting in infarction. Recently, Ross hypothesized that one of the mechanisms by which atherosclerosis occurs involves cytokines derived from monocytes and T lymphocytes (*6*).

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Table 1. Characteristics of Animals at 12 Weeks of Age

Characteristics	SD (*n*=8)	FFR (*n*=14)	FFR+olmesartan (*n*=9)
BW (g)	388±10	378±9	383±6
SBP (mmHg)	132±2	144±3*	136±4
PR (bpm)	318±7	331±6	330±16
FBG (mmol/l)	4.8±0.2	4.7±0.2	4.7±0.2

Values are means±SEM. SD, Sprague-Dawley rats; FFR, fructose-fed rats; FFR+olmesartan, fructose-fed rats treated with olmesartan for 2 weeks. BW, body weight; SBP, systolic blood pressure; PR, pulse rate; FBG, fasting blood glucose. *$p<0.05$ *vs.* SD, FFR+olmesartan.

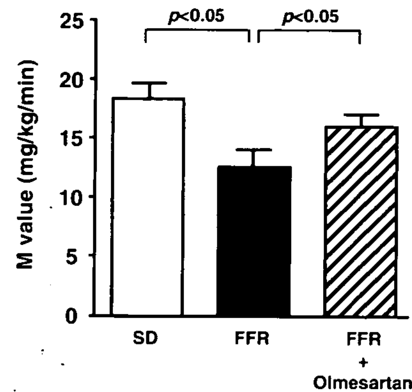

Fig. 1. *Effect of olmesartan on insulin sensitivity (M value) of FFR. Values are the means±SEM. SD, Sprague-Dawley rats (n=8); FFR, fructose-fed rats (n=14); FFR+olmesartan, fructose-fed rats treated with olmesartan for 2 weeks (n=9).*

Tumor necrosis factor (TNF)-α is a multipurpose cytokine that modulates immune functions, induces apoptotic cell death (*7*) and lysis of tumor cells (*8*), and stimulates production of other cytokines (*9*). Recent studies have suggested that TNF-α mediates IR by suppressing insulin signal transduction; TNF-α induces enhancement of serine phosphorylation of insulin receptor substrate (IRS)-1, resulting in inhibition of IRS-1 tyrosine phosphorylation by insulin. TNF-α is expressed in both adipose tissue and skeletal muscle, and many animal models of obesity and IR have been shown to have significantly higher TNF-α mRNA and protein levels than those in lean controls (*9*). We recently reported that IR and hypertension in FFR were concomitant with an increase in TNF-α in skeletal muscle and that an angiotensin II type 1 receptor blocker (ARB) decreased skeletal muscle TNF-α and improved IR in FFR (*10*). However, the relationship between IR and the mechanism of the regulation of TNF-α is not clear.

Recent studies have shown that cyclic adenosine monophosphate (cAMP) inhibits the release of TNF-α in human vascular smooth muscle, monocytes and hepatocytes (*11–13*). However, there have been no reports of the involvement of cAMP in the regulation of TNF-α in skeletal muscles. To evaluate the effects of angiotensin II on insulin resistance in skeletal muscle, TNF-α and cAMP were investigated simultaneously.

Methods

Study-1: Measurement of BP, Pulse Rate (PR) and Insulin Sensitivity in FFR

Six-week-old male Sprague-Dawley rats (Charles River Japan Inc., Yokohama, Japan) were used for the experiments. The care of the animals was in strict accordance with the guiding principles of the Physiological Society of Japan. Before any manipulation, all rats were fed standard rat chow containing 60% vegetable starch, 5% fat and 24% protein (Oriental Yeast Co., Tokyo, Japan). They were maintained on a 12-h light/dark cycle and chow *ad libitum*. The rats were acclimated to handling before randomization and then divided into two groups at the start of the study: those fed standard chow (SD group, *n*=8) or those fed fructose-rich chow containing 60% fructose, 5% fat and 24% protein (#78463; Teklad, Madison, USA) (FFR group, *n*=23) for 6 weeks. The latter group of rats was treated either with 0.1 mg/kg/day of olmesartan medoxomil, an ARB, in 2.5% gum arabic solution (FFR+olmesartan, *n*=9) or with a vehicle (2.5% gum arabic solution) (FFR, *n*=14), and controls were treated with the same vehicle, by gavage, for the last 2 weeks.

BP and PR Measurement

Systolic blood pressure (SBP) and PR were measured in all conscious rats using the indirect tail-cuff method. The averages of six such recordings were taken as the individual SBP and PR. Results obtained using this method were highly correlated with direct measurements.

Euglycemic Hyperinsulinemic Glucose Clamp Technique

At the end of the treatment period, rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The left common carotid artery and the left jugular vein were exposed and then cannulated with a polyethylene tube (PE50; Becton Dickinson and Co., Sparks, USA) for collecting blood samples and for administration of the infusate. After overnight fasting (approximately 12 h), each rat was placed in a foam plastic jacket that allowed movement of all four limbs and forward vision. At the start of the clamp, fasting blood glucose (FBG) was measured, and the initial load of insulin (25 mU/kg of Humalin R, U-40; Shionogi Pharmaceutical Co., Osaka, Japan) was infused by a bolus, followed by an infusion of insulin at a rate of 4 mU/kg/min for 154 min. During the clamp, 12.5% glucose solution was infused as needed to maintain blood glucose at the preinfusion level. Ten microliters of arterial blood was sampled at 7-min intervals for deter-

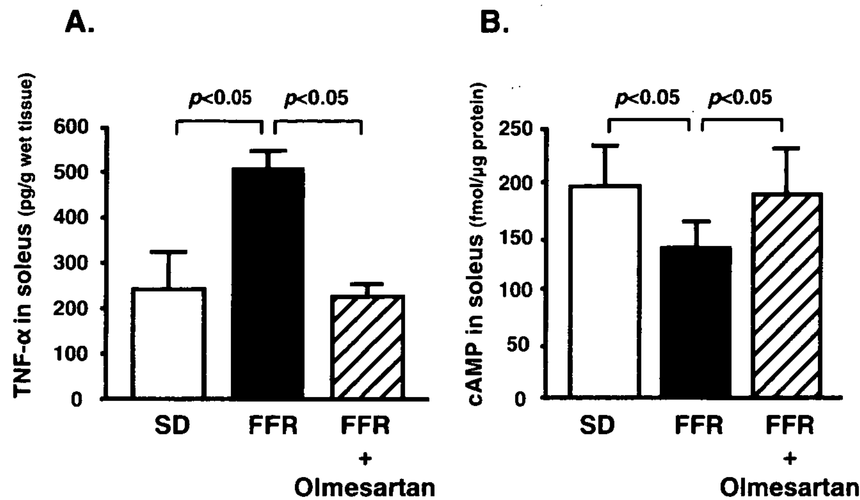

Fig. 2. *A: Comparison of TNF-α levels in the soleus muscle. B: Comparison of cAMP levels in the soleus muscle. Values are the means±SEM. SD, Sprague-Dawley rats* (n = 7)*; FFR, fructose-fed rats* (n = 9)*; FFR+olmesartan, fructose-fed rats treated with olmesartan for 2 weeks* (n = 7)*.*

mination of blood glucose. The average of the rate of glucose infusion for the last 35 min was taken as the index of insulin sensitivity (*M* value) of each rat.

Study-2: Measurement of TNF-α and cAMP in the Soleus Muscle in FFR

Biochemical Measurements of TNF-α and cAMP

Based on the results of Study-1, we prepared another three groups (SD, n=7; FFR, n=9; FFR+olmesartan, n=7), for measurement of TNF-α and cAMP. Rats were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) and perfused with 150 ml of sterile saline through the apex of the left ventricle, and then the soleus muscles were dissected immediately. The soleus muscles were weighed and frozen in liquid nitrogen. TNF-α and cAMP were investigated simultaneously.

To measure TNF-α, the muscles were homogenized with a Polytron (Kinematica, Luzern, Switzerland) in ice-cold 0.1 mol/l Tris-HCl buffer (pH 7.6) containing 1 mol/l NaCl, 2% low endotoxin, fatty acid free bovine serum albumin (BSA; Sigma Co., St. Louis, USA), 2 mmol/l ethylenediaminetetraacetic acid (EDTA disodium salt), 80 trypsin inhibitory units of aprotinin/l and 0.02% NaN_3 (buffer A) at 15% wet tissue wt/vol. The solution was centrifuged at 13,500 × g for 30 min, and the supernatant was subjected to a TNF-α assay (*14*). TNF-α was measured with a rat ELISA kit (Biosource International, Camarillo, USA).

To measure cAMP, the remaining soleus muscles were homogenized with 0.5 ml acidic ethanol (1 mol/l HCl:ethanol, 1:100). Homogenates were transferred to eppendorf tubes and centrifuged at 12,000 × g for 15 min. The supernatant solutions were combined and evaporated to dryness under a stream of nitrogen at 55°C. The residue was dissolved in 50 mmol/l Tris and 4 mmol/l EDTA buffer (pH 7.5) and then frozen at -20°C until assayed. The volume of buffer (0.1 to 2.0 ml) for dissolving the residue was selected so that a 50-μl aliquot of the sample would fall within the range of maximum sensitivity (0.5 to 4.0 pmol) for the cAMP assay. cAMP was assayed using a commercial protein-binding kit (Amersham, Arlington Heights, USA). Except for bovine insulin (Calbiochem, San Diego, USA), all chemicals were obtained from Sigma (*15*).

Study-3: Effects of cAMP on Regulation of TNF-α in the Soleus Muscle

Six-week-old male Sprague-Dawley rats were anesthetized with sodium pentobarbital and perfused with 150 ml of sterile saline through the apex of the left ventricle, and then the soleus muscles were cut longitudinally into small strips (25–35 mg/strip) under sterile conditions (*16*). After dissection, the soleus muscle strips were immediately pre-incubated in 1 ml of Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Life Technologies, Inc., Rockville, USA) containing 2% BSA and then incubated in 1 ml DMEM containing 2% BSA with 0.1 mmol/l dibutyryl cAMP (dBcAMP) as a cAMP analogue, or without dBcAMP, for 4 h. The medium was then subjected to a TNF-α assay.

Statistical Analysis

All data are expressed as the means±SEM. Data were statistically analyzed with one-way analysis of variance (ANOVA) followed by Fisher's PLSD test for multiple comparisons. Regression analyses were used to compare the relationship

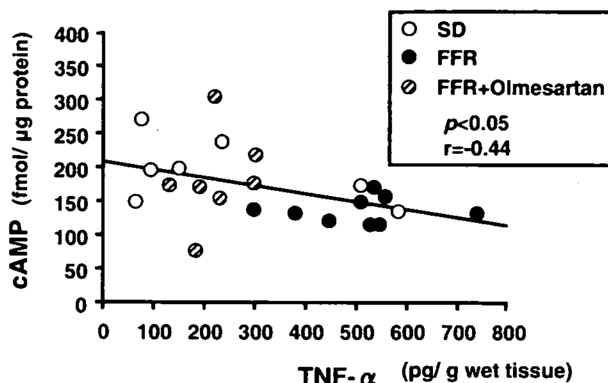

Fig. 3. *Correlation between cAMP and TNF-α levels in the soleus muscle. SD, Sprague-Dawley rats; FFR, fructose-fed rats; FFR+olmesartan, fructose-fed rats treated with olmesartan for 2 weeks.*

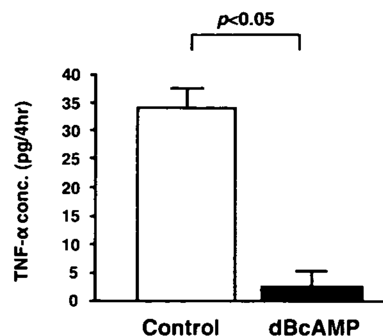

Fig. 4. *Comparison of TNF-α concentrations in the soleus muscles of SD rats. Values are the means±SEM (n=4). dBcAMP, soleus muscle treated with dBcAMP for 4 h.*

between TNF-α and cAMP. Values of $p<0.05$ were considered to indicate statistical significance.

Results

Study-1

As shown in Table 1, SBP in FFR was significantly higher than that in the control rats, and olmesartan significantly lowered SBP in FFR. There were no significant intergroup differences in body weight, PR, and FBG. As shown in Fig. 1, the *M* value was significantly lower in FFR than in the control rats (18.4±1.3 mg/kg/min and 12.6±1.4 mg/kg/min in the control and FFR groups, respectively, $p<0.05$). Olmesartan significantly improved the *M* values of FFR (16.9±1.7 mg/kg/min in the FFR+olmesartan group, $p<0.05$).

Study-2

As shown in Fig. 2a, TNF-α in the soleus muscle increased significantly in FFR compared to that in control rats (242±81 pg/g wet tissue and 503±41 pg/g wet tissue in the controland FFR groups, respectively, $p<0.05$). Olmesartan significantly lowered TNF-α in the soleus muscle (220±23 pg/g wet tissue in the FFR+olmesartan group, $p<0.05$).

As shown in Fig. 2b, cAMP in the soleus muscle decreased significantly in FFR compared to that in control rats (195±48 fmol/μg protein and 138±19 fmol/μg protein in the control and FFR groups, respectively, $p<0.05$). Olmesartan significantly increased cAMP in the soleus muscle (186±66 fmol/μg protein in the FFR+olmesartan group, $p<0.05$). The cAMP level was significantly correlated with the TNF-α level in the soleus muscle (Fig. 3).

Study-3

dBcAMP significantly decreased the secretion of TNF-α from the soleus muscle in SD rats (2.5±2.8 pg/ml/4 h and 34.1±3.3 pg/ml/4 h for incubation with or without 0.1 mmol/l dBcAMP, respectively, $p<0.001$) (Fig. 4).

Discussion

This study confirmed previously reported results showing that feeding healthy rats fructose-rich chow results in IR and hypertension. Skeletal muscle TNF-α is associated with IR (*9*). Treatment with olmesartan resulted in recovery of the *M* value in FFR. We demonstrated that olmesartan significantly increased cAMP and lowered skeletal muscle TNF-α. The cAMP level was significantly correlated with the TNF-α level in FFR. Several possible mechanisms of IR have been proposed. These mechanisms include sympathetic nerve and renin-angiotensin system (RAS) activation and an increase in TNF-α in fat tissue or muscle. Recent studies have suggested that TNF-α mediates IR by suppressing insulin signal transduction; TNF-α induces enhancement of serine phosphorylation of IRS-1, resulting in inhibition of IRS-1 tyrosine phosphorylation by insulin. TNF-α is expressed in both adipose tissue and skeletal muscle, and many animal models of obesity and IR have been shown to have significantly higher TNF mRNA and protein levels than those in lean controls (*9*). We recently reported that IR and hypertension in FFR are concomitant with an increase in TNF-α in skeletal muscle (*10*). We have also reported that TNF-α significantly suppressed [^{3}H]2-deoxyglucose (2-DOG) uptake by insulin and decreased IRS-1 tyrosine phosphorylation in L6 myotubes derived from rat skeletal muscle (*17*). Also, a TNF-α converting enzyme inhibitor that inacivates TNF-α has been shown to improve IR in FFR (*18*).

Several studies have shown the effects of ARBs on IR in insulin-resistant animal models (*10, 19*). A possible mechanism by which ARBs ameliorate IR is the stimulation of

angiotensin type 2 (AT2) receptors and resultant increase in NO production. Olmesartan has been reported to prevent endothelin-1 induced hypertension and the formation of reactive oxygen species (*20*). Recently, Folli *et al.* suggested that angiotensin II impairs insulin signaling *via* the angiotensin type 1 (AT1) receptor by stimulating serine phosphorylation of the insulin receptor b subunit, IRS-1, and the regulatory subunit of phosphatidil inositol 3 (PI3) kinase (*21*). There has been some data indicating that angiotensin II increases TNF-α in many organs or cells other than skeletal muscle, such as cardiac fibroblasts (*22*), monocytes (*23*), and the kidneys (*24*). We recently reported that IR and hypertension in FFR were concomitant with an increase in TNF-α in skeletal muscle and that an ARB decreased skeletal muscle TNF-α and improved IR in FFR (*10*). Okada *et al.* demonstrated that olmesartan improves insulin sensitivity and overproduction of triglyceride in FFR independently of its hypotensive action (*25*). In our experiment, olmesartan decreased TNF-α and increased cAMP to almost the control levels. However, the precise mechanism of IR in FFR is still unknown, and our results, cannot be explained by only angiotensin II blockade. Further investigation of these mechanisms is needed.

We demonstrated that olmesartan significantly increased cAMP and lowered skeletal muscle TNF-α. In fact, we showed that dBcAMP significantly decreased the secretion of TNF-α from the soleus muscle in SD rats. Also, lower levels of cAMP and higher levels of TNF-α were observed in FFR than in control rats. Meng *et al.* demonstrated that hypoxia decreases cellular cAMP levels and enhances macrophage secretion of TNF-α following LPS stimulation (*26*). It has also been reported that exogenous administration of dBcAMP attenuates TNF-α production (*26*), and that dBcAMP inhibits TNF-α production (*27, 28*). In the present *ex vivo* study we examined whether angiotensin II would decrease cAMP or increase TNF-α levels in the soleus muscles. Although TNF-α was increased after 2 to 4 h of incubation, cAMP was not detected either in the incubation medium containing angiotensin II or in that containing vehicle as a control. This was a limitation of our experiment, since cAMP was quickly degraded (*29*), and unlike in the previous *in vivo* study, there was no supply of ATP in the present study. Therefore, we could not detect any cAMP in the medium after 2 to 4 h of incubation, and further experiments will thus be needed to investigate whether cAMP is produced by the soleus muscle in the presence of angiotensin II.

In a previous study, angiotensin II decreased the forskolin-induced increase in cAMP in nonhypertrophic hearts of Dahl salt-sensitive rats (*30*). Angiotensin II has also been found to inhibit stimulation of cAMP by isoproterenol in rat heart myocytes (*31*). Finally, angiotensin II has been shown to inhibit both adenylate cyclase and cAMP accumulation in hepatocytes (*32*), the rat aorta (*33*), and the pituitary gland (*34*). The results of these studies raise the possibility that the adenylate cyclase inhibitory guanine nucleotide regulatory protein (Gi) plays a role in mediating responses to angiotensin II (*35*). In A10 cells, angiotensin II has been shown to decrease the level of cAMP by enhancing the expression of Gi α (*36*).

The fact that olmesartan increased cAMP and decreased TNF-α suggests that a part of TNF-α regulation by angiotensin II might consist of modulation of cAMP through Gi protein activation in FFR. Based on the results of these studies, we assume that one of the mechanisms by which IR is induced by angiotensin II is an increase in TNF-α *via* suppression of cAMP.

In conclusion, the results of this study suggest that TNF-α in skeletal muscle is linked to IR and that olmesartan, an ARB, may improve IR in FFR either by increasing cAMP or decreasing TNF-α. There was a significant negative correlation between levels of cAMP and TNF-α in the soleus muscle. Also, dBcAMP significantly decreased the production of TNF-α in skeletal muscle. These results suggest that olmesartan ameliorates IR in FFR by modulating cAMP and TNF-α in the skeletal muscle, and that one of the mechanisms by which angiotensin II regulates TNF-α might be modulation of cAMP.

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Original Article

Intracardiac fibroblasts, but not bone marrow derived cells, are the origin of myofibroblasts in myocardial infarct repair

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Abstract

Summary: Origin of myofibroblasts in infarcted myocardium was examined by using rats in which bone marrow of green fluorescent protein (GFP)-transgenic mice had been transplanted. GFP was not detected in myofibroblasts at either 3 or 7 days after infarction, suggesting that proliferating myofibroblasts in infarcted myocardium are derived from resident fibroblasts rather than circulating precursor cells of bone marrow origin. **Background:** Myofibroblasts play important roles in the repair process of myocardial infarct, and their origin has been assumed to be interstitial fibroblasts in the heart. However, bone marrow-derived myofibroblasts have recently been identified in pathological fibrosis in extracardiac tissues. In this study, we aimed to determine whether some of the myofibroblasts in infarcted myocardium are derived from circulating precursor cells of bone marrow origin. **Methods and Results:** Bone marrow (BM) of GFP-transgenic mice was transplanted into nude rats, and their coronary arteries were occluded for 60 min and reperfused for 3 or 7 days. Non-BM-transplanted rats served as controls. At 3 days after infarction, some endothelial cells were GFP-positive, indicating that they were of bone marrow origin. Predominant cells in infarcted regions were macrophages and neutrophils, and there were only a small number of vimentin-positive cells and fewer myofibroblasts, both of which were GFP-negative. At 7 days after infarction, there were numerous myofibroblasts in granulation tissue replacing necrotic myocytes, and none of them showed GFP signals, whereas some cells were positive for both GFP and vimentin. Appearance of myofibroblasts and extent of the infarct repair in BM-transplanted and those in non-transplanted rats were similar. **Conclusions:** The findings in this study suggest that proliferating myofibroblasts in infarcted myocardium are derived from resident fibroblasts rather than circulating precursor cells of bone marrow origin. © 2005 Elsevier Inc. All rights reserved.

Keywords: Myofibroblast; Myocardial infarction; GFP-transgenic mice; Bone marrow

1. Introduction

Proliferation of myofibroblasts is observed in myocardial infarcts undergoing repair [1,2], and the myofibroblats disappear by an apoptotic mechanism in association with replacement of necrotic tissue with fibrotic scar [3,4]. Myofibroblasts not only secrete extracellular matrix proteins, cytokines and growth factors but also possess α-smooth muscle actin (α-SMA) and other contractile elements that would allow contraction of granulation tissues [5–7]. These features of myofibroblasts have been shown to play crucial roles in repair of skin wounds [5,6] and are presumably important in repair of myocardial infarct as well. In fact, a recent study by Hayakawa et al. [4] showed that inhibition of apoptosis of granulation tissue cells, most of which were myofibroblasts, in infarcted myocardium significantly attenuated ventricular remodeling and preserved left ventricular function. Thus, myofibroblasts may be a therapeutic target for prevention of heart failure after infarction.

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However, the origin of myofibroblasts in infarcted myocardium remains unclear. There are at least two possible origins of those myofibroblasts: interstitial fibroblasts in the heart [5,6] and circulating precursor cells of bone marrow (BM) origin [8–11]. Myofibroblasts of BM cell origin have recently received attention since they may play significant roles in tissue fibrosis under some pathological conditions [8–11]. The aim of this study was to determine whether some of the myofibroblasts in infarcted myocardium are of BM origin. To test this possibility, we induced myocardial infarction in rats that had received BM transplantation from green fluorescent protein (GFP)-transgenic mice (GFP+mice), and identified myofibroblasts by staining with anti-α-smooth muscle actin (α-SMA) antibodies and by their morphological features.

2. Methods

This study strictly conformed to the Guide for the Care and Use of Laboratory Animals by the Animal Use Committee of Sapporo Medical University.

2.1. GFP+ BM-transplanted chimera rats

GFP+mice [C57BL/6-Tg (Act-EGFP)C14-Y01-FM131Osb], which ubiquitously express enhanced green fluorescent protein [12], were kindly provided by Dr. Okabe, Osaka University, Japan. BM cells were harvested from femurs and tibias of donor GFP+mice by flushing with Iscove's modified Dulbecco's medium (IMDM; Gibco) containing 1% fetal calf serum (FCS; Trace) (FCS/IMDM) using a 25G needle. Collected BM cells were washed with FCS/IMDM, centrifuged at 500 *g* for 5 min, and resuspended with IMDM to prepare 2×10^8 cells/ml GFP+BM cell solution. Recipient nude rats (F344/N Jcl-rnu) at the age of 12 weeks were X-irradiated with a single dose of 7 Gy, and 0.25 ml of GFP+BM cell suspension was slowly injected into the tail vein of each rat under anesthesia at 24 hours after irradiation. Two months after BM cell transplantation, chimerism was confirmed by assay of GFP-labeled peripheral leukocytes (88.5±1.7%) using a flow cytometer (FACScan, Becton Dickinson).

2.2. Surgical preparation and immunohistochemistry

BM-transplanted rats (n=8) and control rats without BM transplantation (n=2) weighing 180~200 g were anesthetized with a mixture of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), intubated, and mechanically ventilated with a rodent respirator. The left coronary artery was occluded by a snare for 60 min and then reperfused to induce myocardial infarction. At 3 days (BM-transplanted rat: n=4) or 7 days (BM-transplanted rats: n=4, control rats: n=2) after myocardial infarction, hearts were perfusion-fixed with 4% paraformaldehyde (PFA) and stored in 0.5% PFA for 24 hrs at 4 °C. Each fixed heart was transversely sectioned into four slices, and the slices were dehydrated with sucrose-added buffered saline and embedded in Tissue-Tek O.C.T. compound. Tissue sections of 10 μm in thickness were prepared

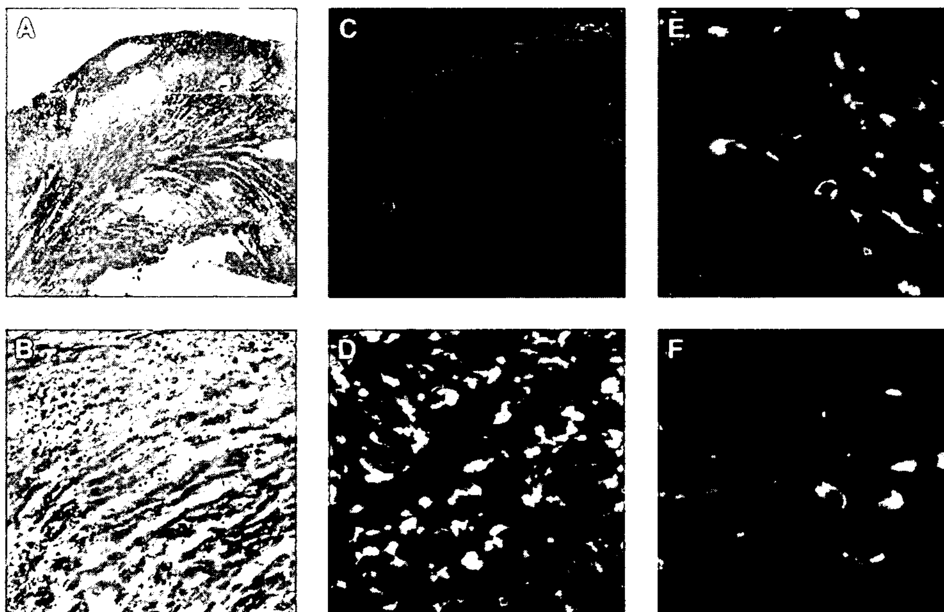

Fig. 1. Myocardial infarct 3 days after 60-min occlusion in a BM-transplanted rat. Panels A and B: hematoxylin-eosin staining. Panels C-F are merged images of rhodamine, Hoechst 33258 and GFP, which were separately taken under confocal laser microscope. α-Smooth muscle actin (SMA) was stained red by anti-SMA antibody and rhodamine-conjugated secondary antibody in panel C, D and E, and vimentin was stained red by anti-vimentin antibody and rhodamine-conjugated secondary antibody in panel F. Nuclei were stained blue with Hochest 33258. Original magnification was ×40 in panel A, ×200 in panel B, ×50 in panel C, ×630 in panel D and ×1000 in panel E and F.

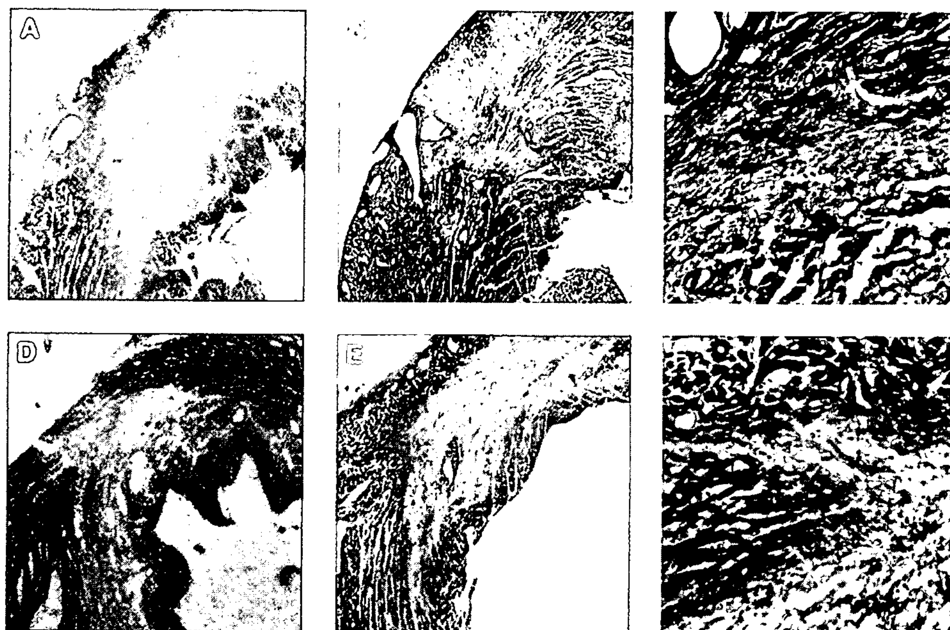

Fig. 2. Myocardial infarct 7 days after 60-min occlusion. Panels A and D: hematoxylin-eosin staining. Panels B, C, E and F: Heidenhain's azan staining. Infarct in a BM-transplanted rat is shown in panels A–C. Infarct in a control rat (without irradiation and BM transplantation) is shown in panels D–F. Original magnification was ×40 in panels A, B, D and E and ×200 in panels C and F.

by a cryostat and incubated with monoclonal anti-α-SMA antibodies (Sigma) or monoclonal anti-vimentin antibodies (Dako) as primary antibodies. Rhodamine-conjugated anti-mouse IgG (Sigma) was used as a secondary antibody. Nuclei were stained with Hochest 33258 (Sigma). The slides were examined by using a confocal laser scanning microscope (Radiance 2100, BioRad). Sections were scanned using 5×/0.25, 10×/0.45, 20×/0.75, 40×/1.0, 60×/1.4 and 100×/1.4 objective lenses. The entire field of each section on the slide was examined using appropriate filter sets against each signal (green: 488 nm, red: 568 nm) for the presence of GFP-positive, α-SMA-positive and vimentin-positive cells

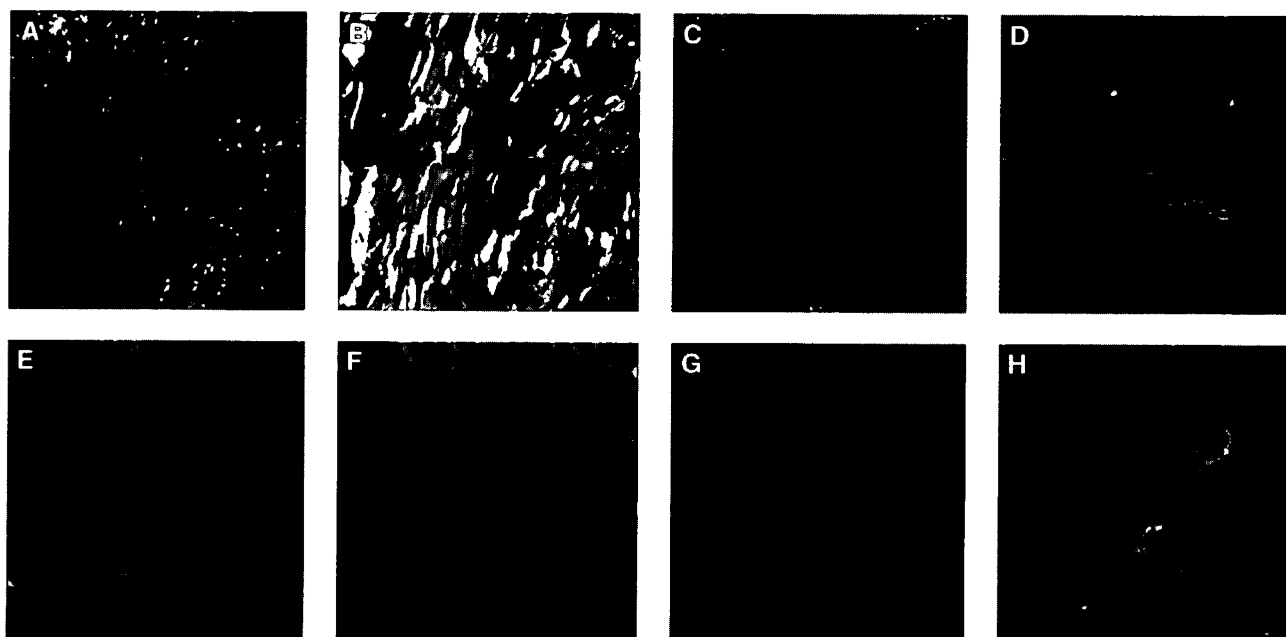

Fig. 3. GFP-positive cells and SMA-positive cells in myocardial infarct 7 days after 60-min coronary occlusion. SMA was stained red by anti-SMA antibody and rhodamine-conjugated secondary antibody, and nuclei were stained blue with Hochest 33258. Merged images of rhodamine, Hoechst 33258 and GFP, which were separately taken under a confocal laser microscope, are shown. Panels A–D: a heart from a BM-transplanted rat. Panels E-H: a heart from a control rat. Panels A, B, E and F: infarcted regions. Panels C, D, G and H: non-infarcted regions remote from infarcted regions. Original magnification was ×200 in panels A and E, ×630 in panels B and F, ×100 in panels C and G and ×400 in panels D and H.

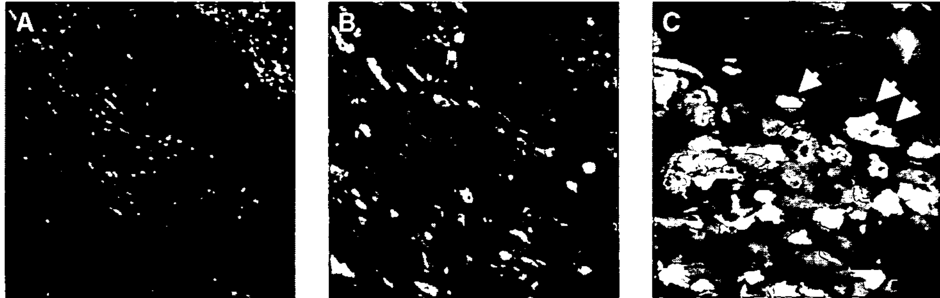

Fig. 4. GFP-positive cells and vimetin-positive cells in myocardial infarct 7 days after 60-min coronary occlusion. Merged images of rhodamine, Hoechst 33258 and GFP, which were separately taken under confocal laser microscope. Vimentin was stained red by anti-vimentin antibody and rhodamine-conjugated secondary antibody. Nuclei were stained blue with Hochest 33258. Three cells with yellow fluorescence (arrows), which indicates the presence of both vimentin and GFP. Original magnification was ×200 in panel A, ×630 in panel B and ×1000 in panel C.

and using UV illumination for nuclei stained with Hochest 33258. Image data obtained by using the confocal microscope were saved as TIFF files, and merged images were made by using Photoshop 7.0 (Adobe Systems Inc.). Histology slides from each heart block were also processed for hematoxylin-eosin staining and Heidenhain's azan staining by standard methods for examination by light microscopy.

3. Results

At 3 days after coronary occlusion, the infarct consisted of a core of necrotic myocytes and a peripheral area of granulation tissues (Fig. 1A) containing neutrophils and macrophages (Fig. 1B). Most of these inflammatory cells infiltrating into the central core of necrotic tissue were GFP-positive (Fig. 1C and D). Most of the signals of α-SMA were observed in microvessels in granulation tissue (Fig. 1E), and myofibroblasts, which are labeled with anti-α-SMA antibodies, in the extravascular space were very few. The number of vimentin-positive spindle-shaped cells was also small but more than that of myofibroblasts, and they were mainly in lateral border zones of infarcts (Fig. 1F). α-SMA-positive myofibroblasts and vimentin-positive cells, which include fibroblasts and myofibroblats, were GFP-negative. However, some of the endothelial cells in the microvasculature were GFP-positive (Fig. 1E), indicating that they were of BM cell origin.

At 7 days after infarction, the necrotic myocytes were almost entirely replaced with granulation tissue and fibrotic tissues in both BM-transplanted and control rats (Fig. 2A–F). In BM-transplanted rats, the number of GFP-positive cells was reduced (Fig. 3A) as compared with the number 3 days after myocardial infarction. There were numerous α-SMA-labeled cells with myofibroblast morphology, aligning along the circumferential axis in the granulation tissue within the infarct zone (Fig. 3B), and none of these myofibroblasts were positive for GFP. In the non-infarcted region, signals of α-SMA were detected only in blood vessels (Fig. 3C, D, G, H). These appearances of the α-SMA-positive cells in the non-infarcted and infarcted regions, and the extent of collagen deposition within infarct zones in the BM-transplanted rats were similar to those in non-BM-transplanted control rats (Figs. 2 and 3). In contrast to α-SMA-positive cells, some of the vimentin-positive cells were GFP-positive at 7 days after infarction (Fig. 4), suggesting that there might be fibroblasts of BM origin at this phase of infarct repair.

4. Discussion

In earlier studies [8–12], the contribution of bone marrow cells to the myofibroblast population was examined by tracking transplanted BM cells in sex-mismatched recipients with y-chromosome as a marker or by tracking BM cells that were tagged with GFP. BM-derived myofibroblasts were found in the lungs, skin, kidneys, adrenal capsules, and intestines of mice that had been irradiated and in intestines of patients with graft-versus-host reactions [8–10]. In livers in which fibrosis had developed after its transplantation, 7~22% of myofibroblasts were estimated to be of BM origin [11]. In contrast, a recent study by Hashimoto et al. [13] showed that fibroblasts but not myofibroblasts in bleomycin-induced pulmonary fibrosis are from BM. In the present study, there were only a few GFP-positive fibroblasts in the subacute infarct repair (Fig. 4C) but no GFP was detected in myofibroblasts proliferating within the myocardial infarct at both acute and subacute stages of infarct repair (Fig. 1D and Fig. 3A and B). The reason why BM-derived fibroblasts in infarcts failed to differentiate into myofibroblasts is unclear, but BM-derived fibroblasts might have been insensitive to TGF-β as were those in bleomycin-induced pulmonary fibrosis [13]. Nevertheless, these findings suggest that origins of myofibroblasts in injured tissue are different depending on the type of injury and type of tissue and that intracardiac resident fibroblasts are the primary origin of myofibroblasts participating in infarct repair.

It is notable that vimentin-positive cells expressing GFP were observed in the 7-day-old infarcts but not in the 3-day-old infarcts. There are two possible interpretations for

this finding. First, the cells positive for both vimentin and GFP were fibroblasts of BM origin, and there is a time lag between differentiation of fibroblasts of BM origin and that of fibroblasts of non-BM origin in infarcted myocardium. This time lag might have been required for expression of cell adhesion molecules and/or capillary formation, which allow homing of BM-derived cells. There is the possibility that such fibroblasts of BM origin show myofibroblast phenotypes at a later time point and contribute to the late phase of infarct repair. The other possible interpretation of the vimetin/GFP doubly labeled cells is that they were actually macrophages expressing high levels of vimentin. However, this possibility is not very likely since numerous macrophages in infarcts 3 and 7 days after infarction were not labeled by the anti-vimentin antibody.

As a limitation in the present study, we cannot exclude the possibility that there were radioresistant BM stromal cells that produced progenitor cells of myofibroblasts. Circulating fibrocytes, which serve as progenitors of myofibroblasts in skin wound and other types of tissue injury [14–16], may be of such a radioresistant BM stromal cell origin. However, the presence of such fibrocyte precursors in BM has not yet been confirmed, and the possibility of other locations of fibrocyte precursors has not been excluded. One method to assess possible contribution of radioresistant BM stromal cells to myofibroblasts in myocardial infarcts would be induction of infarction in transplanted hearts for determination of myofibroblasts labeled with genetic makers of recipients in infarcted myocardium. However, results in such a highly manipulated preparation may not be extrapolated to myocardial infarct repair in general. Another limitation in the present study is use of nude rats as a host of bone marrow transplantation. Nude rats lack functionally mature T cells, which play a major role in rejection of transplanted cells [17]. Although some T cells have been shown to infiltrate infarcted myocardium [18], predominant types of inflammatory cells within infarcts undergoing the repair process are neutrophils and macrophages [19]. However, we cannot exclude the possibility that lack of T cells affected proliferation of myofibroblasts of resident fibroblast origin and those of bone marrow cell origin.

Although the present results suggest that BM-derived cells do not serve as precursors of myofibroblasts in infarcted myocardium, there is no doubt that a variety of BM-derived cells contribute to the repair process of infarcted myocardium. First, macrophages play major roles in phagocytosis of the necrotic myocardium and in cytokine production [2]. TGF-β produced from macrophages is thought to be responsible for differentiation of fibroblasts into myofibroblasts and for collagen secretion [4,5], though TGF-β from other sources, including myofibroblasts themselves, would also participate in infarct repair [4,17,20,21] Second, it has been shown that BM-derived endothelial progenitor cells in circulation contribute to angiogenesis in ischemic tissues [22], and the present study confirmed the presence of BM-derived endothelial cells at the infarct border zone. Although their number was small, they would have contributed to formation of the granulation tissue in infarcted region. Thirdly, participation of other cell types of BM origin in infarct repair by different mechanisms is also possible. Recent studies [23,24] have shown that intravenous administration of BM stromal cells after acute myocardial infarction results in a variety of effects, including VEGF-mediated suppression of myocyte apoptosis and reduction of infarct scar size. However, whether endogenous BM-derived cells have similar effects on infarct remains to be investigated.

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Large-Scale Cohort Study on the Relationship Between Serum Lipid Concentrations and Risk of Cerebrovascular Disease Under Low-Dose Simvastatin in Japanese Patients With Hypercholesterolemia

— Sub-Analysis of the Japan Lipid Intervention Trial (J-LIT) —

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Background The Japan Lipid Intervention Trial was a nationwide cohort study of 52,421 hypercholesterolemic patients treated with open-labeled simvastatin for 6 years under standard clinical practices. Cerebrovascular disease (CVD) is one of the leading causes of death in Japan, but the effect of hypercholesterolemia on CVD has not been well established in Japanese patients. This study aimed to determine the relationship between the risk of CVD and serum lipid concentrations during treatment in Japan.
Methods and Results Patients were treated with 5–10 mg/day of simvastatin and all, including those who discontinued simvastatin for any reason, had their lipid concentrations and incidence of CVD monitored for 6 years. Data of 41,088 patients were analyzed in this study, excluding those who had a history of coronary heart disease or CVD. The risk of cerebral infarction was higher in patients whose mean total cholesterol concentrations during treatment were ≥240 mg/dl, low-density lipoprotein cholesterol concentrations ≥160 mg/dl, triglycerides ≥150 mg/dl and high-density lipoprotein cholesterol concentrations <40 mg/dl. There was no obvious correlation between cerebral hemorrhage and serum lipid concentrations.
Conclusion Improvement of serum lipid concentrations is important for reducing the incidence of cerebral infarction. (*Circ J* 2005; **69:** 1016–1021)

Key Words: Cerebrovascular disease; Lipids; Primary prevention

Cerebrovascular disease (CVD) is one of the major causes of death in Japan[1] and is a leading cause of disability and increased healthcare costs. A number of studies have already demonstrated that lipid-lowering therapy reduces the risk of coronary heart disease (CHD), but its effect on CVD has not been well established in Japan. The Kyushu Lipid Intervention Study demonstrated a correlation between the serum lipid concentration and the incidence of cerebral infarction, but the number of patients was relatively small.[2,3] Several epidemiologic studies have failed to demonstrate an association between cholesterol concentrations and CVD,[4–7] although a post hoc finding in the Scandinavian Simvastatin Survival Study (4S) showed that simvastatin reduced the incidence of CVD by 30%.[8] A pooled analysis of 4 trials conducted primarily in CHD patients showed a 62% lower rate of total CVD attributable to pravastatin.[9] The Heart Protection Study reported that simvastatin reduced the incidence rate of total and ischemic CVD in UK adults with high risk[10] by 25% and 30%, respectively, and the Anglo-Scandinavian Cardiac Outcomes Trial study reported that atorvastatin reduced the incidence of CVD in hypertensive patients by 27%.[11] In Japan, the Oyabe Study reported that the incidence of CVD in Japanese subjects with low concentrations of high-density lipoprotein cholesterol (HDL-C <30 mg/dl) was remarkably higher than that in subjects with high HDL-C (≥60 mg/dl) and the incidence of CVD was higher in the subjects with total cholesterol (TC) concentrations over 220 mg/dl.[12] The Eastern Stroke and Coronary Heart Disease Collaborative Research Group reported that there was a trend toward a decrease in the risk of ischemic CVD and an increase in the risk of hemorrhagic CVD with decreasing cholesterol concentrations in populations from eastern Asia.[13]

The Japan Lipid Intervention Trial (J-LIT) was a nationwide cohort study of 52,421 hypercholesterolemic patients treated with open-label simvastatin (5–10 mg/day), carried out for 6 years by a large number of physicians under standard clinical practices in order to evaluate the relationship

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between cardiovascular disease and lipid concentrations.[14–16] The results clearly showed that normalization of the lipid concentration of hypercholesterolemic patients reduced the risk of coronary events. In the present study, we analyzed the relationship between the risk of CVD and lipid concentrations.

Methods

Subjects

The J-LIT study enrolled 52,421 patients with serum TC concentration ≥220 mg/dl: men aged 35–70 years and post-menopausal women under 70 years old. 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 recent acute myocardial infarction or CVD, uncontrolled diabetes mellitus, serious concomitant hepatic or renal disease, secondary hypercholesterolemia, malignancy or any other illness with a poor prognosis. For this study, 41,088 patients without a history of CHD or CVD were selected.

Study Design

The design of the J-LIT study has been described previously.[17] Patients were treated with open-label simvastatin according to the approved Japanese labeling of Lipovas®. All patients, including those who discontinued simvastatin for any reason, were monitored for 6 years. Their lipid concentrations, adverse events, and incidence of CHD-related events and CVD were recorded. Cholesterol concentrations were determined locally at the study institutions. Dietary and exercise therapies for hyperlipidemia were recommended to patients by the investigators and additional lipid-lowering agents were allowed at the discretion of the physician. No restrictions were placed on the administration of medical treatment for complications. The low-density lipoprotein cholesterol (LDL-C) concentration in patients with 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 type of CVD was determined using clinical symptoms, angiography and/or computed tomography (CT), and classified according to the criteria of the Stroke Committee established by the Japanese Ministry of Education.[19] The pre-specified primary endpoints of the sub-analysis were cerebral infarction, including cerebral thrombosis and cerebral embolism, and cerebral hemorrhage pre-specified in the protocol. The first event or death that occurred during the study period were counted once in each patient, and the events were reviewed and determined by the Endpoint Classification Committee. CVD events were counted once. The follow-up data acquired after the onset of disease other than the endpoints were excluded from the analysis. Each patient was informed of the study purpose, as well as drug efficacy and need for long-term treatment. Written informed consent was not obtained from patients, because commercially available simvastatin preparation was used for the open-label study.

Table 1 Baseline Characteristics and Lipid Profiles of the Patients

N	*41,088*
Males (%)	*31.6*
Age (years)	*57.7±7.9*
Body mass index (kg/m²)	*24.0±3.2*
Hypertension (%)	*45.6*
Diabetes mellitus (%)	*15.1*
ECG abnormal (%)	*12.8*
Coronary heart disease familial history (%)	*4.7*
Smoking habit (%)	*16.5*
Alcohol consumption (%)	*29*
Baseline	
Total cholesterol (mg/dl)	*270±34*
Low-density lipoprotein cholesterol (mg/dl)	*182±33*
Triglyceride (mg/dl)	*196±171*
High-density lipoprotein cholesterol (mg/dl)	*53±15*
During treatment	
Total cholesterol (mg/dl)	*220±30*
Low-density lipoprotein cholesterol (mg/dl)	*134±30*
Triglyceride (mg/dl)	*164±103*
High-density lipoprotein cholesterol (mg/dl)	*55±14*

Statistical Analysis

All data, including those obtained after the termination of simvastatin therapy, were analyzed by survival analysis. The mean lipid concentrations were calculated using the data obtained throughout the treatment period, because the mean lipid concentrations were thought to be important for clinical practice. The data for lipid concentrations acquired after the onset of disease were excluded. For analysis of baseline patient age and lipid profile, continuous variables within and between subgroups were assessed using the paired or unpaired t-test. Patients were classified into 3–6 subgroups based on the mean lipid concentrations during the treatment. TC, TG, LDL-C, and HDL-C concentrations and the ratio of LDL-C/HDL-C were classified into discrete intervals of 20, 150, 20 and 10 mg/dl and 0.5, respectively. The reference categories were set on the subgroups with the lowest concentrations.

We calculated the relative risks, with 95% confidence intervals for each endpoint, of each subgroup relative to the reference category, using the Cox proportional-hazards model with adjustment for gender and age at baseline (as a continuous variable), hypertension, diabetes mellitus, and smoking habit. The effects of each baseline characteristic on each endpoint were assessed, except for the effect of age, which was categorized. Data are expressed as the mean ± SD. For all statistical analyses, a p-value <0.05 was considered to be significant. All statistical calculations were performed using SAS software (version 6.12, SAS Institute Inc, Cary, NC, USA).

Results

Follow-up

A total of 42,360 of the 52,421 patients enrolled in the J-LIT study were eligible for the primary prevention analysis of CHD and their clinical characteristics have been reported previously.[17] In the present study, data were collected from 41,088 patients and 1,272 patients were excluded on the history of CVD. In total, 30,832 patients were followed up by the investigators to the end of the 6th year (mean follow-up, 5.39 years per subject). Baseline characteristics are shown in Table 1. Approximately 46% of patients had hypertension (mean systolic and diastolic

Table 2 Incidence of Cerebrovascular Events During Follow-up Period

	Fatal		*Non-fatal*		*Total*	
	No. of events	*Incidence*	*No. of events*	*Incidence*	*No. of events*	*Incidence*
Cerebral infarction	22	(0.10)	220	(1.00)	242	(1.09)
Cerebral thrombosis	12	(0.05)	139	(0.63)	151	(0.68)
Cerebral embolism	10	(0.05)	81	(0.37)	91	(0.41)
*Cerebral hemorrhage**	22	(0.10)	80	(0.36)	102	(0.46)

**Intracranial. Incidence =/1,000 patients-year.*

Table 3 Relative Risk of Cerebrovascular Disease and Serum Lipid Concentrations During Treatment

	Cerebral infarction					*Cerebral hemorrhage**			
	Population	*Event*	*Relative risk*	*95%CI*	*p value*	*Event*	*Relative risk*	*95%CI*	*p value*
TC (mg/dl)									
<200	9,306	50	1.00			29	1.00		
200–219	12,119	70	1.26	(0.87–1.81)	0.216	34	0.98	(0.60–1.61)	0.940
220–239	10,423	59	1.38	(0.94–2.01)	0.100	17	0.61	(0.34–1.12)	0.112
240–259	5,275	34	1.76	(1.13–2.73)	0.012	11	0.85	(0.42–1.71)	0.638
≥260	3,423	28	2.49	(1.56–3.99)	<0.001	9	1.15	(0.54–2.46)	0.718
LDL-C (mg/dl)									
<120	12,860	74	1.00			32	1.00		
120–139	12,260	76	1.23	(0.89–1.70)	0.200	39	1.41	(0.88–2.26)	0.151
140–159	8,871	36	0.89	(0.60–1.33)	0.575	15	0.82	(0.44–1.51)	0.515
160–179	3,853	30	1.92	(1.25–2.94)	0.003	7	0.95	(0.42–2.16)	0.902
≥180	2,318	24	2.95	(1.85–4.71)	<0.001	6	1.51	(0.62–3.63)	0.363
TG (mg/dl)									
<150	22,413	101	1.00			53	1.00		
150–299	15,577	115	1.54	(1.18–2.02)	0.002	41	0.99	(0.65–1.50)	0.957
≥300	2,532	25	2.31	(1.47–3.63)	<0.001	5	0.73	(0.28–1.86)	0.504
HDL-C (mg/dl)									
<40	4,001	48	1.00			16	1.00		
40–49	11,522	70	0.52	(0.36–0.75)	<0.001	31	0.68	(0.37–1.25)	0.217
50–59	12,162	66	0.49	(0.33–0.71)	<0.001	25	0.54	(0.29–1.03)	0.061
≥60	12,861	57	0.41	(0.28–0.61)	<0.001	28	0.60	(0.32–1.13)	0.117
LDL-C/HDL-C									
<2.0	10,439	55	1.00			25	1.00		
2.0–2.4	9,904	46	0.90	(0.61–1.33)	0.597	24	1.02	(0.58–1.79)	0.943
2.5–2.9	8,682	48	1.11	(0.75–1.63)	0.611	17	0.84	(0.45–1.56)	0.582
3.0–3.4	5,578	37	1.37	(0.91–2.09)	0.136	12	0.95	(0.48–1.89)	0.877
≥3.5	5,559	54	2.13	(1.46–3.11)	<0.001	21	1.75	(0.97–3.14)	0.061
Total	40,546	241				100			

*Cerebral infarction, cerebral thrombosis and cerebral embolism; *Intracranial.*
CI, confidence interval; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol.

blood pressures 150 and 87 mmHg, respectively), and 15% had diabetes mellitus. Hypertension was treated with angiotensin-converting enzyme inhibitors (26%), calcium-channel blockers (53%), β-blockers (18%) and diuretics (6%).

Changes in Serum Lipid Concentrations With Simvastatin Treatment

The mean serum concentrations of TC, LDL-C, and TG at baseline were 270±34, 182±33, and 196±171 mg/dl, respectively, and the mean lipid concentrations during treatment were 220±30 mg/dl, 134±30 mg/dl and 164±103 mg/dl, respectively. The mean serum HDL-C concentration increased from baseline of 53±15 mg/dl to 55±14 mg/dl. The mean percent change from baseline in the TC, LDL-C, TG, and HDL-C concentrations was −18%, −27%, −16%, and +4%, respectively, during the treatment period.

Incidence of CVD During the Treatment Period

Cerebral infarction occurred in 242 patients during the course of the study with an incidence of 1.09 events per 1,000 patients-year (Table 2). Cerebral thrombosis occurred in 151 patients, and cerebral embolism in 91 patients. Cerebral hemorrhage occurred in 102 patients with an incidence of 0.46 events per 1,000 patients-year. The incidence of total CVD was 1.55 events per 1,000 patients-year. CT of the head was performed in 77% of the patients with CVD.

Relationship Between the Relative Risk of Cerebral Infarction and Lipid Concentrations

The relative risk of cerebral infarction was higher in patients with TC concentration ≥240 mg/dl compared to those with TC concentration <200 mg/dl (Table 3) and patients with LDL-C concentration ≥160 mg/dl had higher risk than those with a concentration <120 mg/dl. A group of patients with a mean TG concentration ≥150 mg/dl had a higher incidence of cerebral infarction than those with a mean concentration <150 mg/dl. The incidence of cerebral infarction was lower in patients with a mean HDL-C concentration of 40–49 mg/dl compared with a concentration <40 mg/dl. The relative risk of cerebral infarction was

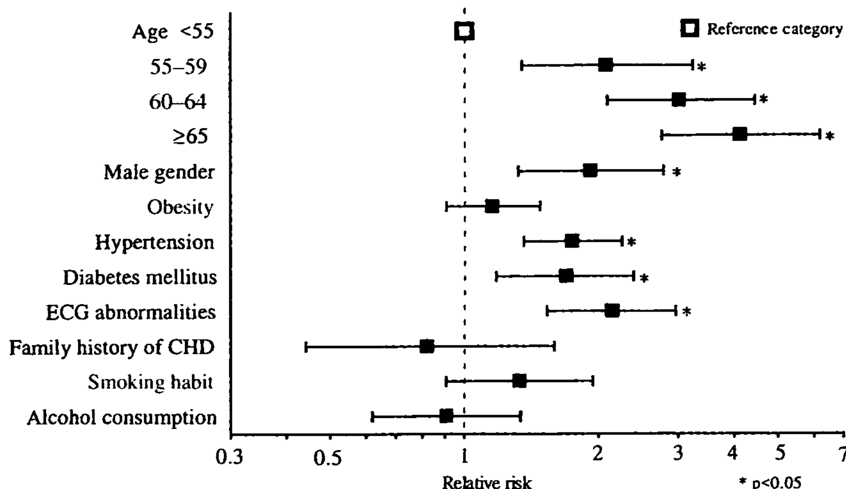

Fig 1. Relationship between the relative risk of cerebral infarction and baseline characteristics of patients maintained on low-dose simvastatin. Data were adjusted for age, sex, hypertension, diabetes mellitus and smoking habit. Bars express the relative risk with a 95% confidence interval. Obesity = body mass index (BMI) ≥25 kg/m². CHD, coronary heart disease.

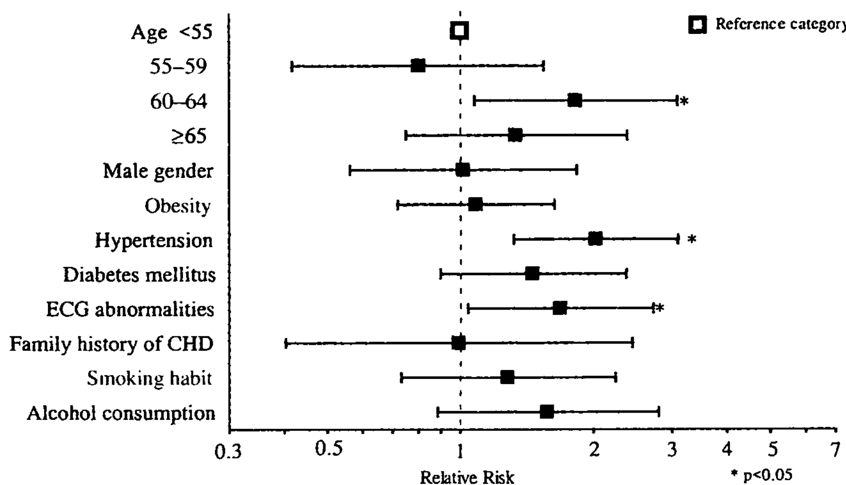

Fig 2. Relationship between the relative risk of cerebral hemorrhage and baseline characteristics of patients maintained on low-dose simvastatin. Data were adjusted for age, sex, hypertension, diabetes mellitus and smoking habit. Bars express the relative risk with a 95% confidence interval. Obesity = body mass index (BMI) ≥25 kg/m². CHD, coronary heart disease.

higher in patients with the ratio of LDL-C/HDL-C ≥3.5 compared with a ratio <2.0.

Relationship Between the Relative Risk of Cerebral Infarction and Baseline Characteristics

The risk of cerebral infarction was analyzed using multiple regression (Fig 1). Age correlated with the incidence of cerebral infarction and male patients had higher risk compared with female patients. Hypertension, diabetes mellitus, and ECG abnormalities were also risk factors for cerebral infarction. Alcohol consumption, cigarette smoking and a family history of CHD were not risk factors.

Relationship Between the Relative Risk of Cerebral Hemorrhage and Mean Lipid Concentrations

There was no clear relationship between cerebral hemorrhage and the concentrations of TC, LDL-C, TG and HDL-C or the ratio of LDL-C/HDL-C (Table 3).

Relationship Between the Relative Risk of Cerebral Hemorrhage and Baseline Characteristics

The risk of cerebral hemorrhage was analyzed by multiple regression (Fig 2), which showed that patients aged 60–64 years had a significantly higher risk of cerebral hemorrhage. Hypertension and ECG abnormalities were also risk factors for cerebral hemorrhage.

Relationship Between the Relative Risk of Cerebral Infarction and Hypertension

Hypertension is known to be a dominant risk factor for cerebral infarction, so the effect of lipid concentrations on cerebral infarction was examined in patients stratified by systolic blood pressure (SBP). Table 4 shows the relationship between cerebral infarction and serum lipid concentrations, comparing a SBP at baseline <140 mmHg and SBP ≥140 mmHg. The influence of serum lipid concentrations on cerebral infarction was observed in both the hypertensive and normotensive groups and was also observed in patients stratified by diastolic blood pressure (<90 mmHg and ≥90 mmHg; data not shown). The serum lipid concentrations are thus independent risk factors of cerebral infarction.

Discussion

We analyzed the relationship between the risk of CVD and lipid concentrations using the J-LIT data. During the 6-year treatment period simvastatin reduced the serum concentrations of TC, LDL-C and TG by 18%, 27% and 16%, respectively, from baseline. The concentration of HDL-C continued to increase during the treatment period. The incidence of CVD in Japanese patients without prior CVD or CHD was 1.55 events per 1,000 patients-year in this study, much higher than for primary CHD (0.91 events

Table 4 Relative Risk of Cerebral Infarction and Serum Lipid Concentrations During Treatment

	SBP at baseline <140 mmHg					*SBP at baseline ≥140 mmHg*				
	Population	*Event*	*Relative risk*	*95%CI*	*p value*	*Population*	*Event*	*Relative risk*	*95%CI*	*p value*
TC (mg/dl)										
<200	4,246	17	1.00			5,012	33	1.00		
200–219	5,807	32	1.55	(0.86–2.79)	0.148	6,246	38	1.05	(0.66–1.67)	0.844
220–239	5,197	25	1.49	(0.80–2.77)	0.208	5,160	33	1.18	(0.72–1.91)	0.515
240–259	2,710	9	1.18	(0.52–2.66)	0.694	2,538	25	1.96	(1.16–3.32)	0.012
≥260	1,796	12	2.66	(1.26–5.63)	0.010	1,604	16	2.15	(1.18–3.94)	0.013
LDL-C (mg/dl)										
<120	5,888	24	1.00			6,896	50	1.00		
120–139	5,899	36	1.68	(1.00–2.81)	0.051	6,302	40	0.96	(0.63–1.46)	0.840
140–159	4,485	17	1.10	(0.59–2.06)	0.757	4,339	19	0.71	(0.42–1.20)	0.201
160–179	2,041	8	1.29	(0.58–2.88)	0.536	1,789	21	2.03	(1.22–3.40)	0.007
≥180	1,274	10	2.91	(1.38–6.13)	0.005	1,031	14	2.70	(1.48–4.91)	0.001
TG (mg/dl)										
<150	11,548	41	1.00			10,762	59	1.00		
150–299	7,062	44	1.72	(1.12–2.65)	0.014	8,419	71	1.51	(1.07–2.14)	0.021
≥300	1,135	10	3.29	(1.61–6.69)	0.001	1,366	15	1.97	(1.09–3.54)	0.024
HDL-C (mg/dl)										
<40	1,908	19	1.00			2,066	29	1.00		
40–49	5,356	30	0.56	(0.31–0.99)	0.047	6,102	40	0.51	(0.31–0.82)	0.005
50–59	5,851	21	0.36	(0.19–0.68)	0.002	6,249	44	0.57	(0.35–0.92)	0.021
≥60	6,641	25	0.38	(0.21–0.71)	0.002	6,143	32	0.42	(0.25–0.71)	0.001
LDL-C/HDL-C										
<2.0	5,125	22	1.00			5,261	33	1.00		
2.0–2.4	4,710	17	0.85	(0.45–1.59)	0.605	5,125	29	0.93	(0.57–1.54)	0.781
2.5–2.9	4,158	20	1.17	(0.64–2.14)	0.617	4,484	28	1.04	(0.63–1.72)	0.890
3.0–3.4	2,731	16	1.46	(0.77–2.80)	0.248	2,820	20	1.20	(0.69–2.10)	0.517
≥3.5	2,863	20	1.81	(1.98–3.35)	0.057	2,667	34	2.23	(1.38–3.60)	0.001
Total	19,756	95				20,560	145			

Cerebral infarction, cerebral thrombosis and cerebral embolism.
SBP, systolic blood pressure. Other abbreviations see Table 3.

per 1,000 patients-year[14]), and indicates that in Japan the prevention of CVD is as important as that of CHD. The risk of cerebral infarction was higher when the mean TC concentration was ≥240 mg/dl and the mean LDL-C concentration ≥160 mg/dl, which are the same values for the risk of CHD. The incidence of cerebral infarction increased in the patients with TG concentration ≥150 mg/dl, whereas the incidence of CHD increased when TG concentration was ≥300 mg/dl, which indicates a closer relationship between cerebral infarction and TG than between CHD and TG. HDL-C inversely correlated with the risk of cerebral infarction. There was no obvious correlation between cerebral hemorrhage and serum lipid concentrations nor could we observe a clear relationship between the incidence of fatal CVD and lipid concentrations. In the preliminary analysis, no different results were obtained when adjusted for SBP. In a meta-analysis of randomized trials,[20] lipid-lowering therapy reduced the overall CVD incidence, but not that of fatal CVD incidence and it was concluded that the incidence of hemorrhagic CVD was not influenced by lipid-lowering therapy. Because hemorrhagic CVD is mostly fatal could partly explain the apparent lack of effect of lipid-lowering therapy on fatal CVD in that analysis and might be applicable to our results.

Our findings are generally consistent with the results from the Oyabe Study, a community-based 10-year follow-up study of Japanese men and women,[12] although the incidence was much less (crude rates for all CVD incidence per 1,000 person-years were 4.30 in men and 1.98 in women in the Oyabe study). Low HDL-C concentrations (<30 mg/dl) led to a significantly and independently increased risk of CVD. The incidence of CVD was higher in the subjects with TC concentrations >220 mg/dl and the relationship of TC to the overall incidence of CVD and that of ischemic CVD in particular showed a U-shaped curve with the bottom between TC values of 180 and 220 mg/dl. In the present J-LIT study, the incidence of ischemic CVD tended to increase in patients with TC concentrations <180 mg/dl, but was not significant (data not shown). The patients who participated in this study were all treated with low-dose of simvastatin, but our results are similar to the result of the Oyabe Study. The Eastern Stroke and Coronary Heart Disease Collaborative Research Group[13] reported that there were trends towards a decrease in the risk of nonhemorrhagic CVD and an increase in the risk of hemorrhagic CVD with decreasing cholesterol concentrations, and in the present study, high TC, LDL-C and TG concentrations and low HDL-C concentrations were risk factors for cerebral infarction. The influence of serum lipid concentrations on cerebral infarction (thrombosis and embolism) was similar in males and females. In the analysis of patients with TC concentrations <180 mg/dl, the incidence of cerebral hemorrhage was not increased, compared with those with a TC concentration ≥180 mg/dl. A relationship between the effect of lipid concentrations and cerebral infarction, analyzed by hypertension, sex, and types of cerebral infarction, was clearly observed in this study.

The antiatherothrombotic properties of statins,[21] apart from their cholesterol-lowering effects, may be the mechanism of the reduction in cardiovascular events. The thrombotic sequelae of plaque disruption may be mitigated by statins through their inhibition of platelet aggregation and maintenance of a favorable balance between prothrombic and fibrinolytic mechanisms.[21] These same antiatherothrom-