

**Fig. 1.** Principles of ELISA system for MDA-LDL. Diluted serum samples were added to microtiterplate walls coated with a monoclonal antibody against MDA-LDL, ML25. MDA-LDL captured by ML25 was detected with an anti-apolipoprotein B monoclonal antibody AB16, labeled with  $\beta$ -galactosidase. Enzyme activity was measured with *o*-nitrophenyl- $\beta$ -galactopylanoside as the substrate.

bent assay (ELISA)<sup>4</sup>). Some studies of adults have described the serum levels of MDA-LDL as increasing in patients with acute coronary syndrome, and were positively correlated with the intima-media thickness of the carotid arteries. Therefore, circulating MDA-LDL might be related with the presence and the severity of atherosclerotic lesions<sup>5,6</sup>).

Recently, statistics reveal that about 10% of Japanese children are obese, and 10-20% have dyslipidemia. The public health problems of these obese children are related to those of children with dyslipidemia because they are caused by an urban lifestyle. Obese children and children with dyslipidemia will develop into obese adults and adults with dyslipidemia, which may result in atherosclerotic disease, thus exacerbating these problems in the future. Therefore, a strategy for the prevention of atherosclerotic disease for children with risk factors of atherosclerosis should be started in Japan.

Serum MDA-LDL may be a useful marker of atherosclerotic lesions in children. However, only a few studies have been performed which describe the levels of serum MDA-LDL in children. The aim of this study was to reveal the levels of serum MDA-LDL in Japanese obese and nonobese school children, and to discuss the relationships of serum MDA-LDL to serum lipids, apolipoproteins, and obesity.

## SUBJECTS AND METHODS

### Subjects

This study examined 137 (36 obese and 101 nonobese) healthy Japanese school boys and 135 (24 obese 111 nonobese) healthy Japanese school girls aged from 10 to 14 years, who received a regular medical examination at school under the administrative guidance of the local government in Niigata Prefecture in September 2000. Informed consent was obtained from the parents of the subjects before participation in this study.

The subjects' body height and weight were measured by school nurses with a portable stadiometer and a digital scale to the nearest 1 mm and 0.1 kg, respectively. Percent of relative weight was calculated based on the table published in 1990 by the Ministry of Education of the Japanese Government. The subjects were divided into two group in this study: the obese group whose percent relative weight was  $\geq +20\%$ , and the nonobese group whose percent relative weight was  $< +20\%$ .

**Table 1.** Clinical characteristics of subjects

	Boys			Girls		
	Obese subjects	Nonobese subjects	All subjects	Obese subjects	Nonobese subjects	All subjects
Number	36	101	137	24	111	135
Age (years)	11.4±1.7	11.5±1.6	11.5±1.6	11.7±1.6	11.8±1.5	11.8±1.5
Body height (cm)	147.5±11.8	146.6±13.1	146.8±12.7	147.5±9.6	147.6±9.6	147.6±9.6
Body weight (kg)	53.6±13.0 ****	38.4±10.4	42.4±12.9	53.5±12.3 ****	38.3±7.6	41.0±10.3
Body height SD score	+0.42±0.98	+0.14±0.99	+0.22±0.99	+0.31±1.26	+0.24±0.91	+0.25±0.98
Percent of relative weight (%)	+34.7±12.5 ****	-2.7±9.2	+7.2±19.4	+32.5±12.3 ****	-4.9±10.3	+1.7±17.9
Body mass index (kg/m/m)	24.2±2.9 ****	17.5±2.1	19.3±3.8	24.2±3.0 ****	17.4±2.0	18.6±3.4
MDA-LDL (IU/l)	68.3±24.1 ***	55.6±16.2	58.9±19.4	62.9±27.5	54.9±17.1	56.3±19.5
Total cholesterol (mg/dl)	186.2±31.1 ***	168.0±26.4	172.8±28.7	179.5±30.7	170.1±24.5	171.8±25.9
HDL cholesterol (mg/dl)	57.8±13.6 **	66.1±13.4	63.9±13.9	57.1±13.3	62.3±11.9	61.4±12.3
LDL cholesterol (mg/dl)	111.7±24.4 ****	90.6±21.0	96.1±23.8	106.5±27.0 *	96.3±21.0	98.1±22.4
Apolipoprotein A1 (mg/dl)	141.1±20.8	143.8±17.4	143.1±18.3	135.1±15.2	138.2±16.6	137.7±16.3
Apolipoprotein B (mg/dl)	85.5±19.5 ****	68.8±14.7	73.2±17.7	80.8±21.7 *	72.6±14.0	74.0±15.9
Apolipoprotein E (mg/dl)	4.2±0.9 **	3.7±0.8	3.8±0.9	4.4±1.1 **	3.8±0.8	3.9±0.8

The values are expressed as mean±SD. MDA-LDL, malondialdehyde-modified low-density lipoprotein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; \*,  $p < 0.05$  vs Nonobese subjects' value using unpaired *t*-test; \*\*,  $p < 0.01$  vs Nonobese subjects' value using unpaired *t*-test; \*\*\*,  $p < 0.001$  vs Nonobese subjects' value using unpaired *t*-test; \*\*\*\*,  $p < 0.0001$  vs Nonobese subjects' value using unpaired *t*-test.

#### Assay for the serum levels of MDA-LDL and other lipids, apolipoprotein

Fasting blood samples were obtained from the antecubital vein of the subjects in the morning. The blood samples were centrifuged at 3000 rpm for 10 min, and the serum was frozen and stored at  $-20^{\circ}\text{C}$  until analysis within one hour.

The serum level of MDA-LDL was measured by the double-antibody sandwich ELISA method using a commercially available kit (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan), its use being based on the same principles as previously reported<sup>7)</sup>. Serum samples were diluted 2000-fold in a dilution buffer containing SDS. Duplicate 100  $\mu\text{L}$  portions of the diluted sample were then added to the wells of plates, which were coated with a monoclonal antibody against MDA-LDL (ML25). ML25 has previously been shown to recognize MDA residue but has not been shown to be specific for MDA-LDL. The reaction was allowed to stand for 2 h at room temperature, and the plates were then washed. A  $\beta$ -galactosidase conjugated monoclonal antibody against Apo B (AB16) was then added. The combination of ML25 with AB16 has been shown to be recognized by MDA-LDL. LDL oxidized by  $\text{Cu}^{2+}$  has been shown to be detectable using this ELISA method. Again, the reaction was allowed to stand for 1 h at room temperature, and the

plates were then washed. One hundred microliters of 10 mmol/L o-nitrophenyl-galactopyranoside as a substrate was pipetted into the wells. After 2 h, the reaction was stopped by adding 100  $\mu\text{L}$  of 0.2 mol/L sodium carbonate (pH 12). Absorbance in the wells was determined at 415 nm with an MPR-4A microplate reader (Tosoh Co., Ltd., Tokyo, Japan). A primary standard was used with preparative MDA-LDL, in which 15% of the total amino groups were modified. We tentatively defined 1 U/L MDA-LDL as the absorbance obtained with the primary standard at a concentration of 1 mg/L. A calibration curve was prepared by diluting a reference serum as a secondary standard from 300-to 9600-fold with a dilution buffer and calculating the amount of MDA-LDL in the samples. Reference sera were prepared from pooled sera from healthy volunteers (Fig. 1).

Serum levels of LDL cholesterol (LDL-C), high density lipoprotein (HDL-C), total cholesterol (TC), apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B), and apolipoprotein E (Apo E) were measured with an automatic analyzer using commercially available kits (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan).

#### Statistical analysis

All statistical analyses were performed using the

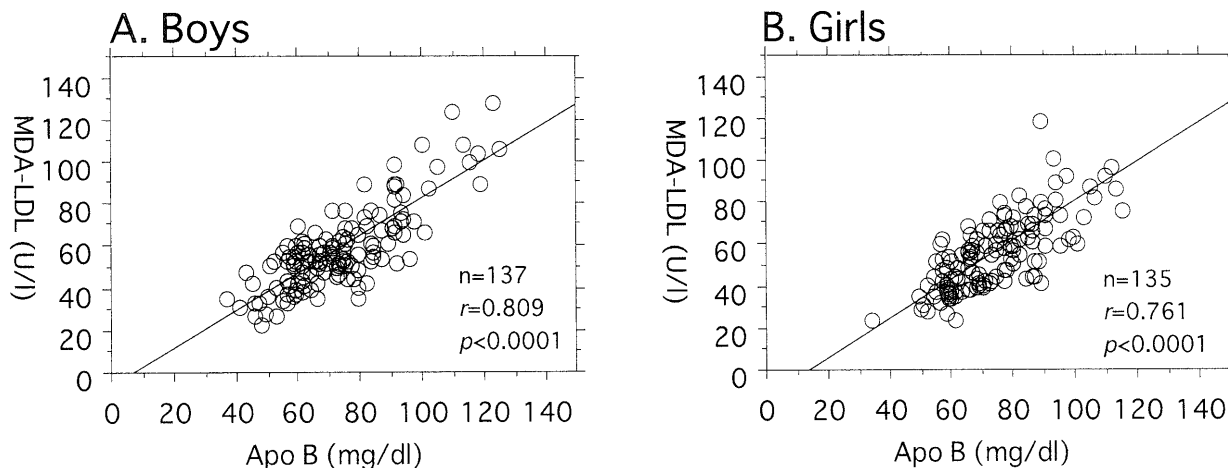


Fig. 2. Correlations between Apo B and MDA-LDL in healthy Japanese boys (A) and girls (B).

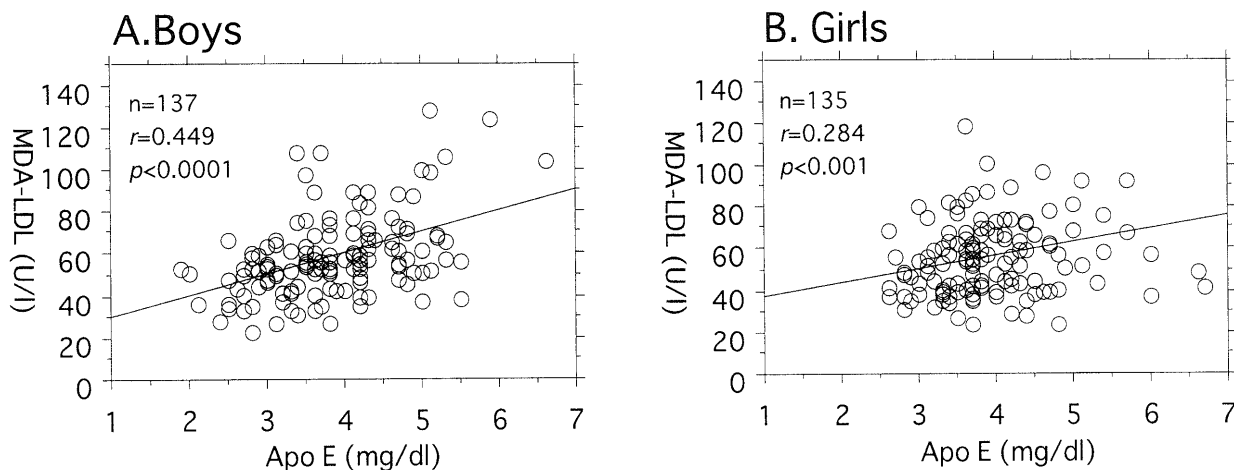


Fig. 3. Correlations between ApoE and MDA-LDL in healthy Japanese boys (A) and girls (B).

StatView program for Macintosh (version 4.5, Abacus Concepts, Berkeley, CA, USA). The test of normality proved that the data demonstrated a normal distribution. The unpaired t-test was used to compare values of the two groups. The relationship between variables was analyzed by simple regression and by stepwise multiple regression. Data were expressed as mean  $\pm$  SD, and  $p<0.05$  was considered statistically significant.

## RESULTS

### Serum levels of MDA-LDL, lipids and apolipoproteins in the subjects

Characteristics of the subjects are shown in Table 1. Of 272 subjects, 36 boys and 24 girls belonged to the obese group, the remaining 101 boys and 111 girls belonging to the nonobese group. The serum levels of MDA-LDL in obese boys were significantly higher than those in nonobese boys ( $p<0.001$ ). The serum levels of MDA-LDL in obese girls had a tendency to be higher than those in nonobese girls ( $p=0.06$ ).

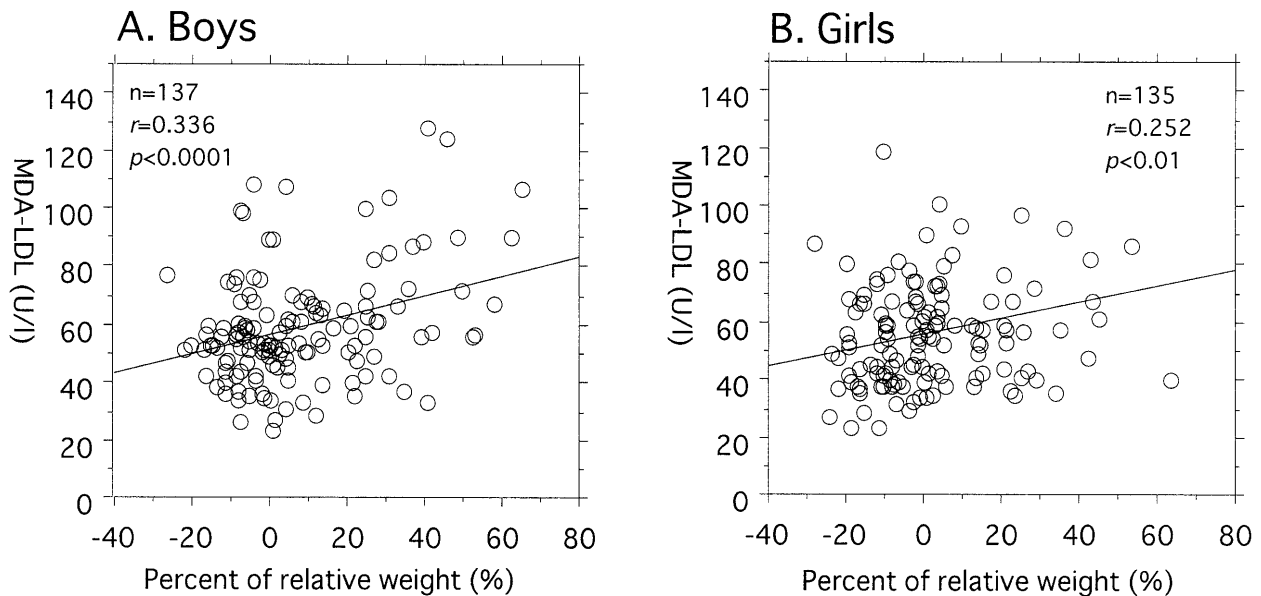


Fig. 4. Correlations between percent of relative weight and MDA-LDL in healthy Japanese boys (A) and girls (B).

However, the serum levels of MDA-LDL of obese subjects were not significantly different from those of nonobese subjects in either sex after adjustment for the serum levels of LDL. Serum levels of MDA-LDL of boys were not significantly different from those of girls in either obese or nonobese subjects. Serum levels of TC, LDL-C, Apo B and Apo E in obese boys were significantly higher than those in nonobese boys, and serum levels of HDL-C in obese boys were significantly lower than those in nonobese boys. Serum levels of LDL-C, Apo B and Apo E in obese girls were significantly higher than those in nonobese girls. The serum levels of HDL-C in obese girls had a tendency to be lower than those in nonobese girls ( $p=0.05$ ). Obese subjects, especially boys, were considered to have a tendency for some dyslipidemia.

#### Relationship of serum MDA-LDL to lipids, apolipoproteins and obesity

Figs. 2 and 3 show that the serum levels of MDA-LDL had a positive correlation with those of Apo B and E in both boys and girls. The serum levels of MDA-LDL showed a positive correlation with those of TC (boys;  $r=0.684$ ,  $p<0.0001$ , girls;  $r=0.614$ ,  $p<0.0001$ ) and LDL-C (boys;  $r=0.764$ ,  $p<0.0001$ , girls;  $r=0.662$ ,  $p<0.0001$ ), and showed a negative correlation with those of HDL-C (boys;  $r=-0.181$ ,  $p<0.05$ , girls;  $r=-0.175$ ,  $p<0.05$ ). Fig. 4 shows that the serum levels of MDA-LDL had a positive correlation with the percent of

relative weight in both boys and girls. The circulating MDA-LDL levels were related to obesity.

#### DISCUSSION

OxLDLs are considered to play key roles in the progression of atherosclerosis<sup>8,9</sup>. OxLDLs become incorporated into macrophages by scavenger receptors and modulate the gene expression involved in the cellular function of endothelial cells and smooth muscle cells in the vessel walls<sup>10</sup>. Recently, a sensitive method to measure circulating MDA-LDL, which is one of the OxLDLs, was established<sup>7</sup>. Some studies on adults have described the relationship between the serum level of MDA-LDL and other serum lipids. The serum levels of MDA-LDL were increased in patients with acute coronary syndrome, and were positively correlated with those of LDL-C and TG, and were inversely correlated with those of HDL-C. In addition, the serum levels of MDA-LDL were positively correlated with the intima-media thickness of the carotid arteries<sup>5</sup>. As a consequence, the serum levels of MDA-LDL presented a clinical significance as a factor in association with the progression of atherosclerosis.

However, only a few studies have described the serum levels of MDA-LDL in children. Iughetti et al. reported the presence of serum MDA-LDL in healthy children, but did not discuss the relationship between

the serum levels of MDA-LDL and the other lipids or obesity<sup>11</sup>). The clarification of clinical features of children with higher serum levels of MDA-LDL might contribute to the primary prevention of atherosclerotic disease. We therefore investigated the serum levels of MDA-LDL and the relationships of serum MDA-LDL to serum lipids and apolipoproteins, and obesity in Japanese school children.

This study showed that the serum levels of MDA-LDL in obese children were higher than those in nonobese children. Also, the serum levels of MDA-LDL were positively correlated with percent of relative weight. This study suggested that the serum levels of MDA-LDL have a relation to childhood obesity. Tanaga et al. examined the levels of MDA-LDL using the same method in Japanese adults with and without coronary artery disease (CAD), and revealed that the levels of MDA-LDL in adults with and without CAD were  $113.4 \pm 49.1$  U/l ( $n=62$ ) and  $85.2 \pm 22.5$  U/l ( $n=42$ ), respectively<sup>5</sup>). The levels of MDA-LDL in adults were higher than those of both the obese and nonobese subjects in this study, which suggested that the serum levels of MDA-LDL were related to aging.

This study also showed that the serum levels of MDA-LDL in school children were positively correlated with those of LDL-C, Apo B, Apo E and percent of relative weight, and were inversely correlated with those of HDL-C. Especially, the serum levels of MDA-LDL levels had a close association with the serum LDL-C and Apo B levels.

There is some controversy about the direct role of obesity in the pathogenesis of atherosclerosis. Gaal LF. et al. reported that the oxidizability of LDL was significantly increased in premenopausal obese women, and suggested that obesity directly affected the oxidation of LDL particles<sup>12</sup>). In this study, the serum levels of MDA-LDL were positively correlated with percent of relative weight, but these associations were eliminated after adjustment for the serum levels of LDL-C or Apo B. These results indicated that obese children can be considered to have a risk factor of the oxidation of LDL particles due to the elevated serum levels of LDL-C. Therefore obesity did not directly affect the oxidation of LDL particles. Visceral fat accumulation, which is a major cause of obesity induced metabolic disorders, leads to decreased serum HDL-C levels and increased serum TG and Apo E levels. This study demonstrated that obese subjects had higher serum MDA-LDL and Apo E levels and lower serum HDL-C levels than nonobese subjects. Also, serum MDA-LDL levels were positively correlated with serum Apo E and HDL-C levels. These results suggested that the disarrangement of

TG and Apo E due to visceral fat accumulation affected the oxidation of LDL particles<sup>13</sup>). Oxidative stress has been reported to be increased in hypertriglyceridemia in several studies. It seems that the detrimental effect of hypertriglyceridemia in obese children is probably due to its enhancing effect on LDL oxidizability. For this reason, obese children are considered to have elevated levels of MDA-LDL through the elevated levels of LDL-C and TG. The finding of elevated serum MDA-LDL levels in obese children suggests that they have a risk factor of atherosclerotic disease.

In conclusion, circulating MDA-LDL was detected in the sera of healthy Japanese school children. The serum levels of MDA-LDL in childhood were lower than those in adults, and were associated with the serum levels of LDL-C, HDL-C, Apo B, Apo E and obesity, as in adulthood. The serum levels of MDA-LDL in children with dyslipidemia and/or obesity might already be elevated. Several questions remain to be answered concerning the relation of the serum levels of MDA-LDL to visceral fat accumulation and adipocytokines as well as the mechanism for increased oxidizability of LDL in obese children.

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