Possible Inhibitory Effect of Lipid Peroxides on Acid Lipase Activity via Low-Density Lipoprotein in Human Mononuclear Leukocytes

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Summary Acid lipase (AL) activity was measured in mononuclear leukocytes (MNL) isolated from 214 subjects. An inverse relation was observed between the AL activity and serum cholesterol (CHO) with age variation: The age groups above 30, which showed low AL activities in their MNL, exhibited high serum CHO; whereas the 20-to-29 age group, which had the highest AL activity among the age groups, showed the lowest CHO value. Moreover, the hypercholesterolemic group with high apo-B levels showed significantly low AL activity in both sexes when compared with the normolipidemic group. Significant negative correlations were also observed in the hypercholesterolemic group between the AL activity and both CHO and apo-B, and between the AL activity and lipid peroxides (LPO). On the other hand, while the addition of low-density lipoprotein (LDL) from patients with type II hypercholesterolemia to the AL assay system and the culture medium of MNL resulted in the inhibition of the AL activity, the inhibitory effect was prevented by preincubation of LDL with anti apo-B serum. These data suggest a certain regulatory mechanism of LDL, the physiological substrate of AL, on the AL activity; this action possibly occurs through lipid peroxides in LDL.

Key Words: acid lipase, low-density lipoprotein, lipid peroxides, mononuclear leukocytes, hypercholesterolemia

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Lysosomal acid lipase plays an important role in the intracellular metabolism of exogenous LDL-associated cholesterol esters (E-CHO), and the genetic deficiency of AL is associated with intracellular accumulation of E-CHO and triglycerides (TG) in various organs of affected patients [1, 2]. Hypercholesterolemia, in which the cell are provided with an increased amount of LDL-associated E-CHO, is established as a major risk factor for atherosclerosis and related occlusive vascular diseases [3, 4]. Since the monocyte-derived macrophages are considered to be a possible progenitor of foam cells in atherosclerotic plaques [5] and since there is a decreased AL activity in MNL from patients with type II hypercholesterolemia [6, 7], the reduced AL activity is suggestive of a risk factor in the development of premature atherosclerosis.

We previously reported that there was a reversible inhibitory effect of apo-B-carrying lipoproteins isolated from hypercholesterolemic rabbits on AL derived from normolipidemic rabbits and that the inhibition was blocked *in vitro* by anti apo-B serum [8]. In the present study, in order to elucidate the regulatory mechanism on AL in relation to blood lipids, AL activity was measured in MNL isolated from 214 subjects, and the results were confirmed by *in vitro* inhibition experiments on the enzyme and by a cell culture study.

MATERIALS AND METHODS

Subjects. Individuals who had fasted for at least 15 h were selected from among inhabitants of Yonashiro-Son, which is located in the central part of Okinawa Island, at the time of their annual health examinations. They were divided into a normal group and a hypercholesterolemic group. The normal group included 166 individuals (63 males and 103 females) with normal laboratory tests. The hypercholesterolemic group consisted of 48 individuals (11 males and 37 females) with serum cholesterol levels above 250 mg/dl and included 11 hypertensive subjects with systolic pressure above 160 mmHg and diastolic pressure above 90. Patients with type IIa or IIb hypercholesterolemia who were seen in the out-patient clinic of the University of the Ryukyus Hospital were free of other complications that would affect lipid metabolism.

Preparation of samples. For the population study of the AL activity, the MNL were isolated from 1 ml of heparinized blood, and AL was extracted from the cell pellets by 0.1% Triton X-100 as described previously [8]. The human lipoprotein fractions were prepared by discontinuous gradient ultracentrifugation at 4°C and 40,000 rpm for 48 h, and the purity of the concentrated fractions was examined electrophoretically [8].

Biochemical analyses. The AL assay was fully described in the previous study [8]. In brief, labeled cholesterol esters at a final concentration of 5 mM were incubated with the enzyme sources for 2 h at 37°C and pH 4.5 in the presence of 1 mM β -mercaptoethanol, 1 mM EDTA, and 0.1% Triton X-100; the fatty acids thereby produced were extracted by the solvent system according to Belfrage and

Vaughan [9]. One unit of enzyme activity was defined as that amount of enzyme catalyzing the release of 1 nmol of fatty acid per hour. In the inhibition study, the substrate was premixed with 10 μ l of lipoproteins and the reaction was started by the addition of a 20- μ l sample. In the experiment for preventing the inhibitory effect of lipoprotein, 2 or 4 μ l of diluted anti-human apo-B goat serum (titer, 4.8 mg/ml; Daiichi Kagaku, Tokyo) was preincubated with 10 μ l of lipoproteins in the antigen vs. antibody ratios as indicated in Table 3 for 2 h at 4°C, followed by the addition of the substrate and the enzyme extract prepared from the MNL of a normolipidemic subject.

Lipid peroxides were measured fluorometrically as thiobarbituric acid reactive substances (TBA) by the method of Yagi [10]. Lipids in sera and lipoproteins were measured manually by use of commercial kits employing enzymatic procedures. Protein was determined by the Folin-Lowry method [11].

Cell culture. The MNL (85–95% lymphocytes and 5–15% monocytes) isolated from a normolipidemic subject were incubated for 72 h in RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (100 μ g/ml), and 5 mg/ml human lipoprotein-deficient serum in a humidified 5% CO₂ incubator at 37°C at a final cell concentration of about 2×10^5 /ml. The cells were then washed with the medium, resuspended in the same, and plated out in 24-well cluster plates with the addition of 1 ml of cell suspension containing about 2×10^5 cells to each well. LDL isolated from patients with type II hypercholesterolemia was added next to make the indicated concentration in the presence or absence of various concentrations of chloroquine, and the cells were then further incubated for 6 h. After that the cells were washed three times with cold phosphate-buffered saline (PBS), and extracted with 0.1% Triton X-100 in PBS.

Other methods. Subjects were judged to be obese who had a body weight of more than 75% of the average body weight, which was set as the theoretical 50% value, of the population group of the same sex, age, and height. This was based on the "Table for Judging Obesity for Japanese" which was determined from 21,530 adults over age 20. The table was published by the Ministry of Public Health Welfare of Japan on August 22, 1986. The significance of correlation coefficients was tested by the two-sided *t*-test.

RESULTS

All data represent the mean values of duplicate or triplicate determinations.

Relation of AL activity to serum cholesterol

An inverse relation was observed between the AL activity and the serum CHO levels with age variation: The age group above 30, which showed low AL activity in their MNL, exhibited high serum CHO; whereas the 20-to-29 age group, which had the highest AL activity of all age the groups, showed the lowest CHO values (Table 1). The AL activity in the hypercholesterolemic group with high apo-B

Table 1. Acid lipase activity of mononuclear leukocytes and serum cholesterol levels in normal and hypercholesterolemic subjects.

Categories			Males				Females				
Age (years)	N	n	AL activity (unit/mg)	T-CHO (mg/dl)	Apo-B (mg/dl)	Obesity n (%)	n	AL activity (unit/mg)	T-CHO (mg/dl)	Apo-B (mg/dl)	Obesity n (%)
Normal group											
20-29	25	13	5.67±3.23	183±32	104±22	3(23)	12	5.19±4.51	182±31	95±19	1(8)
30-39	20	9	2.33±1.30 ^{*3}	200±39	100±30	0	11	4.03±2.26	214±25 ^{*2}	120±22 ^{*3}	2(18)
40-49	13	6	3.31±1.76	204±28	100±20	0	7	2.21±0.75	205±21	103±24	4(57)
50-59	28	8	2.29±1.36 ^{*2}	199±52	103±30	3(38)	20	2.45±2.06 ^{*2}	217±25 ^{*4}	123±29 ^{*3}	11(55)
60-69	39	12	3.66±3.19	214±24 ^{*2}	105±27	4(33)	27	3.26±3.14	223±21 ^{*5}	122±30 ^{*3}	10(37)
70-79	28	11	3.23±3.89	200±39	93±36	4(36)	17	3.49±3.68	221±24 ^{*5}	121±27 ^{*3}	7(41)
80-89	13	4	1.97±0.24 ^{*1}	194±24	112±32	1(25)	9	1.63±1.25 ^{*1}	218±35 ^{*2}	131±22 ^{*5}	2(22)
30-89	141	50	2.93±2.29 ^{*5}	203±35	103±26	12(24)	91	3.02±2.69 ^{*2}	218±24 ^{*5}	122±27 ^{*5}	36(40)
20-89	166	63	3.39±2.83	200±34	103±26	15(24)	103	3.29±3.06	214±25	121±27	37(36)
Hypercholesterolemic group											
25-86	48	11	1.45±0.74 ^{*1}	307±38 ^{*3}	144±37 ^{*3}	7(64)	37	1.47±0.94 ^{*2}	300±47 ^{*3}	150±35 ^{*3}	16(43)

Difference compared to the second decade was significant at ^{*1}*p*<0.05; ^{*2}*p*<0.025; ^{*3}*p*<0.001; ^{*4}*p*<0.005; or ^{*5}*p*<0.001. Difference compared with the normal group was significant at ^{†1}*p*<0.05; ^{†2}*p*<0.025; or ^{†3}*p*<0.01.

Fig. 1. Linear regression of T-CHO (A), E-CHO (B), TBA (C), and apo-B (D) on the AL activity in the hypercholesterolemic group. The open and closed symbols represent male and female, respectively. Correlation coefficients and *p* value are presented in Table 2.

Table 2. Correlations between the acid lipase activity in mononuclear leukocytes and various parameters of plasma cholesterol metabolism in the hypercholesterolemic group.

Parameters		Total (<i>n</i> =48)		Males (<i>n</i> =11)		Females (<i>n</i> =37)	
<i>x</i>	<i>y</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
AL activity	T-CHO	-0.812	<0.001	-0.544	NS	-0.864	<0.001
	E-CHO	-0.831	<0.001	-0.875	<0.001	-0.827	<0.005
	Apo-B	-0.673	<0.001	-0.413	NS	-0.745	<0.001
	TBA	-0.510	<0.001	-0.804	<0.005	-0.469	<0.005
T-CHO	Apo-B	+0.592	<0.001	+0.443	NS	+0.644	<0.005
	TBA	+0.494	<0.001	+0.833	<0.005	+0.520	<0.001

NS, not significant.

levels was significantly lower than the average value in the normal group taken as a whole. Significant negative correlations were also observed between the AL activity and both the CHO and apo-B levels, and between the AL activity and TBA values in the hypercholesterolemic group (Fig. 1). The *r* and *p* values are presented in Table 2. TBA values were 3.52 ± 1.03 for males (*n*=54) and 2.72 ± 1.07 for females (*n*=80) in the normal group, whereas they were 3.96 ± 1.37 for males (*n*=11) and 3.04 ± 1.14 for females (*n*=37) in the hypercholesterolemic group. The TBA values were significantly higher in males than in females in both the normal group (*p*<0.001) and the hypercholesterolemic group (*p*<0.05), and they were ca.

Fig. 2. The inhibitory effect of lipoproteins on AL activity. The percentage of inhibition was calculated based on the control activity of 12.3 units/mg. The moles per ml of lipoproteins added, which were isolated from patients with type IIa (closed symbols) and type IIb (open symbols) hypercholesterolemia, were calculated, assuming estimated molecular weights of 6.0×10^6 , 2.2×10^5 , and 2.5×10^5 for VLDL (squares), LDL (circles), and HDL (triangles), respectively.

10% higher in the hypercholesterolemic group than in the normal group for both sexes, but the difference was not significant.

Effect of lipoproteins on AL activity

Addition of lipoproteins isolated from patients with hypercholesterolemia to the assay system inhibited the AL activity, and the apo-B-carrying lipoproteins were ca. 150 times more potent than high-density lipoprotein (HDL) when compared at the 50% inhibition point (Fig. 2). The inhibition caused by apo-B-carrying lipoproteins was prevented by preincubation of the lipoproteins with anti apo-B serum prior to the enzyme assay, but the anti-serum showed no effect on the inhibition that was observed following the addition of HDL in large concentration (Table 3).

Cell culture study of the effect of lipoproteins on the AL activity

There was a dose-dependent chloroquine inhibition of the lysosomal AL activity of MNL, in which the inhibition was reduced by the presence of $15 \mu\text{g/ml}$ LDL; but the effect of LDL disappeared at $100 \mu\text{M}$ chloroquine. Chloroquine inhibited the AL activity not only in the intact cells but also directly in the cell-free extract (Fig. 3 (A), (C)). Addition of LDL isolated from hypercholesterolemic serum to the culture media inhibited the AL activity at concentrations above $15 \mu\text{g/ml}$ in the absence of chloroquine pertaining to both oleate and linoleate esters as substrates; thus the inhibited fraction represented catalytic

Table 3. Prevention of inhibitory effects of lipoproteins on acid lipase by anti apo-B serum.

	Lipoproteins added			Anti Apo-B** (μ l/ml)	CHO oleate		CHO linoleate	
	Protein (μ g/ml)	Apo-B (μ g/ml)	E-CHO (μ g/ml)	Total* (μ g/ml)	AL activity (unit/mg)	Inhib (%)	AL activity (unit/mg)	Inhib (%)
Control					6.1	0	7.5	0
IIa -LDL	105	68	139	471 (236)	1.8	70	3.0	60
					2.4	61	3.9	48
					2.8	54	4.9	35
-VLDL	96	87	71	815 (136)	2.0	67	3.2	58
					3.2	48	5.2	30
-HDL	101	—	29	254 (1,016)	3.7	39	5.7	24
					4.0	34	5.5	27
IIIb -LDL	97	48	129	443 (221)	2.2	64	3.1	59
					2.6	57	4.2	44
					3.4	44	5.0	33
-VLDL	100	30	2	1,543 (257)	2.4	61	4.5	40
					2.9	52	5.5	27
-HDL	102	—	25	246 (988)	4.1	33	6.0	20
					4.3	30	6.1	19

IIa and IIb: Type IIa (T-CHO 323, TG 148 mg/dl), Type IIb (T-CHO 305, TG 270 mg/dl) hypercholesterolemia. * Total: T-CHO+TG+PL+Protein, which concentrations were also expressed as pmol per ml in parentheses. **The titer of antiserum, which indicates mg of antigen that reacts with 1 ml of antiserum, was 4.8 mg/ml, and the antiserum was diluted to give the weight ratios of antigen vs. antibody, which are shown in parentheses, in the indicated volume. The protein content of HDL was substituted for its apoprotein content.

Fig. 3. The inhibitory effect of LDL on the AL hydrolysis of CHO oleate (A and B) and CHO linoleate (C and D) in cultured human MNL. A and C: Effects of increasing concentration of chloroquine are shown in the absence (■) and presence (□) of 15 $\mu\text{g}/\text{ml}$ LDL, and in the cell-free extract (Δ). B and D: Effects of an increasing concentration of LDL are illustrated in the absence (●) and presence (○) of 100 μM chloroquine, and the difference ($\blacktriangle$) between these two conditions (● and ○) represents the % activity specifically inhibited by chloroquine.

activities in both lysosomes and cytosol (closed circles in Fig. 3 (B), (D)). The inhibition caused by chloroquine, a lysosomotropic agent, was greater for linoleate esters than for oleate esters, as was indicated by the difference between activities in the absence of and in the presence of 100 μM chloroquine (closed triangles in Fig. 3 (B), (D)). In other words, most of the activities inhibited by LDL were related to lysosomal fraction for linoleate esters, whereas they were related to both lysosomal and cytosol fractions for oleate esters.

DISCUSSION

A characteristic feature of the genetic deficiency of lysosomal acid lipase, as seen in Wolman's disease, and cholesterol ester storage disease (CESD) is an intracellular accumulation of E-CHO and TG. By use of fibroblasts isolated from

CESD, in which there has been reported premature coronary atherosclerotic changes [1], it has been clarified that lysosomal AL plays a major role in hydrolyzing LDL-associated E-CHO entering the cell via specific receptors [12]. In animal studies of atherosclerosis, there have been contradictory reports about the E-CHO-hydrolyzing activity of the aorta. In hypercholesterolemic rabbits that had been fed a high CHO-diet, the E-CHO-hydrolyzing activity was occasionally increased [13]. In this case the lipid metabolism may be in a stage that would compensate for a large influx of blood lipids to the cell, thereby causing production of new primary lysosomes and causing the activity of other lysosomal enzymes to also be high [13, 14]. In the advanced and chronic stages of hypercholesterolemia with atherosclerotic lesions in the aorta, lipid-laden lysosomes were deficient in lysosomal E-CHO-hydrolyzing activity [15] and other E-CHO hydrolases in microsomes were also decreased [16].

A major factor influencing the point at which compensation turns to dysfunction of the hydrolase activity may be the level of LPO in the blood and aorta. In chronic hypercholesterolemic conditions the LPO levels are often increased [7, 16-18], which increase correlates with the degree of atherosclerotic changes in the aorta [19, 20]. Since AL is sensitive to oxidation [21, 22], the reduced AL activity in the hypercholesterolemic condition as found in the present and previous [8] studies may be due to an increase of LPO in the cell. Among negative correlations observed between the AL activity and various parameters of the hypercholesterolemic condition, the negative correlation between the AL activity and TBA may be an important finding. Since plasma LPO is associated with the lipid portion of lipoproteins, the prevention by anti apo-B serum of the inhibition of AL activity caused by addition of lipoproteins to the assay system and to the MNL culture medium supports this possibility of LPO involvement. That is, the antiserum may precipitate apo-B-carrying LPO lipoproteins or may interfere with the contact of these lipoproteins with the enzyme molecules. A possible autosomal mutation resulting in reduced enzyme activity has also been suggested by family studies of patients with hypercholesterolemia in which MNL were used [7]. There is evidence that the foam cells found in atherosclerotic lesions are derived from circulating monocytes [5, 23]. The reduced AL activity of MNL in hypercholesterolemic individuals may be a useful warning sign that precedes the onset of symptomatic atherosclerosis.

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