report, BW did not show any correlation with serum concentrations of lipids except HDL-C or apolipoproteins. Thus, the difference in BW in the subjects studied cannot explain the difference in the present results. Because serum concentrations of lipids and apolipoproteins are affected by many factors such as nutrition, BW and so on, weak associations between BW and these parameters may be obscured by these environmental factors. The finding that the correlation of BW with HDL-C was significant only after adjusting for age, gender and BMI percentile may support our notion. Because BW was not a significant predictor of HDL-C in the present schoolchildren, the relationship between BW and HDL-C seems to have less physiological significance than those with adiponectin and uric acid.

Birthweight and insulin resistance

With respect to the relation between BW and insulin resistance, several recent studies have reported different results.[10,13–20] In reports on infants and children, BW and postnatal catch-up growth have been found to have a significant inverse relationship with insulin secretion and insulin resistance.[13–16] In contrast, Whincup *et al.* reported that BW is not associated with insulin secretion or resistance in a study on schoolchildren.[17] In addition, racial difference has been reported in the relationship between BW and insulin resistance.[18] In the present studies, as in a previous study, BW is not associated with insulin secretion or resistance.[17] Recently, a U-shaped relation between BW and fasting insulin was found in Pima Indian children and adolescents.[19,20] Fasting insulin and HOMA2-IR in the present children showed a similar tendency, but this association was not significant (Table 1). Thus, we have demonstrated that BW is not associated with insulin resistance or sensitivity, at least in Japanese schoolchildren.

Study limitations

Pubertal status can influence the biochemical parameters assessed in the present study. The pubertal stage of the present children was not defined, because most children were aged between 9 and 10 years (presumably prepubertal). However, we cannot exclude the possibility that some children were pubertal. Fortunately, the age distributions in the BW groups were similar and age and gender were adjusted for in the statistical analysis. Thus, the effect of pubertal status was likely to have been similar in the present subjects.

Conclusions

BW was related to serum concentrations of adiponectin and uric acid and significantly predicted serum concentrations of adiponectin and uric acid. However, WV was a stronger determinant of serum adiponectin and uric acid levels than BW in Japanese schoolchildren. Thus, it may be important to control weight gain and reduce the prevalence of obesity to prevent the development of ACHD in children, especially in children with LBW.

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Relationship Between Lipid Abnormalities and Insulin Resistance in Japanese School Children

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Objective—Dyslipidemia and insulin resistance (IR) are risk factors for coronary heart disease (CHD) in adults. To help prevent the development of CHD, it may be useful to understand the relationship between lipid abnormalities and IR during childhood.

Methods and Results—IR was assessed by the homeostasis model approximation index. We studied 1175 Japanese school children (642 boys and 533 girls), aged between 7 and 12 years. Obesity was defined by the body mass index standard deviation score (BMISD) (obese: BMISD $\geq$2.0). BMISD was most significantly associated with IR in nonobese children (P=0.000). Associations of IR with lipid-related parameters were affected by BMISD. After being corrected by BMISD, in nonobese children, log triglycerides (TG), apoB and low-density lipoprotein (LDL) size in boys and log TG, LDL size, and high-density lipoprotein (HDL) cholesterol in girls were still significantly associated with IR (P=0.000 to 0.017). In obese children, all parameters except for LDL cholesterol in boys and LDL size in girls were significantly associated with IR (P=0.000 to 0.030). Multiple regression analysis showed that log TG and LDL size in nonobese children, log TG in obese boys and LDL size in obese girls were independently associated with IR. Children with IIb and IV hyperlipidemia had significantly higher IR than those with normolipidemia and IIa, even after correcting for BMISD and age.

Conclusion—Our results suggest that in addition to controlling body weight, it may be important for school children to characterize lipid phenotypes to prevent progression to CHD and/or type 2 diabetes and to identify subjects who are at high risk for these disorders. (***Arterioscler Thromb Vasc Biol.* 2006;26:2781-2786.**)

Key Words: hyperlipidemia ■ insulin resistance ■ obesity ■ school children ■ type 2 diabetes

Dyslipidemia, insulin resistance (IR), and obesity are risk factors for atherosclerotic coronary heart disease (CHD) in adults.[1,2] Pathologically, atherosclerotic changes in coronary arteries originate during childhood, and the extent of atherosclerotic lesions in children and adolescent increases with the number of risk factors.[3,4] In our previous study, we showed the prevalence of dyslipidemia was 1.91% (familial hypercholesterolemia [FHD]: 0.19%, IIa: 0.87%, IIb: 0.26%, IV: 0.20%, low high-density lipoprotein [HDL]: 0.39%) in preschool Japanese children and children with dyslipidemia had more family or genetic background than adults.[5] Okada et al reported that >10% of Japanese school children had hypercholesterolemia (IIa and IIb).[6] Although these studies were performed in different regions, the prevalence of hypercholesterolemia in school children was much higher than that in preschool children. This suggests that the expression of hypercholesterolemia is severely affected by nongenetic factors (environmental factors). Among Japanese school children, the prevalence of type 2 diabetes increased from 0.2 to 7.3 per 100 000 children per year between 1976 and 1995.[7] The increase was attributed to changing dietary patterns and increase rates of obesity among these children.[7] Obese children have a higher prevalence of IR, type 2 diabetes, and dyslipidemia.[8,9] IR is also risk factor for impaired glucose tolerance and type 2 diabetes, even in children.[8] Furthermore, dyslipidemia, IR, and obesity are core phenotypes of the metabolic syndrome.[10–12] Thus, it seems important to clarify the relationship between dyslipidemia, IR, and obesity in children.

The metabolic syndrome is becoming a common disorder even in children, because of the increasing prevalence of obese children.[13–15] To help prevent the future development of CHD or type 2 diabetes, it is reasonable to identify children who are at high risk for these disorders. In the present study, as a first step in detecting a high-risk group, we investigated the relationship between dyslipidemia, IR, and obesity in Japanese school children.

Methods

Subjects

The present study was approved by the Review Board of the University of the Ryukyus. Informed consent was obtained from the

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TABLE 1. Anthropometric and Chemical Characteristics in Non-Obese and Obese Children

	Boys			Girls		
	Non-Obese (n=445)	P Value	Obese (n=197)	Non-Obese (n=389)	P Value	Obese (n=144)
Age, y	9.7±1.6	(P<0.0001)	10.2±0.6	9.8±1.8	(P<0.01)	10.2±0.8
BMISD	0.42±1.03	(P<0.0001)	2.78±0.70	0.19±1.02	(P<0.0001)	2.78±0.77
TC, mg/dL**	177±28	(P<0.01)	184±30	174±26	(P<0.0001)	182±31
TG, mg/dL***	73±41	(P<0.0001)	98±70	73±40	(P<0.0001)	101±55
LDL-C, mg/dL**	100±25	(P<0.001)	109±26	100±23	(P<0.001)	108±27
HDL-C, mg/dL**	63±12	(P<0.0001)	56±11	62±11	(P<0.0001)	54±10
ApoB, mg/dL	73±17	(P<0.0001)	81±18	72±16	(P<0.0001)	82±20
LDL-size, nm	27.14±0.96	(P<0.0001)	26.36±0.82	27.32±0.91	(P<0.0001)	26.42±0.82
Glucose, mg/dL*	88±7	(P<0.0001)	92±6	86±7	(P<0.0001)	91±8
Insulin, μU/mL	7.0±5.8	(P<0.0001)	15.9±9.3	6.7±5.5	(P<0.0001)	20.0±14.3
HOMA-R	1.55±1.33	(P<0.0001)	3.60±2.18	1.46±1.24	(P<0.0001)	4.59±3.67
QUICKI	0.38±0.05	(P<0.0001)	0.33±0.03	0.39±0.05	(P<0.0001)	0.32±0.03

Abbreviations as in text. Values are expressed as mean±SD. *To convert to mmol/L, divide by 18. **To convert to mmol/L, multiply by 0.0259. ***To convert to mmol/L, multiply by 0.0113.

parents of all of the children. We studied 1175 Japanese children (642 boys and 533 girls) aged 7 to 12 years, who underwent screening and were enrolled in a care program for lifestyle-related diseases since 2001 in Okinawa, Japan. Sex maturity stages in the children studied were equal to or less than Tanner Stage 3. The subjects were not patients who visited our hospital. Body mass index (BMI) was calculated as weight [kg]/height^2 [m^2]. BMI standard deviation scores (BMISD) adjusted for age and sex were obtained based on data on Japanese school children provided by the Ministry of Education, Culture, Sports, Science, and Technology (unpublished data). Obesity was defined as BMISD ≥2.0. None of the children studied were receiving therapy for weight reduction or drugs that might affect lipid metabolism. None had a smoking habit. Venous blood was drawn after an overnight fast.

Laboratory Measurements

Serum insulin was measured by 2-step sandwich enzyme-linked immunosorbent assay (SRL, Inc, Hachioji, Japan). Routine chemical methods were used to determine the serum concentrations of total cholesterol (TC), HDL cholesterol (HDL-C), triglycerides (TG), and glucose. Low-density lipoprotein cholesterol (LDL-C) was calculated as [TC − HDL-C − TG/5]. Apolipoprotein B (apoB) was measured by the turbidity immunoassay method.[16] IR and insulin sensitivity were calculated using the homeostasis model approximation index (HOMA-R) and the quantitative insulin-sensitivity check index (QUICKI).[17,18] LDL size was evaluated by electrophoresis in nondenaturing polyacrylamide gradient gels on precast MULTIGEL-LP (2% to 15%) according to the procedure specified by the manufacturer (Daiichi Pure Chemicals Co, LTD, Tokyo, Japan). Standards used for size calibration included latex beads (37 nm) (Dow Chemical Company) and high-molecular-weight standards (Pharmacia). The stained gels were scanned with a laser scanning densitometer to provide a quantitative measurement of the size of the peak and its distance from the origin. Particle diameter was calculated from a plot of the log of the known diameters of the standards (latex beads 37 nm, thyroglobulin 17.0 nm, apoferritin 12.2 nm) on the y-axis against their positions from the origin of the gel (Rf) on the x-axis.

Statistical Evaluation

The significance of differences in clinical and chemical data between nonobese and obese children were determined by the Mann-Whitney *U* test. The distributions of HOMA-R and levels of insulin and triglyceride were markedly skewed. Thus, these parameters were normalized by log-transformation. Pearson and partial correlation coefficients were then computed to assess the associations between log HOMA-R and various parameters. A stepwise multiple regression analysis was performed by entering the independent variable with the highest partial correlation coefficient at each step, until no variable remained with an F value of ≥4. Age-adjusted and BMISD-adjusted differences in parameters among subjects with normal, IIa, IIb, and IV were determined by an analysis of covariance. Parameters in these 4 groups were compared using Scheffe's multiple comparison test. Group differences or correlations with $P<0.05$ were considered to be statistically significant.

Results

As shown in Table 1, adiposity-related differences were found in all of the parameters studied. Obese children showed more atherogenic lipid and apolipoprotein profiles and greater IR than nonobese children. Thus, we separated the data for nonobese and obese children in the following analysis. Tables 2 and 3 show Pearson and partial correlations between IR (log HOMA-R) and the other parameters studied. In nonobese boys, log HOMA-R was correlated with all of the parameters listed ($P=0.000$). In obese boys, log HOMA-R was correlated with BMISD, log TG, apoB and HDL-C ($P=0.000$ to 0.002). After being corrected by age and BMISD, log HOMA-R was correlated with log TG, apoB, and LDL size in nonobese boys ($P=0.000$ to 0.017). In obese boys, all parameters except for LDL-C were correlated with log HOMA-R ($P=0.000$ to 0.010), even after being corrected by BMISD and age. Age, BMISD, TC, log TG, LDL-C, LDL size, and HDL cholesterol (HDL-C) in nonobese girls and age, BMISD, and LDL size in obese girls were significantly correlated with log HOMA-R ($P=0.002$ to 0.003). After being corrected by age and BMISD, log TG, LDL size, and HDL-C in nonobese girls and LDL size in obese girls were significantly correlated with log HOMA-R ($P=0.000$ to 0.024). Because each of these parameters can potentially contribute directly to the regulation of log HOMA-R, we performed a stepwise multiple regression analysis with log HOMA-R as the dependent variable and the other parameters

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TABLE 2. Log HOMA-R and Variables in Boys

	Non-Obese Boys				Obese Boys			
	Pearson Correlation		Partial Correlation		Pearson Correlation		Partial Correlation	
	r	P	r	P	r	P	r	P
Age	0.367	0.000	—	—	−0.040	0.517	—	—
BMISD	0.666	0.000	—	—	0.124	0.001	—	—
TC	0.203	0.000	0.085	0.073	0.133	0.107	0.209	0.003
Log TG	0.260	0.000	0.245	0.000	0.459	0.000	0.459	0.000
LDL-C	0.222	0.000	0.055	0.247	0.050	0.485	0.082	0.252
ApoB	0.294	0.000	0.113	0.017	0.239	0.001	0.273	0.000
LDL-size	−0.286	0.000	−0.166	0.002	−0.138	0.095	−0.178	0.030
HDL-C	−0.228	0.000	−0.082	0.084	−0.224	0.002	−0.183	0.010

Abbreviations as in text. Partial correlation: variables corrected by age and BMISD.

listed in Tables 2 and 3 as independent variables. In nonobese boys, BMISD had the most significant association with log HOMA-R and accounted for 45.8% of the variability in log HOMA-R. Age, log TG, and LDL size had additional effects (6.2%, 2.7%, and 1.3%, respectively) (Table 4). In obese boys, log TG had the most significant association with log HOMA-R and accounted for 20.6% of the variability in log HOMA-R. BMISD had an additional effect (6.6%). In nonobese girls, BMISD had the most significant association with log HOMA-R and accounted for 32.7% of the variability in log HOMA-R. Age, log TG, and LDL size had additional effects (20.8%, 2.1%, and 1.1%, respectively) (Table 4). In obese girls, age had the most significant association with log HOMA-R and accounted for 17.1% of the variability in log HOMA-R. BMISD and LDL size had additional effects (11.3% and 4.5%, respectively).

To elucidate the relationship between lipid phenotypes and IR, we divided school children into normolipidemia (NL) and type IIa (IIa), IIb, and IV hyperlipidemia groups. We defined hyperlipidemia based on serum lipid levels in Japanese school children.[6] When serum concentrations of TC, TG, and LDL-C were >90th percentiles for the respective age-matched and gender-matched values, we considered the children to be hyper TC, hyper TG, and hyper LDL-C (IIa, hyper LDL-C alone; IIb, hyper LDL-C and hyper TG; IV, hyper TG alone). NL was defined as serum concentrations of LDL-C and TG of <90th percentiles. Table 5 shows BMISD-adjusted and age-adjusted chemical parameters in children with NL, IIa, IIb, and IV. In boys, serum concentrations of HDL-C in IIb and IV were significantly lower than those in NL and IIa ($P<0.0001$). LDL sizes in IIb and IV were significantly smaller than that in NL ($P<0.05$ to 0.0001). LDL size in IIb was significantly smaller than that in IIa ($P<0.001$). Serum concentrations of glucose were significantly higher in IIa and IIb than in NL ($P<0.01$). Serum concentrations of insulin and the levels of HOMA-R in IIa, IIb, and IV were significantly higher than those in NL and those in IIb and IV were significantly higher than those in IIa ($P<0.01$ to 0.0001). Differences between IIb and IV were not significant. The levels of QUICKI in IIa, IIb, and IV were significantly lower than that in NL ($P<0.0001$). Those in IIb and IV were significantly lower than that in IIa ($P<0.0001$). In girls, serum concentrations of HDL-C in IIb and IV were significantly lower than those in NL and IIa ($P<0.0001$). The difference between IIb and IV was not significant. LDL size in IIb and IV were significantly smaller than those in NL and IIa ($P<0.05$ to 0.0001). Serum concentrations of glucose were similar in all groups. Serum concentrations of insulin and the levels of HOMA-R in IIb and IV were significantly higher than those in NL and IIa ($P<0.01$ to 0.0001). Differ-

TABLE 3. Log HOMA-R and Variables in Girls

	Non-Obese Girls				Obese Girls			
	Pearson Correlation		Partial Correlation		Pearson Correlation		Partial Correlation	
	r	P	r	P	r	P	r	P
Age	0.468	0.000	—	—	0.414	0.002	—	—
BMISD	0.576	0.000	—	—	0.347	0.010	—	—
TC	−0.146	0.008	−0.078	0.158	−0.020	0.861	0.024	0.832
Log TG	0.319	0.000	0.221	0.000	0.161	0.152	0.098	0.384
LDL-C	−0.124	0.025	−0.081	0.143	−0.011	0.919	0.031	0.784
ApoB	−0.084	0.327	−0.019	0.866	0.056	0.623	0.079	0.483
LDL-size	−0.283	0.000	−0.173	0.002	−0.326	0.003	−0.250	0.024
HDL-C	−0.260	0.000	−0.145	0.008	−0.126	0.264	−0.071	0.529

Abbreviations as in text. Partial correlation: variables corrected by age and BMISD.

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TABLE 4. Stepwise Multiple Regression Models for Predicting Log HOMA-R

Independent Parameters	r	r^2
Boys		
Non-Obese		
Step 1 BMISD	0.677	0.458
Step 2 BMISD, Age	0.721	0.520
Step 3 BMISD, Age, Log TG	0.740	0.547
Step 4 BMISD, Age, Log TG, LDL size	0.749	0.560
Obese		
Step 1 Log TG	0.454	0.206
Step 2 Log TG, BMISD	0.521	0.272
Girls		
Non-Obese		
Step 1 BMISD	0.572	0.327
Step 2 BMISD, Age	0.731	0.535
Step 3 BMISD, Age, Log TG	0.746	0.556
Step 4 BMISD, Age, Log TG, LDL size	0.753	0.567
Obese		
Step 1 Age	0.414	0.171
Step 2 Age, BMISD	0.533	0.284
Step 3 Age, BMISD, LDL size	0.573	0.329

P values of all steps were $P<0.0001$.

ences between IIb and IV were not significant. The levels of QUICKI in IIb and IV were significantly lower than those in NL and IIa ($P<0.0001$). Those in IIb and IV were significantly lower than that in IIa ($P<0.0001$). The difference between IIb and IV was not significant.

Discussion

Because the sample size in our study was large (total number of subject 1175), IR and insulin sensitivity were determined by HOMA-R and QUICKI, respectively. The accuracy and precision of HOMA-R and QUICKI as measures of IR and insulin sensitivity have been determined elsewhere by comparison with euglycemic and hyperglycemic clamps and the intravenous glucose tolerance test.[17,18] Recent data have shown that these indices are reliably sensitive and specific for evaluating IR even in children.[19,20] In the present study, we have shown that: (1) associations of IR (HOMA-R) with parameters of lipids and lipoproteins were affected by BMISD and age in our school children; (2) BMISD had the most significant association with IR in nonobese children. Among lipids and lipoprotein parameters, TG and LDL size were significantly associated with IR. Especially, TG had the most significant association with IR in obese boys. In obese girls, age had the most significant association with IR. Only LDL size among lipids and lipoprotein parameters was significantly associated with IR; and (3) both boys and girls with IIb and IV hyperlipidemia had significantly higher IR than those with IIa and NL.

Insulin regulates many aspects of lipoprotein metabolism. Resistance to the normal actions of insulin causes the hepatic overproduction of TG and apoB, which thereby enhances the secretion of very low-density lipoproteins from the liver.[21] In addition, IR decreases lipoprotein lipase activity, resulting in a delayed clearance of TG-rich lipoproteins.[22] It is generally believed that a delayed clearance of TG-rich lipoprotein is associated with the generation of small dense LDL and lower concentrations of HDL-C.[23,24] IR was significantly correlated with TG, apoB, HDL-C, and LDL size in our school children. Taken together, these findings suggest that IR may play an important role in lipid metabolism even in school children. However, BMISD and age were also significantly associated with IR in our school children. Adiposity, especially the accumulation of visceral fat, increases intraportal free fatty acid (FFA) levels and flux, thereby inhibiting insulin clearance and promoting IR.[25] In addition, an increased or de-

TABLE 5. Chemical Data Adjusted for BMISD and Age Among Boys and Girls With Normolipidemia and Type IIa, IIb, and IV Hyperlipidemia

	NL	*P* Value	IIa	*P* Value	IIb	*P* Value	IV
Boys, n	435		119		32		56
HDL-C, mg/dL	63±12	(ns)	62±12	($P<0.0001$)	50±8[d]	(ns)	53±11[d,h]
LDL-size, nm	27.00±0.95	(ns)	27.00±0.99	($P<0.001$)	26.07±0.97[d]	(ns)	26.58±0.99[a]
Glucose, mg/dL	89±7	($P<0.01$)	91±6	(ns)	93±6[b]	(ns)	90±8
Insulin, μU/mL	7.9±6.5	($P<0.01$)	10.2±7.3	($P<0.0001$)	18.9±11.1[d]	(ns)	16.7±11.8[d,h]
HOMA-R	1.78±1.50	($P<0.01$)	2.32±1.69	($P<0.0001$)	4.38±2.66[d]	(ns)	3.75±2.70[d,h]
QUICKI	0.38±0.06	($P<0.0001$)	0.35±0.04	($P<0.0001$)	0.32±0.03[d]	(ns)	0.33±0.04[d,h]
Girls, n	387		85		27		34
HDL-C, mg/dL	62±11	(ns)	61±10	($P<0.0001$)	45±6	(ns)	52±9[d,h]
LDL-size, nm	27.23±0.92	(ns)	27.20±0.99	($P<0.001$)	26.25±0.89[d]	(ns)	26.65±1.01[b,e]
Glucose, mg/dL	87±8	(ns)	86±9	(ns)	89±8	(ns)	87±7
Insulin, μU/mL	9.1±9.4	(ns)	9.9±10.2	($P<0.001$)	17.8±10.7[d]	(ns)	17.0±16.2[d,g]
HOMA-R	2.04±2.35	(ns)	2.24±2.49	($P<0.01$)	3.92±2.40[d]	(ns)	3.74±3.83[d,f]
QUICKI	0.37±0.06	(ns)	0.37±0.06	($P<0.0001$)	0.33±0.04[d]	(ns)	0.33±0.03[d,h]

n: No of subjects. NL: Normolipidemia. Abbreviations as in Text. a, $P<0.05$; b, $P<0.01$;c, $P<0.001$; d $P<0.0001$: significantly different from NL. e, $P<0.05$; f, $P<0.01$; g, $P<0.001$; h, $P<0.0001$: significantly different from IIa. ns: not significant.

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creased in the secretion of adipocytokines form adipocytes, such as leptin, tumor necrosis factor (TNF)-α, adiponectin, etc., may cause IR.[26–28] An age-related reduction in insulin receptor expression has also been reported.[25] Thus, to exclude effects of adiposity and age, BMISD and age were adjusted for by partial correlation. Because age did not affect the relationship between insulin resistance and lipid-related parameters (data not shown), the partial correlation in Tables 2 and 3 reflected the effect of BMISD. After being corrected by BMISD, correlations between IR and lipid-related parameters were weakened in girls and nonobese boys, and strengthened in obese boys (Tables 2 and 3). Although several parameters were significant after being corrected by BMISD, multiple regression analysis showed that only two (TG and/or LDL size) were independently associated with IR in our school children (Table 4). However, these parameters can only account for 3.3% to 4.5% of the variability in IR in girls and nonobese boys. In contrast, TG in obese boys was the strongest predictor for IR and accounted for 20.6% of the variability in IR. These findings suggest that weight gain (adiposity) can mostly explain the relationship between IR and lipid-related parameters in girls and nonobese boys. However, in obese boys, TG metabolism might be more important for IR than adiposity. Although further studies are needed, genetic factors may exacerbate TG metabolism (overproduction of very low-density lipoprotein or delayed clearance of TG-rich lipoprotein) in obese boys.

The question is whether increased TG or decreased LDL size precedes or follows IR. As mentioned, IR itself can induce hypertriglyceridemia and make LDL size smaller.[21–24] A substantial reduction of serum TG levels with fibrate treatment did not improve IR.[29,30] Improvement of IR reduced small dense LDL particles.[31] To date, no data are available on whether the improvement of LDL size can affect IR. Interestingly, the state of insulin resistance in familial combined hyperlipidemia (FCHL) is associated with the lipid phenotype.[32] Subjects with FCHL based on hyper TG (IV) or combined hyperlipidemia (IIb) are more insulin-resistant than FCHL subjects based on hyper TC (IIa) even after correcting for BMI.[32] As in FCHL, school children with IIb and IV showed more IR and smaller LDL size than those with NL and IIa (Table 5). Because a family study was not performed in the present study, we could not diagnose FCHL in our school children. However, the characteristics of school children with IIb and IV were very similar to those of FCHL patients. In addition, as shown in our previous study, most young children (preschool) with IIb were FCHL based on a familial study.[5] Taken together, these results might extend our previous finding (ie, that most young children with IIb are FCHL) to school children. If our notion is valid, a genetic background that regulates serum TG and/or LDL size such as in FCHL might contribute to the relationship between TG, LDL size, and IR. Weight gain may exacerbate IR and lipid abnormalities in these children.

With respect to the differences between boys and girls, it is well known that sex hormone affect the lipid metabolism. Because most of our children were pre-puberty, we did not measure sex hormone. However, age was strongly associated with IR especially in girls. This may suggest that subtle change of sex hormone may be responsible for the gender differences of our data. Further studies are needed to clarify a complex interplay between sex hormone, BMI, and insulin action.

In conclusion, TG and/or LDL size were significantly associated with IR, and lipid phenotypes (IIb and IV) showed higher IR, but neither of these associations could be fully explained by their BMISD. School children with types IIb and IV showed characteristics similar to those in subjects with FCHL. Thus, it is important for school children to control body weight to prevent progression to the metabolic syndrome and a familial study should be performed in children with IIb and IV to screen for those at high risk for CHD and/ or type 2 diabetes.

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Disclosures

None.

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Serum C-Reactive Protein and Its Relation to Cardiovascular Risk Factors and Adipocytokines in Japanese Children

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Background: C-Reactive protein (CRP) is an independent risk factor for atherosclerotic coronary heart diseases (ACHD) in adults. To help prevent ACHD, it may be useful to understand risk factors during childhood.

Objective: The objective of this study was to investigate serum CRP and its relation to other risk factors for ACHD and adipocytokines (adiponectin, IL-6, and TNF-α) in Japanese children.

Methods: CRP, conventional risk factors for ACHD, and adipocytokines were determined in 568 children (340 boys and 228 girls, aged 7–10 yr). Serum concentrations of adipocytokines were measured by sandwich ELISA.

Results: Children with high CRP concentrations (highest tertile) had higher body mass index (BMI) SD scores, insulin, insulin resistance, uric acid, and adipocytokines and had more atherogenic lipoprotein profiles than other children. However, after being corrected by BMI SD, only high-density lipoprotein cholesterol, apolipoprotein A-I, IL-6, and TNF-α for boys and high-density lipoprotein cholesterol, apolipoprotein B, uric acid, IL-6, and TNF-α for girls were significantly correlated with CRP. IL-6 was the strongest predictive variable for CRP and accounted for 26.2 and 27.7% of the variability in serum concentrations of CRP in boys and girls, respectively. Serum concentrations of IL-6 were partly dependent on BMI SD and TNF-α in both boys and girls.

Conclusion: Although serum concentrations of CRP are partly regulated by adipocytokines and conventional risk factors for ACHD, high CRP levels were associated with atherogenic profiles of cardiovascular risk factors in children. Our findings suggest that it may be important to control body weight to prevent an increase in serum CRP in children. (***J Clin Endocrinol Metab* 91: 2133–2137, 2006**)

C-REACTIVE PROTEIN (CRP) increases nonspecifically in inflammatory disorders. The recent development of a highly sensitive assay for serum CRP concentrations has led to the unexpected finding that elevation of CRP levels within the normal range is associated with an increased risk for atherosclerotic coronary heart diseases (ACHD) in apparently healthy subjects (1–3). Although the underlying mechanism by which CRP contributes to the development of ACHD is not yet clear, Sternik *et al.* (4) reported in an *in vitro* study that CRP induces vasorelaxation independent of the endothelium. In addition, it has been reported that CRP induced apoptosis in human coronary vascular smooth muscle cells (5). However, van den Berg *et al.* (6) recently reported that vasorelaxation induced by CRP may be an artifact caused by the reagent used in their experiment. CRP is correlated with many conventional risk factors for ACHD, such as insulin resistance, obesity, high-density lipoprotein cholesterol (HDL-C), *etc.* (7, 8). Because most of these epidemiological studies were performed in adults, ACHD risk factors acquired later in life, such as smoking, alcohol use, *etc.*, may affect these relationships. In contrast to adults, children rarely drink alcohol or smoke and usually exercise regularly at school. Thus, environmental factors that affect the relationship between CRP and risk factors may have less of an effect in children than in adults. It seems reasonable to consider the relationship between serum CRP and risk factors for ACHD in schoolchildren. Several studies in children are currently available (9–11). Most of these have indicated that adiposity is the major determinant of CRP levels in children and have speculated that cytokines secreted from adipocytes may be responsible for the relationship between serum concentrations of CRP and adiposity. In the present study we investigated serum CRP and factors that influence serum CRP in children to better understand the roles of various risk factors in the development of atherosclerosis.

Subjects and Methods

Subjects

The present study was approved by the review board of University of the Ryukyus. Informed consent was obtained from the parents of all children. We studied 568 Japanese children (340 boys and 228 girls), aged 7–10 yr, who underwent screening and had been enrolled in a care program for lifestyle-related diseases since 2002 in Okinawa, Japan (Table 1). Sex maturity stages in the children we studied were equal to or less than Tanner stage 2. The subjects were not patients who visited our hospital. Body mass index (BMI) was calculated as weight (kilograms)/height (meters)^2. BMI SD scores adjusted for age and sex were obtained based on data for Japanese schoolchildren provided by the Ministry of Education, Culture, Sports, Science, and Technology (Murata, M., unpublished observations). None of the children studied were receiving therapy for weight reduction or drugs that affected lipid metabolism. None had a smoking habit. Venous blood was drawn after an overnight fast.

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Abbreviations: ACHD, Atherosclerotic coronary heart disease; apo, apolipoprotein; BMI, body mass index; CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol; HOMA-R, homeostasis model approximation index; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

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TABLE 1. Clinical and chemical data

	Boys	Girls
No. of subjects	340	228
Median age (range) (yr)	9.2 (6.5–10.4)	9.2 (6.5–10.4)
BMI SD	1.43 ± 1.25 (−1.77–5.06)	1.25 ± 1.29 (−2.04–5.45)
CRP (mg/liter)	1.08 ± 1.50 (0.05–9.34)	0.82 ± 1.28 (0.05–8.82)[d]
Glucose (mg/dl)	91 ± 6 (70–111)	90 ± 7 (48–121)
Insulin (μU/ml)	10.4 ± 9.0 (1.0–54.6)	12.7 ± 10.5 (1.0–65.3)
HOMA-R	2.43 ± 2.33 (0.19–12.40)	2.86 ± 2.48 (0.12–14.03)
TC (mg/dl)	184 ± 29 (118–282)	177 ± 27 (123–281)[b]
TG (mg/dl)	84 ± 70 (20–272)	91 ± 95 (20–367)
LDL-C (mg/dl)	109 ± 26 (51–215)	103 ± 26 (38–193)[b]
HDL-C (mg/dl)	59 ± 12 (31–97)	57 ± 11 (28–92)[a]
ApoA-I (mg/dl)	140 ± 18 (96–198)	132 ± 17 (91–178)[d]
ApoB (mg/dl)	79 ± 17 (35–140)	76 ± 17 (38–161)
Adiponectin (μg/ml)	8.4 ± 3.8 (0.2–22.7)	8.3 ± 4.2 (0.9–24.5)
Uric acid (mg/dl)	4.8 ± 1.0 (2.4–8.4)	4.7 ± 0.9 (1.8–7.0)
IL-6 (pg/ml)	2.24 ± 2.27 (0.24–19.49)	1.99 ± 0.69 (0.19–18.92)
TNF-α (pg/ml)	1.19 ± 0.73 (0.17–6.42)	1.03 ± 0.69 (0.04–4.11)[c]

Values are expressed as mean ± SD (range) unless specified otherwise. To convert glucose to mmol/liter, divide by 18. To convert TC, LDL-C, and HDL-C to mmol/liter, multiply by 0.0259. To convert TG to mmol/liter, multiply by 0.0113.

[a] $P < 0.05$.

[b] $P < 0.01$.

[c] $P < 0.001$.

[d] $P < 0.0001$.

Laboratory measurements

The serum CRP concentration was measured by a highly sensitive immunoturbidimetric assay with the use of reagents and calibrators from Dade Behring Marbura GmbH (Marburg, Germany; the lower limit of detection for the serum CRP concentration was 0.05 mg/liter). IL-6 and TNF-α were measured by ELISAs (R&D Systems, Inc., Minneapolis, MN). The serum adiponectin concentration was measured by sandwich ELISA (Otsuka Pharmaceutical Co., Ltd., Tokushima City, Japan). Serum insulin was measured by a two-step sandwich ELISA (SRL, Inc., Hachioji, Japan). Routine chemical methods were used to determine the serum concentrations of total cholesterol (TC), HDL-C, triglycerides (TG), uric acid, and glucose. Low-density lipoprotein-cholesterol (LDL-C) was calculated as TC − HDL-C − TG/5. Apolipoproteins (apoA-I and apoB) were measured by the turbidity immunoassay method (12). Insulin resistance was calculated using the homeostasis model approximation index (HOMA-R) (13). This equation, which is based on both fasting glucose and insulin, correlates well with insulin dynamics, as measured by the hyperinsulinemic clamp and the iv glucose tolerance test (13).

Statistical evaluation

For statistical analysis, serum concentrations of CRP below the limit of detection were assigned a value of 0.05 mg/liter (lower limit of detection). Gender-related differences were determined by the Mann-Whitney *U* test. Differences in parameters among subjects with low, middle, and high CRP concentrations (tertiles) were determined by the Kruskal-Wallis test. Parameters in these three groups were compared with Scheffé's multiple comparison test. The distributions of HOMA-R and levels of CRP, insulin, TG, IL-6, and TNF-α were markedly skewed. Thus, these parameters were normalized by log transformation. Pearson and partial correlation coefficients were computed to assess the associations between CRP and various parameters. A stepwise multiple regression analysis was performed by entering the independent variable with the highest partial correlation coefficient at each step until no variable remained with an F value of 4 or greater. Group differences or correlations with $P < 0.05$ were considered statistically significant. All statistical analysis was performed using StatView J-5.0 software (SAS Institute, Inc., Cary, NC).

TABLE 2. Clinical and chemical data on boys with different CRP levels

	Low (n = 113)	P value	Middle (n = 114)	P value	High (n = 113)
CRP (mg/liter)	0.14 ± 0.07	<0.0001	0.52 ± 0.18	<0.0001	2.55 ± 1.82[d]
Age (yr)	9.4 ± 1.2	ns	9.7 ± 0.9	<0.05	10.0 ± 0.3[d]
BMI SD	0.71 ± 0.10	<0.0001	1.49 ± 1.17	<0.05	2.09 ± 1.15[d]
Glucose (mg/dl)	90 ± 6	ns	90 ± 7	ns	92 ± 6
Insulin (μU/ml)	7.7 ± 5.2	<0.05	10.9 ± 10.7	ns	13.3 ± 10.9[d]
HOMA-R	1.7 ± 1.2	<0.05	2.5 ± 2.7	ns	3.0 ± 2.6[d]
TC (mg/dl)	183 ± 28	ns	183 ± 28	ns	186 ± 30
TG (mg/dl)	75 ± 48	ns	83 ± 51	ns	95 ± 99
LDL-C (mg/dl)	105 ± 26	ns	108 ± 27	ns	113 ± 26[a]
HDL-C (mg/dl)	64 ± 13	<0.01	59 ± 11	<0.05	55 ± 11[d]
ApoA-I (mg/dl)	144 ± 19	ns	139 ± 19	ns	135 ± 19[c]
ApoB (mg/dl)	76 ± 18	<0.05	78 ± 17	ns	83 ± 17[b]
Adiponectin (μg/ml)	9.3 ± 3.8	ns	8.4 ± 4.1	ns	7.5 ± 3.2[b]
Uric acid (mg/dl)	4.4 ± 0.9	<0.01	4.8 ± 1.1	ns	5.2 ± 0.9[c]
IL-6 (pg/ml)	1.33 ± 0.89	<0.01	2.23 ± 2.39	<0.01	3.16 ± 2.69[d]
TNF-α (pg/ml)	1.04 ± 0.62	ns	1.16 ± 0.62	ns	1.36 ± 0.89[b]

Values are expressed as mean ± SD. ns, Not significant. To convert glucose to mmol/liter, divide by 18. To convert TC, LDL-C, and HDL-C to mmol/liter, multiply by 0.0259. To convert TG to mmol/liter, multiply by 0.0113.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$; [d] $P < 0.0001$, significantly different from low.

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TABLE 3. Clinical and chemical data on girls with different CRP levels

	Low (n = 76)	P value	Middle (n = 76)	P value	High (n = 76)
CRP (mg/liter)	0.09 ± 0.03	<0.0001	0.35 ± 0.17	<0.0001	2.06 ± 1.63[d]
Age (yr)	9.5 ± 1.1	ns	9.5 ± 1.1	ns	9.8 ± 0.7
BMI SD	0.80 ± 1.17	ns	1.09 ± 1.10	<0.001	1.87 ± 1.35[d]
Glucose (mg/dl)	90 ± 6	ns	89 ± 10	ns	90 ± 5
Insulin (μU/ml)	10.6 ± 7.6	ns	10.8 ± 10.8	<0.01	16.7 ± 11.7[c]
HOMA-R	2.4 ± 1.8	ns	2.4 ± 2.6	<0.01	3.8 ± 2.7[b]
TC (mg/dl)	172 ± 23	ns	175 ± 21	ns	184 ± 33
TG (mg/dl)	75 ± 40	ns	85 ± 63	ns	96 ± 50[a]
LDL-C (mg/dl)	99 ± 21	ns	101 ± 20	ns	108 ± 34
HDL-C (mg/dl)	60 ± 10	ns	58 ± 11	<0.05	54 ± 11[b]
ApoA-I (mg/dl)	133 ± 15	ns	132 ± 17	ns	130 ± 18
ApoB (mg/dl)	72 ± 15	ns	74 ± 13	<0.001	84 ± 21[d]
Adiponectin (μg/ml)	8.7 ± 4.1	ns	8.5 ± 4.7	ns	7.7 ± 3.7
Uric acid (mg/dl)	4.4 ± 0.9	ns	4.6 ± 0.9	<0.05	5.1 ± 1.0[c]
IL-6 (pg/ml)	1.25 ± 0.65	<0.01	2.03 ± 2.20	<0.05	2.68 ± 1.44[d]
TNF-α (pg/ml)	0.97 ± 0.66	ns	1.01 ± 0.69	ns	1.10 ± 0.71

Values are expressed as mean ± SD. ns, Not significant. To convert glucose to mmol/liter, divide by 18. To convert TC, LDL-C, and HDL-C to mmol/liter, multiply by 0.0259. To convert TG to mmol/liter, multiply by 0.0113.
[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$; [d] $P < 0.0001$, significantly different from low.

Results

As shown in Table 1, gender-related differences were found in several parameters (CRP, TC, LDL-C, HDL-C, apoA-I, and TNF-α). Thus, we separated the data for boys and girls in the following analysis. To understand the relation between CRP and lipids and other parameters, subjects were divided into tertiles based on CRP concentrations (low, lowest tertile; middle, intermediate tertile; high, highest tertile; Tables 2 and 3). In boys, there were significant graded relationships among the three groups of CRP concentrations and all parameters except for glucose, TC, and TG. For parameters other than BMI SD, HDL-C, and IL-6, significant differences were found in one or two combinations (between low and high and/or between low and middle or between middle and high; Table 2). Table 3 shows the findings in girls. In contrast to the findings in boys, significant differences were not found in LDL-C, adiponectin, and TNF-α, but were found in TG.

Tables 4 and 5 show Pearson and partial correlations between logCRP and the other parameters studied. In boys, logCRP was correlated with all parameters listed except TC ($P = 0.000-0.010$). After being corrected for BMI SD, logCRP was positively correlated with logIL-6 and logTNF-α and was inversely correlated with HDL-C and apoA-I ($P = 0.000-0.049$; Table 4). In girls, logCRP was positively correlated with BMI SD, age, logInsulin, logHOMA-R, logTG, ApoB, uric acid, and logIL-6 and was inversely correlated with HDL-C ($P = 0.000-0.016$). After being corrected for BMI SD, logCRP was positively correlated with apoB, uric acid, logIL-6, and logTNF-α ($P = 0.000-0.031$) and was inversely correlated with HDL-C (Table 5). Because each of these parameters can potentially contribute directly to the regulation of CRP, we performed a stepwise multiple regression analysis with logCRP as the dependent variable and the other parameters listed in Table 4 (HOMA-R was excluded because it was value calculated) as independent variables. In boys, BMI SD had the most significant association with logCRP and accounted for 24.3% of the variability in logCRP. HDL-C had additional effects (2.4%; Table 6, model 1, logIL-6 and TNF-α were excluded). When logIL-6 and logTNF-α were included

TABLE 4. Log CRP and variables in boys

	Simple correlation		Partial correlation	
	r[a]	P	r[b]	P
BMI SD	0.494	0.000		
Age	0.289	0.000	0.045	0.409
Glucose	0.121	0.026	0.050	0.359
Log insulin	0.364	0.000	0.054	0.322
Log HOMA-R	0.362	0.000	0.053	0.331
TC	0.049	0.369	0.010	0.854
Log TG	0.142	0.009	0.051	0.349
LDL-C	0.140	0.010	0.078	0.152
HDL-C	−0.327	0.000	−0.180	0.001
ApoA-I	−0.216	0.000	−0.107	0.049
ApoB	0.182	0.001	0.093	0.087
Adiponectin	−0.223	0.000	−0.012	0.826
Uric acid	0.323	0.000	0.098	0.072
Log IL-6	0.502	0.000	0.403	0.000
Log TNF-α	0.199	0.002	0.218	0.000

[a] Pearson correlation coefficient.
[b] Variables corrected by BMI SD.

TABLE 5. Log CRP and variables in girls

	Simple correlation		Partial correlation	
	r[a]	P	r[b]	P
BMI SD	0.364	0.000		
Age	0.160	0.016	−0.041	0.540
Glucose	0.031	0.647	−0.059	0.539
Log insulin	0.219	0.001	−0.081	0.224
Log HOMA-R	0.209	0.002	−0.082	0.219
TC	0.108	0.104	0.088	0.187
Log TG	0.181	0.005	0.106	0.112
LDL-C	0.092	0.169	0.059	0.377
HDL-C	−0.189	0.004	−0.195	0.003
ApoA-I	−0.049	0.464	−0.004	0.952
ApoB	0.211	0.001	0.151	0.023
Adiponectin	−0.065	0.331	0.090	0.178
Uric acid	0.257	0.000	0.131	0.049
Log IL-6	0.509	0.000	0.453	0.000
Log TNF-α	0.128	0.098	0.167	0.031

[a] Pearson correlation coefficient.
[b] Variables corrected by BMI SD.

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TABLE 6. Stepwise multiple regression models for predicting Log CRP

	Independent parameters	r	r^2
Boys			
Model 1			
Step 1	BMI SD	0.492	0.243
Step 2	BMI SD, HDL-C	0.516	0.267
Model 2			
Step 1	Log IL-6	0.514	0.262
Step 2	Log IL-6, BMI SD	0.599	0.359
Step 3	Log IL-6, BMI SD, HDL-C	0.618	0.382
Step 4	Log IL-6, BMI SD, HDL-C, Log TNF-α	0.627	0.393
Girls			
Model 1			
Step 1	BMI SD	0.363	0.132
Step 2	BMI SD, ApoB	0.389	0.151
Model 2			
Step 1	Log IL-6	0.526	0.277
Step 2	Log IL-6, BMI SD	0.560	0.313
Step 3	Log IL-6, BMI SD, Log TG	0.578	0.335

Model 1, Log IL-6 and Log TNF-α were excluded. Model 2, All parameters were included. For all steps, $P < 0.0001$.

in the model (Table 6, model 2), logIL-6 had the most significant association with logCRP and accounted for 26.2% of the variability in logCRP. BMI SD, HDL-C, and logTNF-α had additional effects (9.7, 2.3, and 1.1%, respectively). In girls, BMI SD had the most significant association with logCRP and accounted for 13.2% of the variability in logCRP. ApoB had an additional effect (1.9%, respectively; Table 6, model 1). When logIL-6 and logTNF-α were included in the model (Table 6, model 2), as in boys, logIL-6 had the most significant association with logCRP and accounted for 27.7% of the variability in logCRP. BMI SD and logTG had additional effects (3.6 and 2.2%, respectively).

To determine the relationship between IL-6 and the other parameters listed in Table 4, we performed a stepwise multiple regression analysis with logIL-6 as the dependent variable and the other parameters as independent variables. BMI SD was most significantly associated with IL-6 and accounted for 14.0 and 15.4% of the variabilities in IL-6 in boys and girls, respectively ($r^2 = 0.140$ and 0.154; $P < 0.0001$). TNF-α and age in boys and TNF-α in girls had additional effects (5.4% in boys and 9.2% in girls).

Discussion

In the present study, we have shown that: 1) boys with high serum concentrations of CRP have more atherogenic clinical and chemical profiles than other children (high levels of BMI SD, insulin, HOMA-R, LDL-C, apoB, uric acid, IL-6, and TNF-α, and low levels of HDL-C, apoA-I, and adiponectin); 2) girls with high serum concentrations of CRP show high levels of BMI SD, insulin, HOMA-R, TC, TG, apoB, uric acid, and IL-6, and low levels of HDL-C; 3) IL-6 had the most significant association with serum concentrations of CRP in children; and 4) BMI SD in children was the most powerful predictor of serum IL-6 concentrations.

In adults, serum concentrations of CRP are increased in subjects with obesity, insulin resistance, hypertension, and/or metabolic syndrome (1–3, 7, 8, 14, 15). These conditions are all well-known risk factors for ACHD. Two recent studies have suggested that serum concentrations of CRP were significant predictors of ACHD even after adjusting for conventional risk factors for ACHD, including serum lipid levels, smoking status, and BMI (3, 14). To date, there have been several large-scale studies of CRP levels in schoolchildren (9–11). Cook *et al.* (9) reported that serum concentrations of CRP were associated with BMI, heart rate, systolic blood pressure, fibrinogen, and HDL-C, but not with other lipid parameters. Ford (11) showed that serum concentrations of CRP were associated with BMI, systolic blood pressure, and TG, but not with glycosylated hemoglobin or glucose. Wu *et al.* (10) reported that BMI, TG, and HDL-C were associated with serum concentrations of CRP in schoolchildren in Taiwan. A common finding among these reports is that BMI was the most powerful predictor of serum concentrations of CRP in schoolchildren. In contrast to our study, adiponectin, IL-6, TNF-α, and uric acid were not determined in these previous reports. When we removed cytokines and uric acid from our statistical analysis, in agreement with previous reports, BMI SD was the most powerful predictor of CRP in our children. After being corrected for BMI SD, age, HOMA-R, TG, LDL-C, apoB, and adiponectin in boys and age, HOMA-R, and TG in girls were no longer correlated with CRP. When we added uric acid to the statistical analysis, it was correlated with CRP in both boys and girls. However, a significant correlation was only found in girls after being corrected for BMI SD. Based on a recent report, human vascular smooth muscle cells and human umbilical vein endothelial cells are also sources of CRP production (16). CRP mRNA expression in human vascular smooth muscle cells, and human umbilical vein endothelial cells and the release of CRP into cell culture medium were both up-regulated by uric acid (16). Although additional studies are needed, our data for girls suggest that vascular cell damage induced by uric acid may begin in childhood.

With respect to cytokines, IL-6 and TNF-α themselves have been reported to be risk factors for ACHD and type 2 diabetes mellitus, even after adjusting for BMI (17, 18). To date, IL-6 and TNF-α are believed to mediate the relationship between BMI and CRP in children, because IL-6 and TNF-α are the main inducers of the hepatic production of CRP and are expressed in and secreted from adipose tissue (19–21). To the best of our knowledge, no previous epidemiological evidence is available on the relation between CRP and these cytokines in children. In the present study IL-6 was the most significant predictor of CRP in both boys and girls. TNF-α was not a significant predictor of CRP in girls. BMI SD was the most powerful predictor of serum IL-6 in children. Mohamed-Ali *et al.* (22) reported that in humans, although IL-6 and TNF-α were both expressed in sc adipose tissues, only IL-6 was released from sc adipose tissues. However, in adults, serum concentrations of these cytokines were closely related to obesity, particularly central obesity (21). These findings suggest that sc fat may be responsible for the production of IL-6 in children, and the accumulation of visceral fat may be less evident in girls than in boys.

In conclusion, although serum concentrations of CRP are partly regulated by adipocytokines and conventional risk factors for ACHD, high CRP levels were associated with atherogenic profiles of cardiovascular risk factors in children. IL-6 was the most powerful predictor of serum CRP in chil-

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dren. BMI SD in both boys and girls was the most significant predictor of IL-6. These findings suggest that it may be important to control body weight to prevent an increase in serum CRP in children.

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Relation between insulin resistance and fast-migrating LDL subfraction as characterized by capillary isotachophoresis

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Abstract **The proportion of the electronegative low density lipoprotein [LDL(−)] subfraction, which is atherogenic, is increased in type 2 diabetes but is not reduced by glycemic control. Therefore, we evaluated the ability of a new technique, capillary isotachophoresis (cITP), to quantify charge-based LDL subfractions and examined the relation between insulin resistance and the cITP fast-migrating (f) LDL levels. Seventy-five 10-year-old boys were included. The two cITP LDL subfractions, fLDL and major LDL subfractions, were proportional to the LDL protein content within the range of 0.1–0.8 mg/ml LDL protein. Levels of cITP fLDL were positively correlated with triglyceride (TG) levels and negatively correlated with LDL size. Insulin resistance as assessed by the homeostasis model assessment (HOMA-IR) was positively correlated ($P < 0.01$) with cITP fLDL levels ($r = 0.41$). The relation between HOMA-IR and cITP fLDL levels depended on TG levels but was independent of body mass index and LDL size. cITP lipoprotein analysis is an accurate and sensitive method for quantifying charge-based LDL subfractions in human plasma, and insulin resistance is related to cITP fLDL independent of LDL size.**—Zhang, B., T. Kaneshi, T. Ohta, and K. Saku. **Relation between insulin resistance and fast-migrating LDL subfraction as characterized by capillary isotachophoresis.** ***J. Lipid Res.*** **2005.** 46: **2265–2277.**

Supplementary key words children • plasma lipoprotein subfraction • low density lipoprotein size • electronegative low density lipoprotein

Electronegative low density lipoprotein [LDL(−)] subfraction in plasma has been shown to have various atherogenic properties [reviewed by Sánchez-Quesada, Benitez, and Ordonez-Llanos (1)]. Although in vitro oxidized LDL can only be taken up by scavenger receptors, LDL(−) can also be taken up by LDL receptors to induce vascular cell adhesion molecule-1 expression through the activation of nuclear factor κB and adaptor protein 1 (2).

Both type 1 and type 2 diabetic patients have been shown to have an increased proportion of LDL(−) (3, 4). However, LDL(−) in type 1 and type 2 diabetes seems to be of different origins. In type 1 diabetes, nonenzymatic glycosylation has been shown to contribute to the increased proportion of LDL(−): glycemic optimization decreased both the glycated LDL and the proportion of LDL(−) (3, 4). However, in type 2 diabetes, glycemic control decreased glycated LDL but had no significant effects on the proportion of LDL(−) (4, 5). It is not clear whether or not insulin resistance contributes to LDL(−) generation in type 2 diabetes.

Insulin resistance is known to be associated with increased levels of triglycerides (TGs) (6). Because LDL(−) separated by anion-exchange chromatography techniques has been shown to contain higher TG content than the major LDL subfraction (7–9), it is possible that there may be a relation between insulin resistance and LDL(−). However, this has not yet been examined. In addition, it would be interesting to know whether or not the relation between insulin resistance and LDL(−) depends on TG levels.

Insulin resistance is also linked to plasma levels of small, dense LDLs (pattern B lipoprotein phenotype), which are associated with an increased risk of coronary heart disease (CHD) (6). In normolipidemic (NL) subjects, LDL(−) is distributed predominantly in small, dense LDL subfractions (10). Therefore, it would also be interesting to determine whether or not the relation between insulin resistance and LDL(−) depends on the size of LDL. Clarifying these points should be important considering that glycemic control failed to decrease the proportion of LDL(−) in type 2 diabetic patients (4).

Chromatography has the advantage that the separated LDL(−) fraction can be collected to characterize the composition of LDL(−) (11–13). However, for routine

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analysis, it has the disadvantage that LDL needs to be separated from other plasma proteins (e.g., by ultracentrifugation) for analysis, because protein absorption is monitored by ultraviolet light detection at 280 nm (12, 13). Therefore, it is time-consuming, and although it determines the proportion of LDL(−), it is not able to determine the absolute amount of LDL(−) in plasma.

Capillary isotachophoresis (cITP) is a new technique for separating plasma lipoprotein subfractions based on their electric charges (14–18). The two cITP LDL subfractions, fast-migrating (f) and slow-migrating (s) LDL, represent the LDL(−) and major LDL subfractions, respectively. We and others (14, 15, 19–22) have previously shown that the absolute amount of lipoprotein subfraction can be determined by reference to an internal marker. In cITP analysis, because lipoproteins are stained by the lipophilic dye 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-ceramide and monitored by laser-induced fluorescence detection (excitation, 488 nm; emission, 510 nm), lipoproteins can be analyzed without prior separation from other plasma proteins. However, it is not clear whether or not cITP LDL subfractions in plasma are equivalent to those in LDL separated by ultracentrifugation. Therefore, in the present study, we compared cITP LDL subfractions in plasma with those in LDL separated by ultracentrifugation. It is also not clear whether or not fLDL as determined by the method used in cITP analysis is related to LDL(−) as determined by the detection method used in chromatography. Therefore, we examined the linearity of the relation between levels of cITP LDL subfractions and protein contents of LDL to evaluate the ability of cITP lipoprotein analysis to quantify LDL subfractions. In addition, although cITP separates plasma lipoproteins into subfractions based on their electric charge, there is still no direct evidence that cITP fLDL represents an electronegative fraction of LDL. Therefore, we examined the changes in the distribution of cITP LDL subfractions during the in vitro oxidation of LDL to clarify whether or not cITP fLDL is related to the electronegativity of LDL, because oxidation is known to increase the negative charge of LDL.

Many studies have shown that atherosclerosis may start in childhood and is related to blood lipid levels measured in early life (23, 24), and both insulin resistance and lipid levels are related to age and gender. Therefore, to clarify whether or not insulin resistance contributes to the generation of LDL(−), we examined the relation between insulin resistance and cITP fLDL subfraction and the interaction of TG levels and LDL size in 10 year old nondiabetic boys, who have no CHD and have fewer conventional risk factors such as smoking, hypertension, etc.

Since Sánchez-Quesada et al. (10) reported that LDL(−) as measured by ion-exchange chromatography is distributed predominantly in the dense LDL subclass in NL subjects but is increased in the light LDL subclass in patients with hyperlipidemia, it would be interesting to know the relationship between cITP fLDL and LDL subclasses as measured by density. Therefore, we examined the distribution of cITP fLDL in light and dense LDL subclasses in NL, hypercholesterolemic (HC), and hypertriglyceridemic (HTG) subjects.

METHODS

Subjects

Seventy-five 10-year-old Japanese boys who underwent screening for lifestyle-related diseases in Okinawa, Japan, were included in the study. The study was approved by the Review Board of the University of the Ryukyus and the Ethics Committee of Fukuoka University. Informed consent was obtained from the parents of all of the children. Fasting blood was drawn from the vein. Serum was separated by low-speed centrifugation (1,000 *g*, 20 min, 4°C). Levels of lipids, lipoproteins, and apolipoproteins were measured in fresh serum. For lipoprotein particle and cITP analysis, aliquots of serum in small volumes were protected with N_2 gas, snap-frozen in liquid nitrogen, and stored at −80°C for <2 months before analysis.

Determination of serum levels of lipids, lipoproteins, and apolipoproteins, LDL size, and insulin resistance

Serum total cholesterol (TC), TG, and high density lipoprotein-cholesterol (HDL-C) levels were measured by enzymatic methods. Low density lipoprotein-cholesterol (LDL-C) was calculated as TC − (HDL-C + TG/5). Serum levels of apolipoprotein A-I (apoA-I), apoA-II, and apoB were determined by the turbidity immunoassay method. LDL size was evaluated by electrophoresis on nondenaturing polyacrylamide gradient gels on precast MULTIGEL-LP (2–15%) according to the procedure specified by the manufacturer (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), as described previously (25). Insulin resistance as assessed by homeostasis model assessment (HOMA-IR) was calculated according to the equation HOMA-IR = fasting glucose (mg/dl) × fasting insulin (μU/ml)/405 (26). A quantitative insulin sensitivity check index (QUICKI), which correlates well with insulin sensitivity from the glucose clamp technique, was determined according to the equation QUICKI = 1/[log(fasting glucose) + log(fasting insulin)] (27).

Determination of lipoprotein subfractions by cITP

cITP of lipoproteins in serum, to which was added EDTA-Na_2 at a final concentration of 1 mM before analysis, was performed on a Beckman P/ACE MDQ system (Beckman-Coulter, Inc., Tokyo, Japan) according to the method of Bottcher et al. (14) with some modifications, as described previously (19–22). All of the reagents used for cITP analysis were purchased from Sigma-Aldrich (Tokyo, Japan) unless indicated otherwise. For routine analysis, 6 μl of serum was diluted with 14 μl of leading buffer (LB) consisting of 10 mM HCl (product number 84428; Fluka, Tokyo, Japan) and 18 mM ammediol (2-amino-2-methyl-1,3-propanediol; product number A9074) (LB1, pH 8.8), prestained with 10 μl of 0.1 mg/ml NBD C6-ceramide (product number N1154; Molecular Probes, Inc., Eugene OR), which was prepared by dissolving 1 mg of NBD C6-ceramide in 1 ml of anhydrous methanol (product number 322415) and diluting 10-fold with anhydrous ethylene glycol (product number 324558) for 1 min at room temperature, and mixed with 50 μl of a mixture containing LB with 0.35% hydroxypropylmethylcellulose (product No. H4649) (LB2), spacers, and 5-carboxy-fluorescein (product number C0537) as an internal marker. For experiments to examine the ability of cITP to quantify LDL subfractions, 10 μl of LDL isolated by ultracentrifugation containing 1–8 μg of LDL protein was added to 6 μl of apoB-depleted EDTA plasma (plasma proteins and HDL) and 4 μl of LB1 and used for prestaining. The

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spacers included were N-(2-acetamido)-2-aminoethanesulfonic acid (product number A7949), D-glucuronic acid (product number 271632), 1-octanesulfonic acid sodium salt (product number O0133), 3-(N-tris[hydroxymethyl]methylamino)-2-hydroxypropanesulfonic acid (product number T0432), N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (product number T9659), L-serine (product number S260-0), L-glutamine (product number G320-2), L-methionine (product number M6039), and glycine (product number G7403). The terminating buffer (TB) contained 24 mM β-alanine (product number 239720) and 13 mM ammediol (TB1) and was adjusted to pH 10.5 (TB2) by adding 47 μl of saturated barium hydroxide (product number 11783) solution to 2 ml of TB1. Saturated barium hydroxide solution was prepared under an atmosphere of N_2, kept at 30°C in a water bath, and used within 1 week of preparation. A dimethylpolysiloxane-modified fused silica capillary (AT-1™; part number 14130) was purchased from Alltech Japan, Inc. (Tokyo, Japan). The sample was injected for 18 s at 20 pounds per square inch into a 30 cm long capillary (inner diameter, 180 μm), and separation was performed at a constant 30 μA for 1 min and 10 kV for 7 min with pressure on both sides (10 pounds per square inch). The separated zones were monitored with argon-laser-induced fluorescence detection (excitation, 488 nm; emission, 520 nm). Each peak was identified, and the peak area in relative fluorescence units was analyzed using 32 Karat Software version 5.0 (Beckman-Coulter, Inc., Tokyo, Japan). The peak area for each cITP lipoprotein subfraction relative to that of the internal marker was presented as the level of cITP lipoprotein subfraction (19–22), unless indicated otherwise.

Preparation of apoB-depleted plasma and isolation of LDL

Plasma containing 1 mM EDTA from a healthy volunteer (female, 32 years old) was used for experiments to examine the ability of cITP to quantify LDL subfractions. Whole plasma was used for cITP analysis and LDL isolation by ultracentrifugation immediately after separation, and some was divided into aliquots in small volumes (150 μl), covered with N_2 gas, and stored at −80°C for 1 day. Plasma depleted of apoB-containing lipoproteins was obtained from frozen whole plasma and used within the day of separation without storage.

ApoB-containing lipoproteins in EDTA plasma were precipitated by the phosphotungstate-Mg^{2+} method, as described previously (19). LDL was isolated from fresh EDTA plasma from the same volunteer by sequential ultracentrifugation. The plasma was first adjusted to a density of 1.019 g/ml with solid KBr and subjected to ultracentrifugation in a TLA-100.3 rotor in a Beckman TL-100 Tabletop Ultracentrifuge for 3 h at 100,000 rpm (541,000 g) and 10°C. The bottom fraction was collected by cutting the tubes, overlaid with liquid KBr (d = 1.019 g/ml), and ultracentrifuged again to remove any contaminating lipoproteins. The density of the bottom fraction was then increased to 1.063 g/ml with solid KBr, and the sample was subjected to ultracentrifugation for 3 h at 100,000 rpm (541,000 g) and 10°C. The top fraction was collected by cutting the tubes and dialyzed in a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) at 4°C against 0.85% NaCl and 0.01% EDTA overnight to remove KBr. LDL was used the day after separation without storage.

Oxidative modification of LDL by incubation with copper

Ultracentrifugally isolated LDL was dialyzed against PBS (pH 7.4) to remove EDTA before oxidation. Oxidation of LDL (0.5 mg/ml) was started by incubation with 10 μM freshly prepared $CuSO_4$ (with water) at 37°C in a shaking water bath. Aliquots were withdrawn at 0, 0.5, 1, 2, and 3 h of oxidation, and oxidation was stopped by cooling the aliquots on ice and adding EDTA-Na_2 (1 mM) and butylated hydroxytoluene (1 mM). Ten microliters of oxidized LDL was immediately used for cITP separation without removing $CuSO_4$, because cITP patterns were not affected by $CuSO_4$ (data not shown).

Agarose gel electrophoresis of LDL was performed as described by Noble (28) using commercial kits (Gel Universal/8; Corning, Chiba, Japan). One microliter of plasma control and 0.5 mg/ml oxidized LDL were applied and subjected to 1% agarose gel electrophoresis for 40 min at 90 V. Lipoproteins were stained with fat red 7B.

Conjugated dienes were determined by measuring absorbance at 234 nm using an ultraviolet-visible recording spectrophotometer (UV-160A; Shimadzu, Kyoto, Japan). Thiobarbituric acid-reactive substances (TBARS) were assayed by the fluorometric method of Yagi (29). ApoB fluorescence was measured at 430 nm (excitation, 360 nm) using a spectrofluorophotometer (RF-5000; Shimadzu).

Separation of light and dense LDL subfractions by heparin-Mg^{2+} precipitation

To examine the distribution of cITP fLDL in light and dense LDL subclasses, blood was drawn from 8 NL, 8 HC (LDL-C ≥ 140 mg/dl), and 8 HTG (TG ≥ 150 mg/dl) subjects. Serum was frozen with liquid nitrogen immediately after separation and preserved at −80°C under N_2 gas. Serum levels of TC, TG, HDL-C, and LDL-C were measured by enzymatic methods. Six microliters of serum was used to measure lipoprotein subfractions by cITP as described above. The light LDL subclass (d = 1.019–1.044 g/ml) was separated from the dense LDL subclass (d = 1.044–1.063 g/ml) using the heparin-Mg^{2+} precipitation method as described by Hirano, Saegusa, and Yoshino (30), with modifications. Briefly, one part of 300 U/ml heparin sodium (Novo-Heparin Injection 1000; Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) was mixed with one part of 180 mM $MgCl_2$. One part of the heparin-Mg^{2+} solution was then mixed with one part of serum. After the mixture was put on ice for 25 min, it was centrifuged at 4°C and 15,000 rpm for 15 min in a high-speed microrefrigerated centrifuge (MTX-150; Tomy Seiko Co., Ltd., Fukuoka, Japan). Twelve microliters of the supernatant was subjected to cITP analysis to determine the levels of cITP fLDL and sLDL in the small, dense LDL subclass contained in serum depleted of the large, light LDL subclass. Levels of cITP fLDL and sLDL in the large, light LDL subclass were calculated from those determined in whole serum and serum containing the light LDL subclass. The proportion of cITP fLDL was calculated from levels of cITP fLDL and sLDL.

Statistical analysis

All statistical analyses were performed using the SAS (Statistical Analysis System) software package (version 8.2; SAS Institute) at the Fukuoka University. Correlations between variables were examined by Spearman correlations and regression analysis. Variables among tertiles of HOMA-IR and among NL, HC, HTG subjects were compared by ANOVA and Scheffe's multiple comparison test (31). Differences in variables between large and dense LDL subclasses were examined by ANOVA. All P values are two-tailed. The significance level was considered to be 5% unless indicated otherwise.

RESULTS

Table 1 shows the anthropometric and biochemical characteristics of the 10 year old boys studied. To show the distribution of continuous variables, mean values, median

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TABLE 1. Anthropometric and biochemical characteristics of the 10-year-old boys studied

Characteristic	Mean ± SD	Median	(Lower Quartile, Upper Quartile)
Height (cm)	140 ± 6	140	137, 144
Body weight (kg)	47 ± 8	48	42, 52
BMI (kg/m^2)	23.9 ± 2.6	23.6	21.9, 25.8
SBP (mmHg)	108 ± 12	108	100, 120
DBP (mmHg)	62 ± 12	60	54, 70
Glucose (mg/dl)	93 ± 6	92	89, 96
Insulin (μU/ml)	13.6 ± 8.0	12.1	7.8, 17.6
HOMA-IR	3.1 ± 1.9	2.9	1.7, 4.2
QUICKI	0.14 ± 0.01	0.14	0.14, 0.15
TC (mg/dl)	186 ± 24	185	166, 204
TG (mg/dl)	67 ± 36	58	39, 86
HDL-C (mg/dl)	60 ± 9	60	53, 65
LDL-C (mg/dl)	113 ± 22	109	96, 129
ApoA-I (mg/dl)	135 ± 15	134	125, 145
ApoA-II (mg/dl)	31.2 ± 3.8	30.9	28.9, 34.0
ApoB (mg/dl)	78 ± 15	78	67, 88
LDL size (nm)	26.8 ± 0.7	26.8	26.3, 27.2

apoA-I, apolipoprotein A-I; BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high density lipoprotein-cholesterol; HOMA-IR, insulin resistance as assessed by homeostasis model assessment; LDL-C, low density lipoprotein-cholesterol; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.

values, and lower and upper quartiles are given. As shown, the mean serum TG level was much higher than the median value, indicating that serum TG levels were not normally distributed.

Figure 1 shows typical electropherograms of plasma lipoprotein subfractions as characterized by cITP for a boy with low HOMA-IR (Fig. 1A) and a boy with high HOMA-IR (Fig. 1B). As shown, plasma lipoproteins were separated into eight fractions by cITP: three HDL fractions [peaks 1–3: fast (f)-, intermediate (i)-, and slow (s)-migrating HDL], a chylomicron/remnant fraction (peak 4), a VLDL/intermediate density lipoprotein (IDL) fraction (peak 5), two LDL fractions (peaks 6 and 7: fLDL and sLDL), and a minor LDL fraction (peak 8).

In **Fig. 2**, cITP LDL subfractions analyzed directly in plasma are compared with those in LDL separated by ultracentrifugation. Figure 2A shows the cITP lipoprotein profile of a female NL volunteer. The cITP HDL fraction was identified by precipitation of apoB-containing lipoproteins (19). As shown in Fig. 2B, peaks 4–7 in Fig. 2A are not seen in apoB-depleted plasma, indicating that peaks 1–3 in Fig. 2A represent HDL subfractions. We identified LDL subfractions by adding ultracentrifugally isolated LDL to apoB-depleted plasma. As shown in Fig. 2C–G, the added LDL subfractions had the same migration time and a similar cITP pattern as those separated from the whole plasma. However, the proportion of fLDL(−) (peak 6) to the major LDL (peak 7, sLDL) seemed to be lower in ultracentrifugally isolated LDL than in plasma LDL, suggesting that more of the cITP fLDL subfraction was lost than cITP sLDL during ultracentrifugation.

One difference between the cITP and chromatographic methods for measuring LDL subfractions is that the cITP method monitors the lipid content of LDL subfractions and the chromatographic method monitors the protein

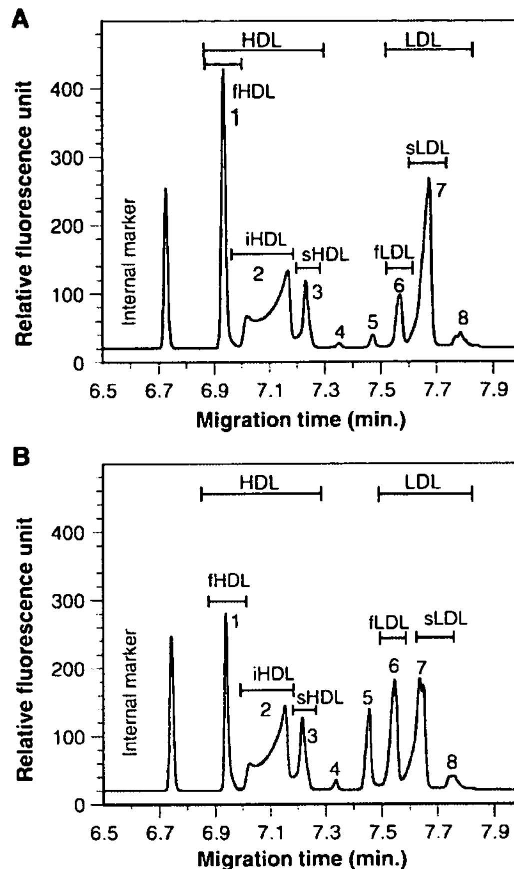

Fig. 1. Lipoprotein profiles as determined by capillary isotachophoresis (cITP) in plasma from a boy with low insulin resistance as assessed by homeostasis model assessment (HOMA-IR; 1.5) (A) and a boy with high HOMA-IR (6.5) (B). Peaks 1–3, fast (f)-, intermediate (i)-, and slow (s)-migrating HDL; peak 4; chylomicron/remnant fraction; peak 5, VLDL/intermediate density lipoprotein (IDL); peaks 6 and 7, fLDL and sLDL; peak 8, a minor LDL fraction.

content of LDL subfractions. In Fig. 2C–G, different amounts of LDL protein were added to apoB-depleted plasma to examine the linearity of the relation between cITP LDL subfractions and the protein content of LDL. As shown, both the cITP fLDL (peak 6) and sLDL (peak 7) subfractions increased with an increasing amount of LDL protein. The relative peak areas of cITP fLDL (r = 0.998) and sLDL (r = 0.997) subfractions were directly proportional to the amount of LDL protein between 0.1 and 0.8 mg/ml LDL protein content. This result indicates that cITP is an accurate and sensitive method for quantifying charge-based LDL subfractions.

To prove that cITP fLDL is related to the electronegativity of LDL, we tested the hypothesis that the changes in cITP fLDL paralleled the changes in the negative charge of LDL, as indicated by the electrophoretic mobility of LDL in agarose electrophoresis during the in vitro oxidation of LDL. Subjecting LDL to lipid peroxidation by incubation at 37°C in PBS containing 10 μM $CuSO_4$ resulted

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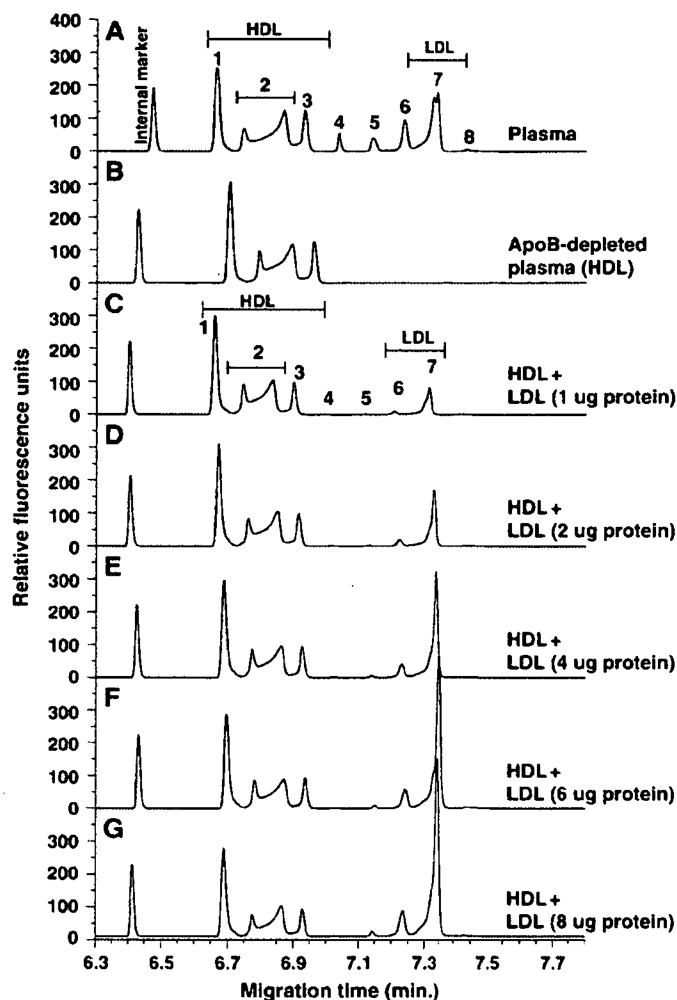

Fig. 2. Electropherograms of plasma lipoproteins (A), HDL fraction obtained by precipitation of apolipoprotein B (apoB)-containing lipoproteins from plasma (B), and ultracentrifugally isolated LDL containing 1 mg (C), 2 mg (D), 4 mg (E), 6 mg (F), and 8 mg (G) of protein in 10 ml of solution that was added to the HDL fraction for cITP separation.

in a rapid increase in conjugated dienes and TBARS (data not shown) and a progressive increase in the electrophoretic mobility on agarose gels (**Fig. 3A**) and apoB fluorescence (data not shown). As shown in Fig. 3A, the increase in the negative charge of oxidized LDL was visible on agarose gels after an oxidation period of 1 h. Changes in cITP LDL subfractions during oxidative modification were monitored by taking aliquots of oxidized LDL at different times and adding them to apoB-depleted EDTA plasma for cITP separation. The electropherograms of oxidized LDLs are shown in Fig. 3B. cITP sLDL (peak 7) gradually decreased from an oxidation time of 0.5 h (Fig. 3Bb–e), whereas cITP fLDL (peak 6) gradually increased from 0.5 to 1.5 h (Fig. 3Bb–d). This result indicates that at the initial stage of oxidation, cITP sLDL subfraction (peak 7) was converted to cITP fLDL subfraction (peak 6), and the increase in cITP fLDL paralleled that in the electrophoretic mobility on agarose gels. From 1.5 to 3 h of oxidation, cITP fLDL (peak 6) decreased, whereas a new cITP LDL subfraction (peak 5), which appeared after 1 h of oxidation (Fig. 3Bc), increased progressively (Fig. 3Bd–f). This result indicates that during the oxidative modification of LDL, although sLDL (peak 7) was converted to fLDL (peak 6), fLDL (peak 6) was further converted to a new, more negatively charged LDL subfraction (peak 5). Therefore, the distribution of cITP LDL subfractions changed gradually during the oxidative modification of LDL, with LDL subfractions shifting toward more negative charges. These results indicate that cITP fLDL represents an in vivo LDL(−) subfraction.

Levels of cITP fLDL were positively correlated with TG levels ($r = 0.65$, $P < 0.01$) and negatively correlated with LDL size ($r = -0.43$, $P < 0.01$) in all of the boys, suggesting that enrichment of LDL with TG increases the electronegative fraction of LDL and that LDL(−) is associated with small, dense LDLs.

As shown in **Table 2**, body mass index (BMI), fasting glucose concentration, fasting insulin concentration, and HOMA-IR were significantly higher and QUICKI was significantly lower in the middle and high HOMA-IR tertiles than in the low HOMA-IR tertile, as assessed by ANOVA and Scheffe's multiple comparison test. Also, levels of TG and cITP VLDL/IDL and cITP fLDL fractions were significantly higher, and HDL-C levels, cITP fHDL levels, and

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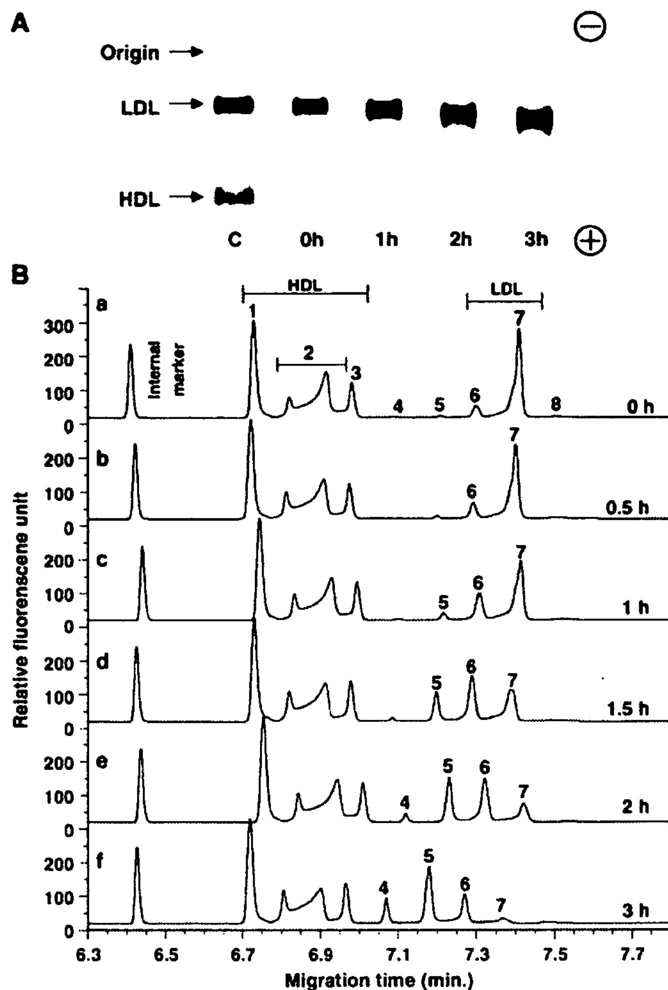

Fig. 3. A: Agarose electrophoresis of the time course to the oxidation of LDL (0.5 mg/ml) catalyzed by Cu^{2+} (10 μM). Lane C represents plasma of the female volunteer who donated blood for the isolation of LDL. B: Electropherograms of ultracentrifugally isolated LDL that was subjected to Cu^{2+}-catalyzed oxidation for 0 h (a), 0.5 h (b), 1 h (c), 1.5 h (d), 2 h (e), and 3 h (f) and added to apoB-depleted plasma (HDL fraction) for cITP separation.

LDL size were significantly lower, in the high HOMA-IR tertile than in low HOMA-IR tertile (Table 2). These results indicate that HOMA-IR is related to the altered distribution of not only cITP HDL subfractions but also LDL subfractions.

Figure 4A, B shows the plots of cITP fLDL versus TG levels and cITP fLDL versus LDL size, respectively, in the low and high HOMA-IR groups. As shown in Fig. 4A, cITP fLDL levels did not differ between the low and high HOMA-IR groups after adjusting for TG levels, suggesting that the relation between HOMA-IR and cITP fLDL levels depended on TG levels. As shown in Fig. 4B, the regression line of cITP fLDL levels versus LDL size in the high HOMA-IR group was shifted to higher values compared with that in the low HOMA-IR group (i.e., cITP fLDL levels were higher in the high HOMA-IR group than in the low HOMA-IR group after adjusting for LDL size). These results indicate that the relation between HOMA-IR and cITP fLDL levels was independent of LDL size.

To clarify the relationship between LDL subclasses as measured by density and cITP, the distributions of cITP fLDL were examined in NL, HC, and HTG subjects. **Table 3** shows the anthropometric characteristics and lipoprotein profiles in NL, HC, and HTG subjects. HC and HTG subjects were similar to NL subjects with respect to age but had higher BMI and lower HDL-C levels (Table 3). HC subjects had significantly higher levels of TC and LDL-C than both NL and HTG subjects, and HTG subjects had significantly higher TG levels than both NL and HC subjects (Table 3). HC and HTG subjects also had higher levels of TG and levels of TC and LDL-C than NL subjects, respectively (Table 3).

Hirano, Saegusa, and Yoshino (30) established a method for quantifying small, dense LDL-C levels by depleting large, light LDLs (d < 1.044 g/ml) from serum using heparin-Mg^{2+} precipitation and subsequently measuring the LDL-C levels in the supernatant by a direct homogenous assay. Large, light LDL levels were calculated from LDL-C levels in whole serum and serum depleted of large, light LDLs (30). In the present study, we used cITP analysis to quantify LDL subfractions in whole serum and serum depleted of large, light LDLs. cITP analysis has the advantage that LDL subfractions can be measured in the presence of lipoprotein particles other than LDL because

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TABLE 2. BMI, serum levels of lipids, lipoproteins, and apolipoproteins, and lipoprotein subfractions as determined by cITP according to tertiles of HOMA-IR

Variable	Tertiles of HOMA-IR			ANOVA
	Low (n = 25)	Middle (n = 25)	High (n = 25)	*P* Value
BMI (kg/m^2)	22.3 ± 2.2	24.3 ± 2.7[a]	25.0 ± 2.3[a]	<0.01
Glucose (mg/dl)	90 ± 4	95 ± 4[a]	95 ± 6[a]	<0.01
Insulin (μU/ml)	6.4 ± 1.7	12.1 ± 1.5[a]	22.0 ± 7.9[a,b]	<0.01
HOMA-IR	1.4 ± 0.4	2.8 ± 0.3[a]	5.2 ± 1.9[a,b]	<0.01
QUICKI	0.16 ± 0.01	0.14 ± 0.00[a]	0.13 ± 0.00[a,b]	<0.01
TC (mg/dl)	182 ± 8	186 ± 21	188 ± 24	NS
Log (TG)	3.8 ± 0.5	4.0 ± 0.4	4.4 ± 0.4[a,b]	<0.01
HDL-C (mg/dl)	63 ± 9	61 ± 10	56 ± 7[a]	<0.01
LDL-C (mg/dl)	109 ± 3	114 ± 19	114 ± 23	NS
ApoA-I (mg/dl)	138 ± 4	134 ± 18	134 ± 13	NS
ApoA-II (mg/dl)	30.1 ± 3.8	31.1 ± 3.7	32.2 ± 3.3	NS
ApoB (mg/dl)	74 ± 6	77 ± 11	82 ± 16	NS
LDL size (nm)	27.0 ± 0.7	26.9 ± 0.5	26.5 ± 0.6[a]	<0.05
cITP lipoprotein subfractions (peak area relative to an internal marker)				
fHDL	1.86 ± 0.30	1.64 ± 0.33	1.62 ± 0.34[a]	<0.05
iHDL	2.44 ± 0.31	2.44 ± 0.34	2.48 ± 0.30	NS
sHDL	0.50 ± 0.11	0.51 ± 0.09	0.53 ± 0.11	NS
VLDL/IDL	0.20 ± 0.20	0.22 ± 0.20	0.36 ± 0.17[a,b]	<0.01
fLDL	0.66 ± 0.23	0.65 ± 0.24	0.89 ± 0.20[a,b]	<0.01
sLDL	1.99 ± 0.54	2.08 ± 0.49	1.98 ± 0.51	NS

cITP, capillary isotachophoresis; fHDL, fast-migrating HDL; IDL, intermediate density lipoprotein; iHDL, intermediate-migrating HDL; sHDL, slow-migrating HDL. Data are presented as means ± SD.

[a] $P < 0.05$, vs. low tertile, assessed by ANOVA and Scheffe's multiple comparison test.

[b] $P < 0.05$, high tertile vs. middle tertile, assessed by ANOVA and Scheffe's multiple comparison test.

HDL, TG-rich lipoprotein (TRL), and LDL are separated into discrete fractions (14, 15, 19–22).

Figure 5 shows typical cITP lipoprotein profiles in whole serum and serum depleted of large, light LDLs by heparin-Mg^{2+} (30) in NL, HC, and HTG subjects. As shown, cITP sLDL (peak 7) in serum depleted of large, light LDLs (Fig. 5B, D, F) was greatly reduced compared with that in whole serum LDL (Fig. 5A, C, E) in NL (Fig. 5A, B), HC (Fig. 5C, D), and HTG (Fig. 5E, F) subjects, whereas cITP fLDL (peak 6) in serum depleted of large, light LDLs was only slightly reduced (Fig. 5). These results indicate that the distribution of cITP LDL subfractions was different between whole serum and light LDL-depleted serum. cITP HDL subfractions (peaks 1–3) were similar in whole serum and serum depleted of large, light LDLs, as expected (Fig. 5). As shown in Fig. 5E, F, the VLDL/IDL subfraction (peak 5) in the HTG subject was also reduced in serum depleted of large, light LDLs compared with that in whole serum, indicating that heparin-Mg^{2+} precipitation also affects TRL. Hirano, Saegusa, and Yoshino (30) did not address the effects of heparin-Mg^{2+} precipitation on TRL because changes in TRL did not affect their measurement of LDL-C levels in whole serum or serum depleted of large, light LDLs. Similarly, because cITP completely separates HDL, TRL, and LDL subfractions, the effects of heparin-Mg^{2+} precipitation on TRL also do not interfere with the measurement of cITP LDL subfractions. Therefore, distributions of cITP fLDL subfractions in different LDL density subclasses can be compared among

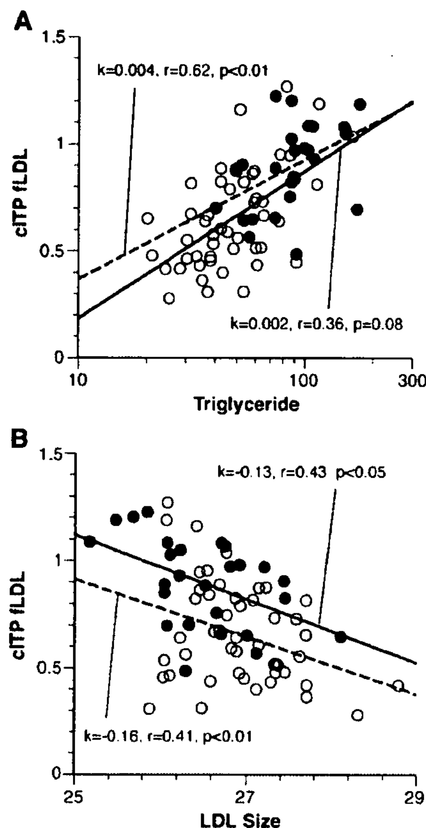

Fig. 4. Correlations between cITP fLDL and triglyceride (A) or LDL size (B) in the low (open circles) and high (closed circles) HOMA-IR groups.

NL, HC, and HTG groups while ignoring the effects of heparin-Mg^{2+} precipitation on lipoprotein particles other than LDL.

Figure 6A shows the cITP fLDL levels in NL, HC, and HTG groups according to LDL density subclasses separated by precipitation method (30). In whole serum (Fig. 6A, left panel), both the HC and HTG groups had significantly higher cITP fLDL levels than the NL group (indicated by asterisks). Levels of cITP fLDL in the small,

TABLE 3. Anthropometric characteristics and lipoprotein profiles of NL, HC, and HTG subjects

Variable	NL (n = 8)	HC (n = 8)	HTG (n = 8)
Age (years)	76 ± 5	72 ± 9	76 ± 4
BMI (kg/cm^2)	17.9 ± 1.6	22.7 ± 3.4[a]	21.7 ± 2.2[a]
TC (mg/dl)	193 ± 21	243 ± 11[a]	209 ± 28[a,b]
TG (mg/dl)	54 ± 13	117 ± 20[a]	198 ± 44[a,b]
HDL-C (mg/dl)	85 ± 13	47 ± 5[a]	48 ± 7[a]
LDL-C (mg/dl)	86 ± 14	160 ± 10[a]	119 ± 28[a,b]

HC, hypercholesterolemic; HTG, hypertriglyceridemic; NL, normolipidemic.

[a] $P < 0.05$ vs. NL, assessed by ANOVA and Scheffe's multiple comparison test.

[b] $P < 0.05$, HTG vs. HC, assessed by ANOVA and Scheffe's multiple comparison test.

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