Table 2. Number of Blood Pressure Categories by Sex

	Normotensives (*n* (%))	Hypertensives (*n* (%))		Total (*n*)
		Treated	Nontreated	
Men	2,774 (62.8)	450 (10.2)	1,191 (27.0)	4,415
Women	4,620 (67.1)	860 (12.5)	1,407 (20.4)	6,887
Total	7,394 (65.4)	1,310 (11.6)	2,598 (23.0)	11,302

Normotensives: normal blood pressre without treatment. Treated hypertenives: subjects with treatment if any blood pressure. Nontreated hypertensives: hypertension without treatment.

mogenic assay autoanalyzer (Behringwerke; interassay CV: 3.8%).

We examined the association between BP categories and other risk factors in Japan.

Statistical Analysis

Analysis of variance (ANOVA) was used to calculate variance among BP categories, and mean values of lipids, fibrinogen, factor VIIc, BMI, and PAI were shown after age adjustment in each sex. Scheffe's test was used in comparison with the values of variables. The χ^2 test was used for BP categories and other categories such as lipid status, blood glucose status, MS, smoking status, and drinking status, and the Mantel-Haenszel test was used for adjustment for age. These analyses were done using SAS software version 8.2 (SAS Institute, Cary, USA).

Results

The general characteristics of the subjects are shown in Table 1. The mean ages were 55.2 years in men and 55.3 years in women. SBP and DBP were higher in men (131.4 mmHg and 79.2 mmHg) than in women (128.1 mmHg and 76.3 mmHg).

The proportions of the normotensives, treated hypertensives, and nontreated hypertensives were 62.8%, 10.2%, and 27.0% in men, and 67.1%, 12.5%, and 20.4% in women, respectively (Table 2).

Age-adjusted mean values of risk factors categorized by BP status were calculated using ANOVA. In both men and women, total cholesterol was lower in normotensives and higher in nontreated hypertensives, whereas triglyceride was lower in normotensives than in treated or nontreated hypertensives. Blood glucose, factor VIIc, and BMI were lower in normotensives than in treated or nontreated hypertensives in both men and women. As for HDL cholesterol, the mean value was higher in normotensives than in treated or nontreated hypertensives in women, but there was no tendency shown in men (Table 3).

In men, according to Scheffe's test, triglyceride, blood glucose, factor VIIc, BMI, and PAI were significantly higher in treated hypertensives than in normotensives; and total cholesterol, triglyceride, blood glucose, and BMI were significantly higher in nontreated hypertensives than in normotensives. In women, total cholesterol, triglyceride, blood glucose, fibrinogen, factor VIIc, BMI, and PAI were significantly higher in treated hypertensives than in normotensives; and total cholesterol, triglyceride, blood glucose, Lp(a), fibrinogen, factor VIIc, and BMI were significantly higher in nontreated hypertensives than in normotensives (Table 3).

Associations of BP categories with lipid status, blood glucose status, MS, smoking status, and drinking status are shown in Table 4. The proportions of dyslipidemia, IGT, and MS were significantly higher in treated and nontreated hypertensives than in normotensives in men and women using both the χ^2 test and the Mantel-Haenszel test adjusted for age. The same tendency was seen for smoking in men and women. The proportion of drinkers was lower in normotensives than in the treated and nontreated hypertensives in men, whereas in women the proportion of drinkers was lower in hypertensives with treatment than in normotensives and nontreated hypertensives.

Discussion

The JMS Cohort Study (*17*, *18*), a population-based cardiovascular cohort study using a mass screening examination system, was started in 1992 in a Japanese population. The study subjects were drawn from 12 rural districts in Japan, and the overall response rate was 63%. We examined cardiovascular risk factors in relation to hypertension categories in the present study using baseline data from the JMS Cohort Study.

There may be some bias in this study, such as detection bias, in that a single BP measurement may not reflect actual hypertension, and measurement bias, in that there is not enough information about antihypertensive treatment. Single measurement of BP is substantially considered to underestimate the strength of the relationship between BP and CVD. Single measurement of cholesterol has substantially underestimated the strength of the relationship between cholesterol and the risk of coronary heart disease (*20*), and the same framework might be applicable to BP. Single measurement of BP for epidemiological studies may markedly overestimate the true prevalence of hypertension, and a diagnosis of hypertension is difficult (*21*). However, in many epidemiological studies, single measurement of BP was used to determine hypertension.

Table 3. Age-Adjusted Means of Risk Factors

	Normotensives		Hypertensives				$p^{\#}$
			Treated		Nontreated		
	Mean[$]	SEM	Mean[$]	SEM	Mean[$]	SEM	
Men							
Total cholesterol (mg/dL)	183.1	0.7	186.6	1.6	188.4	1.0†	<0.01
HDL-chotesterol (mg/dL)	48.7	0.3	48.6	0.6	49.4	0.4	0.32
Triglyceride (mg/dL)[§]	102.5	(101.5–103.6)	122.1	(119.0–125.3)*	117.1	(115.3–119.0)†	<0.01
Blood glucose (mg/dL)	102.8	0.6	109.8	1.5*	110.2	0.9†	<0.01
Lipoprotein(a) (mg/dL)[§]	12.9	(12.6–13.2)	11.5	(10.9–12.1)	11.5	(11.1–11.8)	<0.01
Fibrinogen (mg/dL)	244.3	1.4	240.2	3.4*	241.0	2.2	0.32
Factor VIIc (mg/dL)	107.9	0.5	111.4	1.4*	109.9	0.8	0.02
Body mass index (kg/m^2)	22.4	0.1	24.2	0.1*	23.8	0.1†	<0.01
Physical activity index	35.8	0.2	34.9	0.5*	35.4	0.3	0.09
Women							
Total cholesterol (mg/dL)	194.3	0.5	200.9	1.2*	203.1	0.9†	<0.01
HDL-chotesterol (mg/dL)	53.2	0.2	51.9	0.4*	51.5	0.3†	<0.01
Triglyceride (mg/dL)[§]	90.0	(89.4–90.7)	108.3	(106.5–110.1)*	105.2	(103.8–106.6)†‡	<0.01
Blood glucose (mg/dL)	98.9	0.3	105.2	0.8*	103.3	0.6†‡	<0.01
Lipoprotein(a) (mg/dL)[§]	14.7	(14.5–15.0)	13.3	(12.8–13.7)	14.9	(14.5–15.3)†	0.01
Fibrinogen (mg/dL)	248.4	1.0	256.1	2.3*	250.5	1.9†‡	0.01
Factor VIIc (mg/dL)	113.7	0.5	117.1	1.0*	117.0	0.8†	<0.01
Body mass index (kg/m^2)	22.6	0.0	24.8	0.1*	23.9	0.1†‡	<0.01
Physical activity index	31.7	0.1	30.9	0.2*	31.5	0.1	<0.01

[$]Mean and SEM were shown with adjustment for age using ANOVA. [§]Geometric mean (±SEM). [#]$p<0.05$, ANOVA adjusted for age. *$p<0.05$, normotensives *vs.* treated hypertensives; †$p<0.05$, normotensives *vs.* nontreated hypertensives, ‡$p<0.05$, treated hypertensives *vs.* nontreated hypertensives. *, † and ‡ using Scheffe's test.

Hypertension is an important condition affecting health in Japan as well as in Western countries; hypertension contributes to CVD, such as stroke, ischemic heart diseases, heart failure, and high mortality. In prospective cohorts for the populations, there was a five-fold difference in stroke risk between the highest and lowest of the six BP categories, but relative stroke risks were greater in middle age than in old age even though absolute stroke risks were higher in old age (*8*). In other cohorts for a population in East Asia, BP was an important determinant of stroke risk, whereas cholesterol concentration was less important. The authors concluded that the association between BP and stroke seems stronger in East Asia than in Western populations, and a population-wide reduction of 3 mmHg in DBP eventually decreased the number of strokes by about one-third (*22*).

In case-control studies for the incidence of stroke in hypertensive patients, higher levels of BP were related to the onset of stroke (*7*, *23*, *24*) and myocardial infarction (*6*), and a considerable proportion of the stroke incidence among treated hypertensive patients may be prevented by achieving BP control (*7*). Bulpitt *et al.* reported that the optimal level of BP control for survival was treated SBP of <134 mmHg in men and <149 mmHg in women, and treated DBP of <95 mmHg in men and women in a prospective cohort study in hypertensive patients (*11*).

Many intervention trials have proven that antihypertensive drugs will help reduce the incidence of CVD. In a meta-analysis, the effect of antihypertensive drugs was significant for stroke and CVD in men and women; and, in terms of relative risk, the treatment benefit did not differ between men and women (*25–27*). Meanwhile, arterial stiffness increased with age according to the severity of hypertension, after adjustment for BP (*28*).

Even if hypertensive patients receive antihypertensive treatment, such treatment seems less effective if it does not achieve the target BP, for example 140/90 mmHg. In the present study, actually, elevation in treated hypertensives was seen in some cardiovascular risk factors, such as total cholesterol, blood glucose, fibrinogen, BMI, and PAI, in comparison with normotensives. Antihypertensive treatment does not modify the patient's physical condition and does not reduce BMI. A similar analysis of blood glucose in Japanese patients was performed in a previous study, and serum insulin and BMI were also higher in treated hypertensives than in normotensives, although hypertension was defined by SBP ≥160 mmHg and/or DBP ≥95 mmHg (*1*).

We found a different tendency between men and women with regard to HDL cholesterol. Although HDL was known to

Table 4. The Rates of Status Categorized with Metabolic Status, Smoking and Drinking

	Normotensives (*n* (%))	Hypertensives (*n* (%))		$p^{\#}$	$p^{\#\#}$
		Treated	Nontreated		
Men					
Lipid status					
Dyslipidemia†	865 (31.2)	169 (37.6)	465 (39.0)	<0.01	<0.01
Nomal	1,909	281	726		
Blood glucose status					
IGT	671 (24.2)	143 (31.8)	397 (33.3)	<0.01	<0.01
Nomal	2,103	307	794		
Metablic syndrome					
MS§	141 (5.2)	94 (22.4)	246 (21.2)	<0.01	<0.01
Non-MS§	2,556	326	913		
Smoking status					
Current	1,494 (54.0)	161 (35.9)	565 (47.7)	<0.01	0.03
Ex-	706	185	355		
Non	565	103	265		
Drinking status					
Current	1,983 (73.3)	337 (78.6)	898 (78.4)	<0.01	<0.01
Ex-	93	28	34		
Non	630	64	214		
Women					
Lipid status					
Dyslipidemia†	1,345 (29.1)	404 (47.0)	646 (45.9)	<0.01	<0.01
Nomal	3,275	456	761		
Blood glucose status					
IGT	832 (18.0)	261 (30.0)	382 (27.7)	<0.01	<0.01
Nomal	3,788	599	1,025		
Metablic syndrome					
MS§	154 (3.4)	203 (24.8)	225 (16.4)	<0.01	<0.01
Non-MS§	4,371	616	1,149		
Smoking status					
Current	292 (6.4)	31 (3.7)	54 (3.9)	<0.01	0.04
Ex-	150	18	28		
Non	4,097	793	1,293		
Drinking status					
Current	1,192 (26.7)	151 (19.2)	325 (24.2)	<0.01	0.31
Ex-	63	12	27		
Non	3,217	625	990		

*χ^2 test, **Mantel-Haenzel test adjusted for age (10 years). †Dyslipidemia: total cholesterol ≥220 mg/dL and/or triglyceride ≥150 mg/dL. §Metabolic syndrome: BMI ≥25 kg/m^2 as an essential combined with 2 or more of the following components: triglycerides ≥150 mg/dL and/or HDL cholesterol <40 mg/dL; systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg; fasting blood glucose ≥110 mg/dL. IGT, impaired glucose tolerance; MS, metabolic syndrome; BMI, body mass index; HDL, high-density lipoprotein.

be affected by drinking alcohol (*29*, *30*), the results was identical after adjustment for age and alcohol drinking status. Higher HDL cholesterol in normotensives in women was due to a lower BMI level. That was because the difference in HDL cholesterol in women was not detected after adjustment for BMI in addition to age and drinking status. Yamamoto *et al.* reported that low HDL was related to high triglycerides and BMI in large-scale cross-sectional data, and this tendency was similar to ours (*31*). Serum insulin was higher in hypertensives with or without treatment in our data (data not shown because of the smaller number of subjects). Of course, lowering BP reduces the incidence of CVD, but it is more important to control BP or modify lifestyle. In a clinical setting, home BP has been used to evaluate BP control in recent years, but even now, neither home nor office BP values are adequately controlled (*32*).

We examined metabolic profiles such as dyslipidemia and IGT in relation to the different BP categories. In recent years it has been proposed that hypertension is part of a cluster of metabolic risk factors and is such as MS (*33*), and that MS is related to other CVD risk factors and manifestations, such as pulse wave velocity and sleep apnea syndrome (*34–37*). The Japanese criteria for MS were applied using BMI $\geq$25 kg/m^2 to define obesity in the present study, because waist circumference was not measured in most of the subjects. The proportion of hypertensive subjects with MS was also higher than that of normotensives with this syndrome. Even if we could have controlled BP, we could not control all other risk factors for CVD. Shapo *et al.* reported results similar to ours, *i.e.*, higher levels of total cholesterol, triglycerides, and glucose tolerance in hypertensives with or without medication than in normotensives, and higher levels in untreated than treated hypertensives. In our study, after adjustment for age, the findings were identical (*13*). In other studies, two-thirds of the hypertensive population were aware of the diagnosis, but only a quarter of the hypertensives were adequately controlled (*4*, *14*, *16*). Our data showed a similar tendency.

We thought a limitation of the present study was that only the relationship between BP categories and cardiovascular risk factors was investigated, and not the relationships between BP categories and CVD as true outcomes. We have been conducting a follow-up study for stroke and myocardial infarction, and some data will be shown in the future.

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Original Article

Comparison of C-reactive Protein Levels between Serum and Plasma Samples on Long-term Frozen Storage after a 13.8 Year Interval: The JMS Cohort Study

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BACKGROUND: C-reactive protein (CRP) is an acute phase reactant, and has been reported as a predictor of cardiovascular diseases. Measurements of high sensitive CRP in thawed samples are possible and the values are thought to remain stable even after frozen storage. However, the long-term stability of CRP values has not been documented. We measured the values of CRP before and after long-term storage, and examined the difference in determined values.
METHODS: High sensitive CRP was measured before and after long-term storage of samples from 99 men and women among the JMS Cohort Study subjects. We selected subjects who underwent measurement of high sensitive CRP at the baseline by stratified sampling methods using baseline CRP values. CRP was measured in serum samples at the baseline and in thawed plasma samples after an average storage period of 13.8 years.
RESULTS: Geometric means of CRP were 0.25 mg/L and 0.59 mg/L before and after storage, respectively. The CRP values were significantly higher after long-term frozen storage than at the baseline ($p<0.0001$). The both values of logarithm CRP were significantly correlated using Pearson's correlation ($r = 0.920$, 95% confidence interval: 0.883-0.945).
CONCLUSION: CRP values increased after long-term frozen storage. The CRP values showed a high correlation between before and after long-term storage.
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Key words: C-reactive Protein, Stability, Time, Plasma, Japanese

C-reactive protein (CRP) is an acute phase reactant and a marker of inflammation in the human body. Some studies have reported high sensitive CRP (hsCRP) is a marker of cardiovascular diseases not only in western countries[1-9] but also in Japan.[10] Danesh et al[9] reviewed the relationship between CRP and coronary heart disease (CHD). The odds ratio of the top third to the bottom third was about 1.5 on a meta-analysis.

Because some cross-sectional studies reported that CRP correlate with obesity, high triglycerides, low high-density lipoprotein (HDL) cholesterol and abnormal glucose metabolism, the relationship between CRP and metabolic syndrome has received attention in recent years.[11-15] In some other studies, CRP was a predictor of future metabolic syndrome.[16-19] Ridker et al[20] indicated that the interrelationship between hsCRP and metabolic syndrome strongly predicts CHD and CVD death. A recent statement from the Centers for Disease Control and Prevention and the American Heart Association concluded that it is reasonable to measure CRP as an adjunct to the measurement of established risk factors in order to assess the risk of coronary heart disease.[21]

It is necessary to use stored samples in nested-case control

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studies or cohort studies, when we examine new risk factors. Many cohort studies[1,3,5,10] and nested case-control studies[4,6,7,9] have examined the relationship between CRP and CVD using frozen samples stored for many years.

Variability in the measurement of CRP under various situations, including freeze/thaw experiments did not affect the levels of CRP over a short period of time.[22,23] However, the long-term stability of CRP values in frozen samples has not been examined previously. In the present study, we measured CRP values before and after long-term storage, and examined the long-term variability of CRP values in frozen samples.

METHODS

HsCRP was measured before and after long-term storage of samples obtained from 99 men and women among the JMS Cohort Study subjects. A detailed description of the JMS Cohort Study was reported previously.[24] The subjects were population-based and were followed for incidences of stroke and myocardial infarction. CRP was measured with non-thawed serum samples at the baseline in 6,759 participants (2,573 men and 4,186 women). After an average storage period of 13.8 years, we selected 99 samples (45 men and 54 women) by the stratified random sampling method using baseline CRP values. Distribution of CRP values is highly skewed, and we intended to select samples showing various levels of CRP at the baseline.

Blood samples were obtained in the morning after an overnight fast, and drawn from the antecubital vein of seated subjects with minimal tourniquet use. Tubes were centrifuged at 3,000 g for 15 minutes at room temperature within two hours after sampling. After separation, the serum samples were stored at 4℃ in refrigerated containers if analyses were to be performed within two days. Plasma samples were stored in refrigerated containers with dry ice for a maximum of 6 hours, and then frozen as rapidly as possible at −80℃ for storage until laboratory tests were performed.

At the baseline, CRP values were measured from serum samples using highly sensitive nephelometry, a latex particle-enhanced immunoassay (NA Latex CRP Kit, Dade Behring, Tokyo, Japan). The value in the calibrator was assigned using certified reference Material 470 (IRMM, Geel, Belgium), and international plasma protein reference material to achieve international standardization for the assay of CRP. The function of the assay was satisfactory.[25].The lower limit of detection of CRP in the assay is 0.030 mg/L and undetectable CRP values were recorded as 0.015 mg/L.

We stored frozen plasma samples with trisodium citrate at −80℃ after one freeze/thaw procedure to measure coagulate factors at the baseline, and measured CRP levels using the same methods after an average storage period of 13.8 years. At this time, the assay is sufficiently sensitive to detect 0.050 mg/L of CRP, therefore, undetectable CRP values were recorded as 0.025 mg/L.

Distributions of CRP and triglycerides were skewed, and those were expressed as the geometric means and ± standard deviation (SD). Variables were expressed as mean ± SD except CRP and triglycerides, and categorical data were in proportion. We analyzed the correlation between the two measurements of CRP using Pearson's correlation coefficients with logarithm CRP and 95% confidence interval. T-test was performed between the two measurements. Statistical analysis was performed with SPSS® (Version 14.0J; Chicago, IL, USA).

RESULTS

The general characteristics of the subjects were shown in Table 1. The mean and standard deviation of age was 53.6 ± 11.9 years, and those of body mass index were 23.2 ± 3.2 kg/m². Levels of CRP ranged from undetectable to 40.0 mg/L before storage and from undetectable to 31.3 mg/L after storage (Figure 1). Medium levels of CRP were 0.23 mg/L and 0.57 mg/L, and geometric means of CRP were 0.25 mg/L and 0.59 mg/L before and after storage, respectively. The levels of CRP after storage were significantly higher than those before interval ($p<0.0001$, paired t-test) (Table 2). Distributions of CRP values were similar in both sexes in each measurement, and there was no significant difference in CRP values between men and women at each time point. Correlation was significant between the values of logarithm CRP using Pearson's correlation coefficient ($r=0.920$, confidence interval: 0.883-0.945) (Figure 2).

DISCUSSION

We showed the intra-individual variability of CRP values obtained before and after 13.8 years of frozen storage. We found a close relationship between the two measurements, but CRP values examined after long-term storage were significantly higher than those at the baseline.

Our colleagues reported previously that CRP values were lower in Japanese than in western subjects, were less than the lowest detection value (0.03 mg/L) in more than 10% of the subjects in the general population,[26] and that intra-individual correlation coefficient over 5 years was 0.43.[27] In the present study, we selected 99 subjects among those who underwent CRP examination at the baseline using the stratified sampling method. Distribution of CRP was highly skewed, and the values of CRP would be mainly in the lower level when random sampling was performed. We tried to examine reproducibility after long-term storage using a wide spectrum of the CRP values; from less than the minimum to more than 30 mg/L.

It has been reported that CRP value remains stable in a frozen sample.[22,28] Macy et al[22] reported several types of intra-individual variability using samples collected at a single time point. They compared sample types; serum, SCAT-1 plasma, SCAT-2 plasma, EDTA plasma, and citrate plasma samples in six individuals, CRP values were lower in sodium citrate tubes than in other types of

Table 1. General characteristics of the subjects with two measurements of C-reactive protein

	n	Mean	SD
Age (years)	99	53.6	11.9
Body mas index (kg/m²)	81	23.2	3.2
Systolic blood pressure (mmHg)	81	126.0	19.5
Diastolic blood pressure (mmHg)	81	74.9	11.4
Total cholesterol (mg/dL)	99	198.1	34.8
Triglycerides (mg/dL)*	99	110.0	(58.7-206.1)
HDL-cholesterol (mg/dL)	99	46.9	12.2
Sex (Men/Women)	45/54		
Smoking**			
Current	16 (20)		
Ex-smoker	15 (19)		
Non-Smoker	50 (62)		
Alcohol drinking**			
Current	41 (51)		
Ex-drinker	3 (4)		
Drinker	37 (46)		

*: Geometric mean (± standard deviation)
**: Number (percentage)

Table 2. The values of C-reactive protein before and after 13.8 years interval.

		Percentale				
	n	1	25	50	75	99
CRP, first (mg/L)	99	0.015	0.130	0.230	0.470	40
CRP, second (mg/L)	99	0.025	0.307	0.568	0.960	31.3

		Geometric mean	Standard deviation*
CRP, first (mg/L)	99	0.251	(0.04-1.40)**
CRP, second (mg/L)	99	0.587	(0.17-1.99)

CRP, first: measured at the baseline
CRP, second: measured after defrosted with 13.8 years interval
*: Geometric mean ± standard deviation
**: $p<0.0001$ by paired t-test

tubes. They also compared values in a freeze/thaw experiment and thawed samples once, twice, three, and four times using the same 5 types of tubes. There were no significant differences in the mean value of CRP and CRP remained stable throughout multiple freeze/thaw cycles. Aziz et al[23] confirmed that stability of CRP levels did not differ between serum and plasma samples, or after 7 freeze-thaw cycles. They also found that there were no significant differences in CRP levels with up to a 6 hour delay in specimen processing. Nisson et al[28] reported in 34 patients admitted to a coronary care unit using 10 year frozen samples that hsCRP showed a predictable slope in the regression between serum and citrate plasma ($[hsCRP]_{citrate}=0.902 \times [hsCRP]_{serum} - 0.1695$).

We stored samples at 4℃ within 2 hours after processing specimens, after that hsCRP was measured using serum samples within few days. Otherwise, only one thaw-freeze cycle was performed before long-term storage of plasma samples. It was considered that neither our measurement procedure nor storage critically affected the levels of CRP.

We first measured CRP values using serum sample at the baseline, and second using thawed plasma sample after long-term storage. According to the study by Macy et al,[22] the fact that the CRP values of the second measurement were higher than those of the first was not due to the different types of sample tubes. The variability of differences between the two measurements could not be

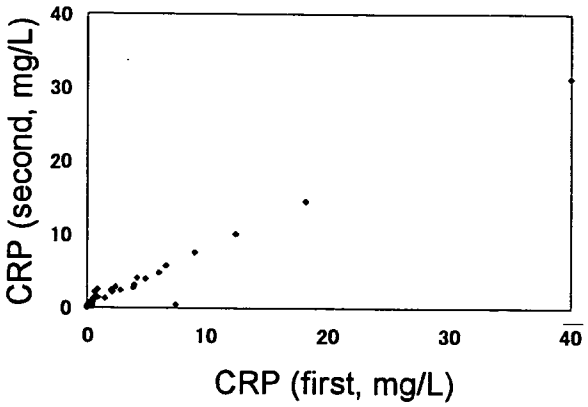

Figure 1. Plots of raw C-reactive protein measurements before and after storage for 13.8 years.
Samples were measured at the baseline (CRP, first), and after thawing following long-term frozen storage (CRP, second).

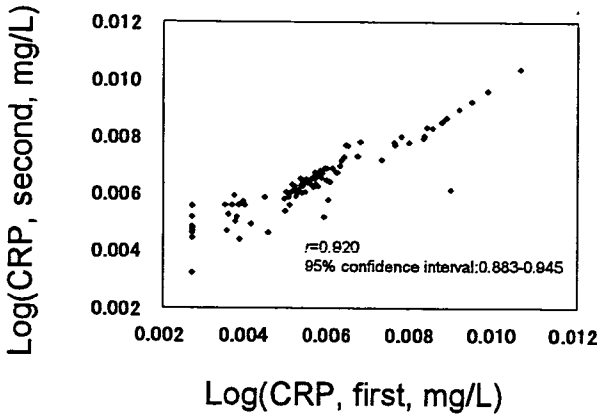

Figure 2. Plots of logarithm of C-reactive protein measurements before and after storage for 13.8 years.
Samples were measured at the baseline (CRP, first), and after thawing following long-term frozen storage (CRP, second).

explained by regression to the mean phenomenon because we selected samples using stratified methods, but only the lower values of CRP at the first measurement showed an increase, while higher CRP levels did not decline. We speculated that differences between the two measurements were caused by evaporation during long-term frozen storage, and especially affected low CRP values. Another possibility is that progressive changes in the precipitability of some proteins might occur. A linear relationship was seen in both Figures 1 and 2, and the values converged toward a high level of CRP.

To our knowledge, this is the first study to examine the stability between before and after long-term interval of frozen storage. Some limitations exist in the present study. First, we selected samples randomly from groups stratified by CRP value at the baseline, not simple random samples. Second, the second measurement was performed after freeze/thaw procedure once at the baseline to measure coagulating factors. Third, we could not confirm the effect of evaporation, because other measurements, such as elctrolyte: potassium, sodium or chloride, were not done. The samples were stored in tempered tubes to prevent evaporation. Nevertheless, evaporation might have occurred during storage, and CRP values in the second measurement might have increased. We could confirm the linear relationship between the two measurements due to stratified selection. We could not estimate how CRP values change during long-term frozen storage, but the results of the present study might characterize the reliability of the long-term storage data. It is considered reasonable to use CRP values of frozen samples in epidemiological studies to estimate risk ratios, such as odds ratio or hazard ratio. However, we should be careful when determining the cut-off level of CRP values as a risk factor based on values obtained using frozen samples after long-term storage.

In conclusion, the CRP values showed a high correlation between before and after long-term storage. In our data, the values after long-term storage were significantly higher than those before storage, although we could not confirm the effect of evaporation. Other studies to examine differences before and after storage for various periods should be conducted in future.

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BRIEF REPORT

Serum Ghrelin and Carotid Atherosclerosis in Older Japanese People with Metabolic Syndrome

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Ghrelin may play a role in the development of atherosclerosis. However, the effect of serum ghrelin on carotid intima-media thickness (cIMT) (well-established as a surrogate marker to atherosclerosis) in metabolic syndrome (MS), particularly among relatively older subjects, has still not been thoroughly investigated. A total of 101 subjects >60 years of age (mean age, 72.3 years) with MS were enrolled in the study to investigate the relationship between serum total ghrelin and B-mode ultrasonographic cIMT levels. There were significantly positive correlations between cIMT and both age and systolic BP, but cIMT was significantly inversely correlated to ghrelin levels. In the multiple regression analysis for cIMT adjusted by other measured parameters, ghrelin was a significant and independent factor along with age and systolic BP. These findings suggest that decreased ghrelin levels may be related to carotid atherosclerosis among older subjects with MS. © 2006 IMSS. Published by Elsevier Inc.

Key Words: Carotid intima-media thickness, Risk factor, Blood pressure, Elderly.

Introduction

Ghrelin, an endocrine peptide newly identified mainly in stomach epithelium, stimulates food intake in humans (1). In individuals with a negative energy balance, such as a low-calorie diet, regular exercise or anti-obesity medication, serum ghrelin levels are increased (1). Furthermore, ghrelin may play a role in energy balance, lipid-lipoprotein/glucose metabolism and blood pressure (BP) regulation (2,3). Indeed, low serum ghrelin levels have been reported to be associated with insulin resistance, elevated BP and type 2 diabetes (2). Also, cardioprotective effects of ghrelin have been suggested (1,4,5). These results imply a beneficial role of this hormone in the development of atherosclerosis.

On the other hand, metabolic syndrome (MS) is an important factor in accelerating the atherosclerotic process (6). However, there have been few studies examining the influence of ghrelin on atherosclerosis in MS (6). At present, a considerable number of studies using carotid intima-media thickness (cIMT) have been accumulated; therefore, cIMT is considered a well-established clinical surrogate marker of atherosclerosis (7). Despite the present status of cIMT, there have been no studies to assess the influence of ghrelin on atherosclerosis using this marker. Thus, it is crucial to determine the relationship between ghrelin and atherosclerosis in MS using cIMT. Here we examine the relationship between serum ghrelin and ultrasonographic cIMT levels in MS, particularly in a population of older subjects, which may reflect the potential long-term effects of ghrelin in addition to some conventional atherosclerotic risk factors.

Subjects and Methods

Altogether, 101 subjects (male:female, 31:70; age range, 60 to 85 years; mean age, 72.3 ± 7.6 years (mean ± SD) participated in the study, which was conducted according to the

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principles of the Declaration of Helsinki. Each subject gave informed consent. All subjects were recruited from outpatients and community volunteers. Subjects with gastric surgery were excluded. None of the subjects modified their regular diet or exercise habits or were using any medications.

According to the recent World Health Organization (WHO) (8) and NCEP-ATP III report (9) with minor modifications (10), MS was defined as the presence of at least three of the following five conditions: 1) obesity identified by body mass index (BMI) >25.0 kg/m^2, 2) elevated BP identified by systolic BP >130 mmHg and/or diastolic BP >85 mmHg, 3) hypertriglyceridemia identified by fasting serum triglyceride values of >1.70 mmol/L, 4) low high-density lipoprotein (HDL) cholesterolemia identified by fasting serum HDL cholesterol values <1.04 mmol/L in men and <1.30 mmol/L in women, and 5) elevated glucose levels identified by fasting plasma glucose values >6.11 mmol/L. There were 28 male and 9 female smokers defined as current smokers. BMI was calculated as weight (kg)/height (m^2) in each subject. We measured BP in the right upper arm of seated patients using a standard sphygmomanometer. Overnight fasting plasma glucose, serum total cholesterol and triglyceride values were measured using enzymatic methods, and HDL cholesterol values were measured using a homogenous method. Plasma insulin (immunoreactive insulin) values were measured using the enzyme immunoassay (EIA). Serum total ghrelin values were measured using the EIA kit (Peninsula Laboratories Inc., San Carlos, CA). An ultrasonographic measurement of cIMT using a 7.5-MHz linear type B-mode probe (SSD-900, Aloka Co. Ltd., Tokyo, Japan) was performed by an ultrasonographic specialist. Each subject was examined in the supine position. Because the internal and external carotid arteries could not be fully detected in some patients, the intima-media thickness of the common carotid artery was bilaterally measured in segments free of plaque (plaque was defined as the presence of wall thickening at least 50% greater than the adjacent thickness) with longitudinal and transverse scans. After three measurements of cIMT were taken at the thickest site and at two other points, 10 mm upstream and 10 mm downstream from the thickest site, these measurements were averaged. All assessments were blind to the subjects' clinical characteristics.

All values are expressed as mean ± SD. The correlations between cIMT levels and various parameters were, respectively, examined by Pearson's correlation test. The relationship between cIMT levels and other measured parameters including serum ghrelin levels were also examined by multiple regression analysis; $p<0.05$ was considered significant.

Results

The average levels of each parameter in all subjects were calculated: BMI, 23.3 ± 3.2 kg/m^2; systolic BP, 145.8 ± 15.3 mmHg; diastolic BP, 81.2 ± 8.6 mmHg; total cholesterol, 5.25 ± 0.78 mmol/L; triglyceride, 1.66 ± 0.62 mmol/L; HDL cholesterol, 1.34 ± 0.37 mmol/L; glucose, 6.11 ± 1.22 mmol/L; insulin; 9.8 ± 10.0 μU/mL; ghrelin, 169.9 ± 147.8 pg/mL; cIMT, 0.96 ± 0.22 mm. In the simple correlation test, ghrelin was significantly inversely correlated to BMI ($r=-0.220$, $p<0.05$) but not correlated to other parameters such as BP, lipid-lipoprotein, glucose and insulin. In the simple correlation test, cIMT levels significantly positively correlated to both age ($r=0.455$, $p<0.0001$) and systolic BP ($r=0.310$, $p<0.01$) and inversely to ghrelin ($r=-0.256$, $p=0.01$). These significant correlations for cIMT remained after adjustment of the other parameters (Table 1).

Discussion

The effects of ghrelin on increased cIMT, which is a surrogate marker to the development of atherosclerosis, remain to be fully elucidated. The present study suggests that ghrelin might play a role in increased cIMT, independently with age and systolic BP, in older subjects with MS. Positive associations between cIMT and both age and BP in this study are basically consistent with prior findings of studies on MS (10,11). There were weaker associations between the other parameters such as lipid-lipoprotein/glucose and cIMT in this study. Although it has been described that subjects with hyperlipidemia and diabetes mellitus generally have increased cIMT (14), a nonsignificant association between lipid-lipoprotein/glucose and cIMT has been found elsewhere (15). Although fasting insulin has also been reported to be related to carotid atherosclerosis (16), several studies have found no association between insulin and cIMT levels (17–19), as with our results. This disagreement may be partly explained by differences in the study populations.

Table 1. Multiple regression analysis for carotid intima-media thickness with clinical parameters including ghrelin

Parameters	β-coefficient	p value
Age (years)	0.375	<0.0001*
Gender, male	0.050	0.702
Smoking, current smoking	0.105	0.406
Body mass index (kg/m^2)	0.109	0.241
Systolic blood pressure (mmHg)	0.224	0.020*
Diastolic blood pressure (mmHg)	−0.186	0.074
Total cholesterol (mmol/L)	0.169	0.076
Triglyceride (mmol/L)	−0.137	0.122
HDL cholesterol (mmol/L)	−0.029	0.752
Glucose (mmol/L)	0.111	0.212
Insulin (μU/mL)	0.144	0.108
Ghrelin (pg/mL)	−0.192	0.026*

HDL, high-density lipoprotein.
*$p<0.05$, statistical significance.

Our study population tended to include subjects who did not have markedly higher levels of lipid-lipoprotein/glucose and who were relatively older, so the influence of these characteristics might be considered.

Because reduced ghrelin levels raise BP levels (2), systolic BP, which is raised by ghrelin, could be associated with increased cIMT. Also, ghrelin may be affected by some metabolic factors such as obesity, lipid-lipoprotein, glucose and insulin (20). For example, obesity has been reported to be inversely related to ghrelin levels (21) and an inverse correlation of BMI to ghrelin was found in our study population. Insulin has been reported to suppress ghrelin levels independently of BMI (20,22). However, our results suggest that ghrelin was significantly associated with cIMT, independent of these metabolic parameters. To date, it has been reported that ghrelin acts as a vasodilator and shows anti-inflammatory actions via various mechanisms concerning cytokine production (6,12,13), oxidative stress (23) and sympathetic activity (13). In subjects with MS, it has been more recently demonstrated that ghrelin reverses endothelial dysfunction by increasing nitric oxide bioactivity (6). These data, together with our results, suggest that the increased ghrelin levels themselves may be potentially beneficial to the reduction of atherosclerosis among older subjects with MS. Additionally, decreased ghrelin levels are thought to be associated with aging (2). Considering our results, ghrelin, even at its lower levels in our older subjects, may have a significant effect on cIMT levels. The mechanisms between ghrelin and atherosclerosis remain to be further explored in future studies.

There were some limitations to this study. Because of its cross-sectional design, we have not stated any cause and effect relationship in the results. Actually, there are few patients with MS among older people in Japan, so this study was done with a relatively small sample size. Due to the above definition of MS, our study population may be somewhat heterogeneous. Although lifestyle factors such as diet and exercise are related to ghrelin levels (1), we unfortunately did not collect such detailed information from each subject.

In conclusion, our study suggests that decreased serum ghrelin levels might be associated with increased cIMT, independent of age and systolic BP, in relatively older subjects with MS. Further research with a perspective design and a larger sample size is needed to clarify the causal role of ghrelin in carotid atherosclerosis.

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Severe Decrease in Serum HDL-Cholesterol During Combination Therapy of Bezafibrate and Pioglitazone

Fibrate often produces a rise in HDL-cholesterol (C) mediated by the increased expression of apolipoprotein A-I by the activation of peroxisome proliferator-activated receptor (PPAR)α. It is also well known that pioglitazone increases HDL-C by activation of PPARγ. Many diabetic patients with hypertriglyceridemia receive combination therapy of fibrate and pioglitazone; however, there have been a very small number of reports describing a severe decrease in serum HDL-C with this combination therapy[1, 2]. We encountered a profound decrease in HDL-C with combination therapy of bezafibrate and pioglitazone in a man aged 61 who had type 2 diabetes mellitus, hyperlipidemia, and impaired renal function. Initial serum levels of total cholesterol (TC), triglycerides (TG), HDL-C, and creatinine (Cre) were 257 mg/dL, 217 mg/dL, and 38 mg/dL, and 1.6 mg/dL, respectively, in October 2000 before starting the combination therapy. He suffered from diarrhea, nausea, appetite loss, and general fatigue for 7 days before admission to our hospital on June 27, 2005. Until admission, he was taking 400 mg/day of bezafibrate and 15 mg/day of pioglitazone. Serum HDL-C levels with this combination therapy were around 10 mg/dL. On the admission day, serum fasting levels of TC, TG, HDL-C, glucose, HbA1c, blood urea nitrogen (BUN), Cre, albumin, and hemoglobin were 114 mg/dL, 207 mg/dL, 4 mg/dL, 104 mg/dL, 3.8%, 30 mg/dL, 3.2 mg/dL, 3.1 g/dL, and 8.2 g/dL, respectively. On the 14th hospital day after stopping both bezafibrate and pioglitazone, HDL-C increased to 16 mg/dL, and on the 28th hospital day HDL-C returned to 35 mg/dL (**Fig. 1**), and serum levels of TC, TG, BUN, Cre, albumin, and hemoglobin were 173 mg/dL, 77 mg/dL, 72 mg/dL, 3.2 mg/dL, 3.7 g/dL. and 8.4 g/dL, respectively.

One of the causes of the decrease in HDL-C on admission was insufficient food intake and poor general condition; however, taking such conditions into consideration, his 4 mg/dL HDL-C level was too low. The precise mechanism of fibrate-, pioglitazone-, or this combination-induced hypo-HDL-cholesterolemia is, as yet, unclear in the literature. However, it is supposed that polymorphisms in the promoter regions of several genes regulated by PPARα and/or PPARγ involved in the HDL metabolic pathway may contribute to the HDL-C level at baseline and may account for dramatic change in HDL metabolism[3, 4]. Furthermore, since this patient had decreased renal function when starting bezafibrate, which should be prescribed very carefully in patients with decreased renal function because of its renal excretion and renal impairment, the serum concentration of bezafibrate may increase.

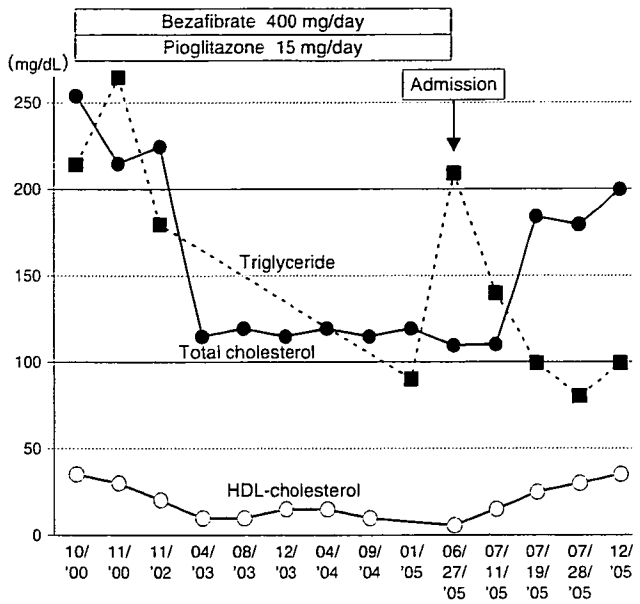

Fig. 1. Lipid pattern in relation to the combination therapy of bezafibrate and pioglitazone.

It is unclear whether the profound decrease in HDL-C in this case occurred by bezafibrate, or pioglitazone, or their combination. However, it would be advisable to ensure that HDL-C has been documented before starting fibrate and/or pioglitazone and that HDL-C is rechecked shortly after beginning these drugs.

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RESEARCH ARTICLE

Adeno-associated virus vector-mediated interleukin-10 gene transfer inhibits atherosclerosis in apolipoprotein E-deficient mice

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Inflammation is a major contributor to atherosclerosis by its effects on arterial wall biology and lipoprotein metabolism. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that may modulate the atherosclerotic disease process. We investigated the effects of adeno-associated virus (AAV) vector-mediated gene transfer of IL-10 on atherogenesis in apolipoprotein E (ApoE)-deficient mice. A murine myoblast cell line, C2C12, transduced with AAV encoding murine IL-10 (AAV2-mIL10) secreted substantial amounts of IL-10 into conditioned medium. The production of monocyte chemoattractant protein-1 (MCP-1) by the murine macrophage cell line, J774, was significantly inhibited by conditioned medium from AAV2-mIL10-transduced C2C12 cells. ApoE-deficient mice were injected with AAV5-mIL10 into their anterior tibial muscle at 8 weeks of age. The expression of MCP-1 in the vascular wall of the ascending aorta and serum MCP-1 concentration were decreased in AAV5-mIL10-transduced mice compared with AAV5-LacZ-transduced mice. Oil red-O staining of the ascending aorta revealed that IL-10 gene transfer resulted in a 31% reduction in plaque surface area. Serum cholesterol concentrations were also significantly reduced in AAV5-mIL10-transduced mice. To understand the cholesterol-lowering mechanism of IL-10, we measured the cellular cholesterol level in HepG2 cells, resulting in its significant decrease by the addition of IL-10 in a dose-dependent manner. Furthermore, IL-10 suppressed HMG-CoA reductase expression in the HepG2 cells. These observations suggest that intramuscular injection of AAV5-mIL10 into ApoE-deficient mice inhibits atherogenesis through anti-inflammatory and cholesterol-lowering effects.
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Keywords: *IL-10; AAV; atherosclerosis; cholesterol*

Introduction

The inflammatory reaction involves complex interactions between inflammatory cells (lymphocytes and macrophages) and vascular endothelial and smooth muscle cells. The disturbance of vascular wall integrity and homeostasis by inflammation is thought to be a major contributor to atherosclerosis. Therefore, an anti-inflammatory strategy may be a promising approach to prevent and treat atherosclerotic disease. Another critical feature of atherogenesis is lipid accumulation. Several large-scale clinical trials have demonstrated that lipid reduction therapy involving HMG-CoA reductase inhibitor (statin) is useful for atherosclerotic disorders, such as ischemic heart disease.[1,2] Recent studies have indicated that statins have pleiotropic effects on the atherogenic process, including an anti-inflammatory effect.[3] On the other hand, proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6, have profound effects on lipid metabolism.[4] These findings suggest that there are complex interactions between inflammation and lipid metabolism.

IL-10, which is secreted by a wide variety of cells such as lymphocytes and macrophages, is a key inhibitor in a number of inflammatory responses,[5] including the production of proinflammatory cytokines and chemokines and the expression of endothelial adhesion molecules. IL-10 expression has been identified in early and advanced atherosclerotic plaques[6,7] and is thought to have potential antiatherogenic effects. Indeed, recent studies have shown that IL-10-transgenic mice fed a high-fat diet exhibit a decrease in atherogenesis.[8] Conversely, IL-10-deficient mice were found to suffer from more severe atherosclerosis, and the atherogenic tendency of these mice was ameliorated by the plasmid-mediated introduction of IL-10.[9] IL-10 is thought to have a protective role in human atherosclerotic disease as well.[10,11]

Despite the tremendous interest in the effects of cytokines on inflammation and lipoprotein metabolism, there have been few studies that have examined the

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influence of IL-10 on these processes *in vivo*.[12,13] As atherogenesis is a chronic process, the long-term expression of IL-10 is required in order to assess its effects on this disease. In this study, we have used adeno-associated virus (AAV) vectors for IL-10 gene transfer to investigate the antiatherosclerotic effects of IL-10 *in vivo*, because these vectors can transduce skeletal muscle and permit the sustained expression and systemic delivery of therapeutic proteins following a single intramuscular administration.[14]

Results

IL-10 expression in C2C12 cells

We first verified the integrity of our vectors *in vitro*. Differentiated C2C12 cells, murine myoblasts, were transduced with AAV encoding murine IL-10 (AAV2-mIL10) at various dosages and cultured for 48 h. The concentration of IL-10 in the conditioned medium was found to increase in a vector dose-dependent manner (Figure 1a). Western blot analysis demonstrated the presence of an 18-kDa product, the size expected for murine IL-10, in the conditioned medium of AAV2-mIL10-transduced C2C12 cells, but not in the conditioned medium of AAV2-LacZ-transduced cells (Figure 1b).

To evaluate the biological activity of secreted IL-10, we examined the influence of conditioned medium from AAV2-mIL10-transduced C2C12 cells on cytokine production by J774 cells, murine macrophages. As shown in Figure 1c, treatment with lipopolysaccharide (LPS) increased the production of the cytokines, IL-6, TNF-α, and monocyte chemoattractant protein-1 (MCP-1), from J774 cells. These increases were significantly inhibited by the addition of conditioned medium from AAV2-mIL10-transduced C2C12 cells, and the production of these cytokines was completely restored in the presence of anti-mIL-10 antibody (1 μg/ml). Unstimulated J774 cells did not exhibit any change in cytokine expression when exposed to the conditioned medium.

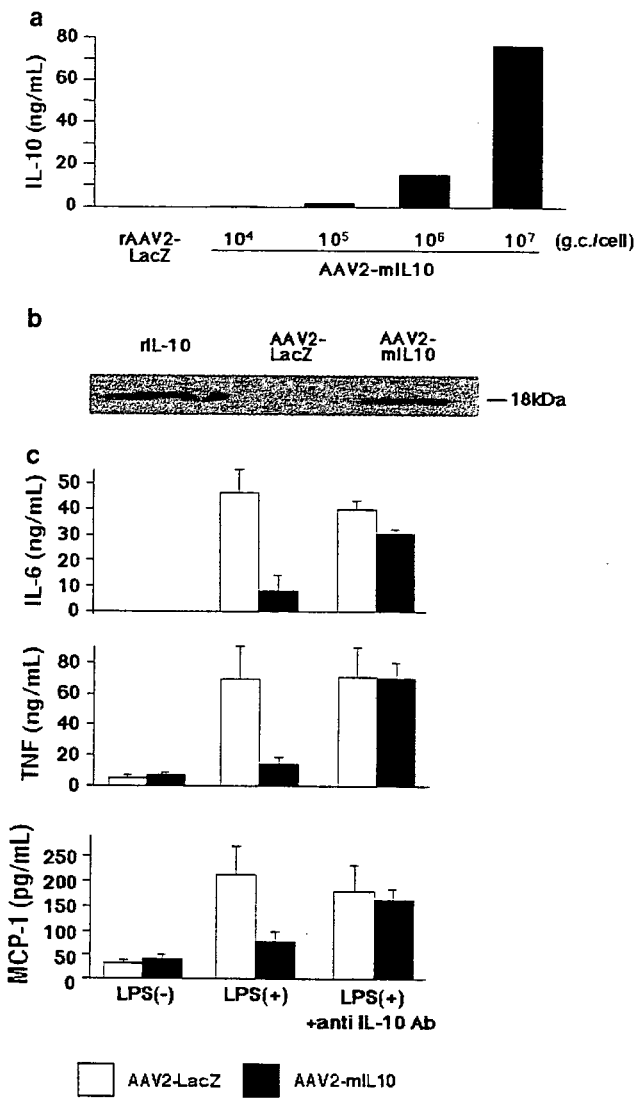

Figure 1 Transduction of the IL-10 gene into C2C12 cells with AAV2-mIL10. (a) Concentration of IL-10 in the conditioned medium of AAV2-mIL10-transduced C2C12 cells. The IL-10 concentration was measured by ELISA 48 h after transduction with the indicated number of genome copies per cell (g.c./cell). (b), Western blotting with an anti-IL-10 antibody was performed after immunoprecipitation of the conditioned medium of AAV2-mIL10- and AAV2-LacZ-transduced C2C12 cells. Recombinant mouse IL-10 (rIL-10) was used as a positive control. (c) LPS-stimulated J774 cells were incubated with the conditioned medium of AAV2-mIL10- (solid bars) or AAV2-LacZ (open bars)-transduced C2C12 cells for 24 h in the presence or absence of anti-IL-10 antibody (1 μg/ml). IL-6, TNF-α, and MCP-1 concentrations were analyzed by ELISA. Data are means ± s.e.m. (n = 4).

IL-10 expression in apolipoprotein E-deficient mice

We next injected AAV2-mIL10 and AAV5-mIL10 into the anterior tibial muscle of apolipoprotein E (ApoE)-deficient mice. The serum concentration of IL-10 increased in a vector dose-dependent manner, and the efficacy of transduction was higher in AAV5-mIL10-treated mice than in AAV2-mIL10-treated mice at the same vector dose (1×10^{13} genome copies/body) (Figure 2). When 1×10^{12} genome copies/body of AAV5-mIL10 were injected, the serum IL-10 levels (1.2–4.9 ng/ml) were maintained at a higher than physiological range (up to 160 pg/ml) for 8 weeks. Moreover, the serum IL-10 levels at 14 months were 398.3 ± 146.6 pg/ml.

Effect of IL-10 on MCP-1 expression

We then investigated the anti-inflammatory effects of IL-10 in ApoE-deficient mice by focusing on the expression of MCP-1, a potent chemokine implicated in atherogenesis. ApoE-deficient mice transduced with AAV5-mIL10 at 4 weeks old were evaluated at 8 weeks old. Few atherosclerotic lesions were detected by oil red-O staining at this time point. An immunohistochemical analysis of MCP-1 in the aortic sinus of ApoE-deficient mice revealed that MCP-1 expression was modestly suppressed in AAV5-mIL10-transduced mice, whereas it was clearly observed in the vascular wall of AAV5-LacZ-transduced mice (Figure 3a). Moreover, 8 weeks after gene transfer, the serum concentration of MCP-1 in AAV5-mIL10-transduced mice was significantly reduced compared with that in AAV5-LacZ-transduced mice (Figure 3b).

Effects of IL-10 on atherogenesis

We evaluated the lesion area in ApoE-deficient mice fed an atherogenic diet 8 weeks after gene transfer. As shown in Figure 4, the aortic sinus of mice transduced with AAV5-mIL10 revealed a significant decrease in oil

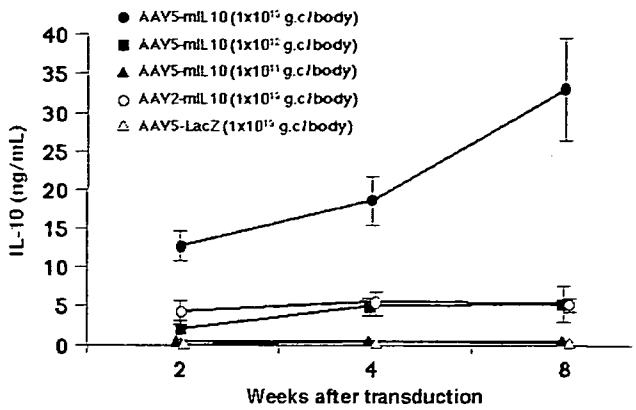

Figure 2 Serum concentration of IL-10 after transduction of AAV-mIL10 into the anterior tibial muscle of ApoE-deficient mice. ApoE-deficient mice at 8 weeks of age were inoculated with AAV2-mIL10 (1×10^{13} g.c./body), AAV5-mIL10 ($1 \times 10^{11} \sim 1 \times 10^{13}$ g.c./body), or AAV5-LacZ (1×10^{13} g.c./body) by injection into the anterior tibial muscle. At 2, 4, and 8 weeks after injection, the serum IL-10 concentration was measured. Data are means ± s.e.m. (n = 3–7).

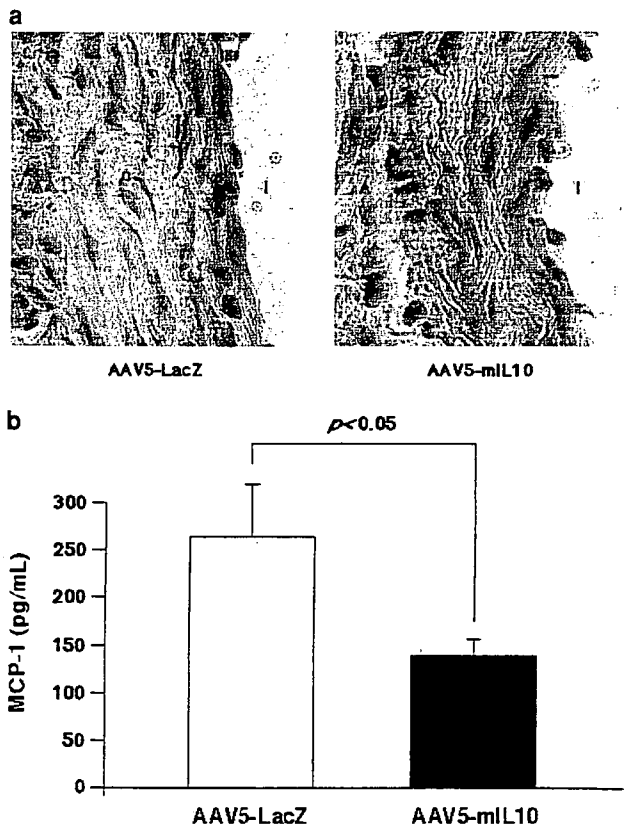

Figure 3 Systemic and local MCP-1 expression in ApoE-deficient mice. (a) Immunohistochemical staining of the aortic sinus segment in ApoE-deficient mice was performed 4 weeks after inoculation with AAV5-mIL10 (1×10^{12} g.c./body). Enhanced MCP-1 expression was observed in the vascular wall of AAV5-LacZ mice, but was suppressed in AAV5-mIL10-transduced mice. I, intima; A, adventitia. (b) The serum MCP-1 concentration in ApoE-deficient mice was measured 8 weeks after inoculation with AAV5-mIL10 or AAV5-LacZ. Means and s.e.m. for each group are presented as histograms (n = 6 for LacZ, n = 13 for IL-10).

red-O-positive areas compared to that of mice transduced with AAV5-LacZ. The systemic overexpression of IL-10 resulted in a 31% reduction in plaque surface area

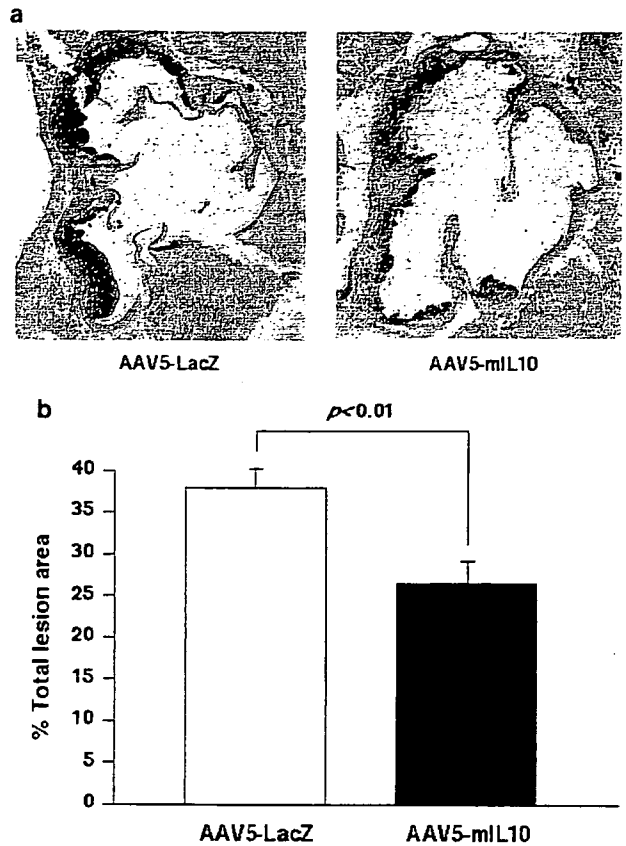

Figure 4 The inhibitory effect of IL-10 on atherogenesis in ApoE-deficient mice. (a) At 8 weeks after inoculation with AAV5-mIL10 (1×10^{12} g.c./body), the proximal aortas were removed, sectioned, and stained with oil red-O. (b) Oil red-O-positive areas were analyzed in comparison with the total cross-sectional vessel wall area. The average values for five sites from each animal were used for analysis. Means and s.e.m. for each group are presented as histograms (n = 5 for LacZ, n = 9 for IL-10).

(AAV5-IL-10, 26.5 ± 1.9% *versus* AAV5-LacZ, 37.7 ± 2.2% of total cross-sectional vessel wall area, $P < 0.01$). Figure 5 shows that serum MCP-1 concentration correlates with the extent of atherosclerotic lesion formation, suggesting that a decrease in MCP-1 expression is related to a decrease in atherosclerotic lesion formation.

Effect of IL-10 on lipids

We investigated the effects of IL-10 expression on the level of serum lipids. Total cholesterol levels were significantly reduced in the AAV5-mIL10-transduced mice (931 ± 432, 1074 ± 419 mg/dl) compared to the AAV5-LacZ-transduced mice (2212 ± 640, 1840 ± 421 mg/dl, 4 weeks and 8 weeks after gene transfer, respectively). Triglyceride level in the AAV5-IL10-transduced mice 8 weeks after gene transfer was also reduced (171.7 ± 67.3 mg/dl) compared to that in the AAV5-LacZ-transduced mice (291.6 ± 172.4 mg/dl, $P < 0.05$).

Nonlinear regression fitting to a sigmoidal dose curve revealed a high correlation between the serum cholesterol level and IL-10 concentration ($r = 0.857$), with an estimated EC_{50} of 5.3 ng/ml (Figure 6a). In addition, the serum cholesterol concentration positively correlated with the atherosclerotic lesion area ($r = 0.728$, $P < 0.01$; Figure 6b). IL-10 gene transfer did not affect body

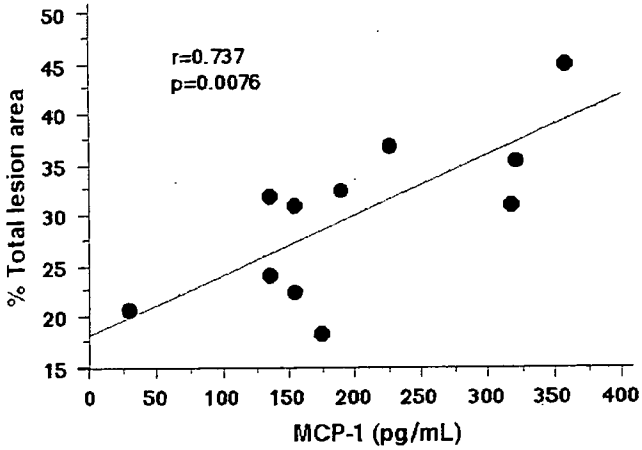

Figure 5 Correlation between serum MCP-1 concentration and extent of atherosclerotic lesion formation. The serum MCP-1 concentration positively correlated with oil red-O-positive surface area in ApoE-deficient mice 8 weeks after inoculation with AAV5-mIL10 ($r=0.737$, $P=0.0076$).

weight, food intake, blood sugar levels, or blood pressure (data not shown).

To assess whether IL-10 causes changes in the lipoprotein profile of apoE-deficient mice, plasma lipoproteins were subjected to agarose gel electrophoresis. No differences in the patterns of lipoprotein expression were observed in AAV5-mIL10-transduced mice and AAV5-LacZ-transduced mice (data not shown).

To further understand the mechanism by which the serum cholesterol level is decreased in AAV5-mIL10-transduced mice, we evaluated cholesterol levels of HepG2 cells, human hepatocytes, incubated in the absence of lipoprotein. As shown in Figure 7, the level of cholesterol in HepG2 cells was significantly decreased by the addition of IL-10 in a dose-dependent manner. The level of intracellular cholesterol was also significantly decreased by the addition of HMG-CoA reductase inhibitor, fluvastatin, to these cells. When HepG2 cells were incubated in the presence of lipoprotein, the level of cholesterol in the conditioned medium was not decreased by the addition of IL-10 (data not shown). These data suggest that IL-10 reduces *de novo* cholesterol synthesis, but does not stimulate cholesterol uptake by hepatocytes. Furthermore, we estimated the effect of IL-10 on the expression of HMG-CoA reductase. Interestingly, IL-10 significantly decreased mRNA levels of HMG-CoA reductase ($P<0.01$), while fluvastatin, enzyme inhibitor, did not alter the expression of the enzyme itself (Figure 8).

Discussion

IL-10, a pleiotropic cytokine produced by Th2-type T cells, B cells, monocytes, and macrophages, has potent anti-inflammatory properties. The main finding of the present study is that AAV vector-mediated IL-10 gene transfer to ApoE-deficient mice following a single intramuscular administration inhibits atherosclerotic lesion formation through the inhibition of MCP-1 expression and the reduction in the level of serum cholesterol.

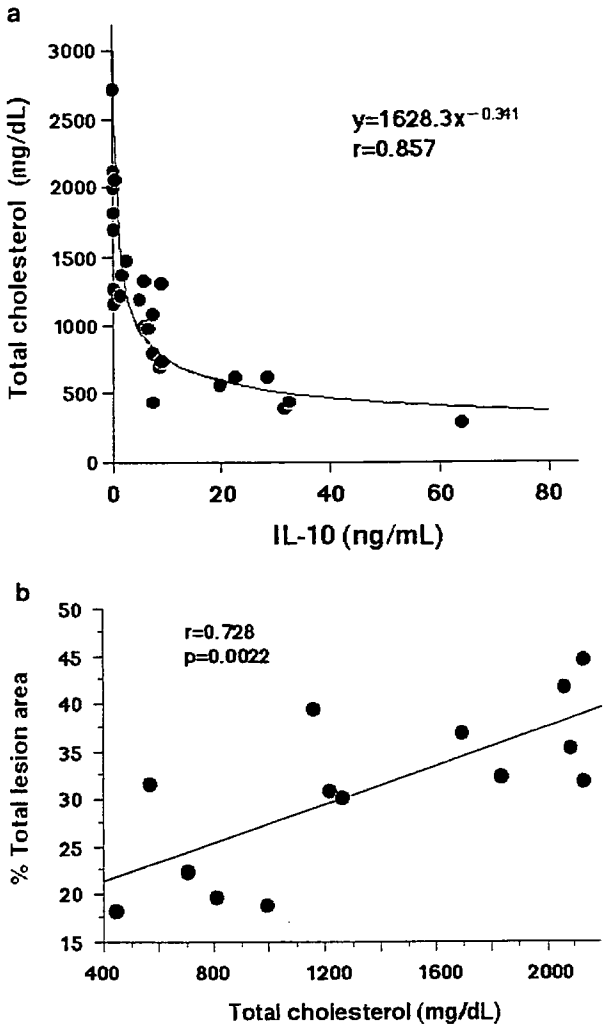

Figure 6 (a) Curve-fitting of the serum cholesterol concentration against the serum IL-10 concentration 8 weeks after inoculation of AAV5-mIL10 yielded a close fit ($r=0.857$) to a dose–response curve ($y=1628.3\times^{-0.341}$), with an EC_{50} of 5.3 ng/ml. (b) The serum cholesterol level positively correlated with atherosclerotic lesion surface area 8 weeks after inoculation with AAV5-mIL10 ($r=0.728$, $P=0.0022$).

Differentiated C2C12 cells transduced with AAV2-mIL10 were verified by Western blot analysis and enzyme-linked immunosorbent assay (ELISA) to express IL-10. The biological activity of the secreted IL-10 was also confirmed. Conditioned medium from C2C12 cells transduced with AAV2-mIL10 significantly inhibited the production of IL-6, TNF-α, and MCP-1 by J774 cells in response to LPS treatment. Based on these *in vitro* observations, we used recombinant AAV constructs to evaluate the effects of IL-10 on atherogenesis in ApoE-deficient mice. Intramuscular injection of AAV5-mIL10 into ApoE-deficient mice resulted in long-term systemic IL-10 expression. The serum IL-10 concentration was sustained at levels higher than 398.3 ± 146.6 pg/ml ($n=6$) up to 14 months after gene transfer (1×10^{12} genome copies/body).

Although double-stranded AAV genomes probably remain extrachromosomal in mouse myofibers, their tight association with chromatin allows their persistence and stable expression over periods of several months.[15–17] This particular feature of AAV vectors might be advanta-