# Low density lipoproteins develop resistance to oxidative modification due to inhibition of cholesteryl ester transfer protein by a monoclonal antibody

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**Abstract** **Although numerous studies have investigated the relationship between cholesteryl ester transfer protein (CETP) and high density lipoprotein (HDL) remodeling, the relationship between CETP and low density lipoproteins (LDL) is still not fully understood. In the present study, we examined the effect of the inhibition of CETP on both LDL oxidation and the uptake of the oxidized LDL, which were made from LDL under condition of CETP inhibition, by macrophages using a monoclonal antibody (mAb) to CETP in incubated plasma. The 6-h incubation of plasma derived from healthy, fasting human subjects led to the transfer of cholesteryl ester (CE) from HDL to VLDL and LDL, and of triglycerides (TG) from VLDL to HDL and LDL. These net mass transfers of neutral lipids among the lipoproteins were eliminated by the mAb. The incubation of plasma either with or without the mAb did not affect the phospholipid compositions in any lipoproteins. As a result, the LDL fractionated from the plasma incubated with the mAb contained significantly less CE and TG in comparison to the LDL fractionated from the plasma incubated without the mAb. The percentage of fatty acid composition of LDL did not differ among the unincubated plasma, the plasma incubated with the mAb, and that incubated without the mAb. When LDL were oxidized with $CuSO_4$, the LDL fractionated from the plasma incubated with the mAb were significantly resistant to the oxidative modification determined by measuring the amount of TBARS and by continuously monitoring the formation of the conjugated dienes, in comparison to the LDL fractionated from the plasma incubated without the mAb. The accumulation of cholesteryl ester of oxidized LDL, which had been oxidized for 2 h with $CuSO_4$, in J774.1 cells also decreased significantly in the LDL fractionated from the plasma incubated with mAb in comparison to the LDL fractionated from the plasma incubated without the mAb. These results indicate that CETP inhibition reduces the composition of CE and TG in LDL and makes the LDL resistant to oxidation. In addition, the uptake of the oxidized LDL, which was made from the LDL under condition of CETP inhibition, by macrophages also decreased.**—Sugano, M., S. Sawada, K. Tsuchida, N. Makino, and M. Kamada. **Low density lipoproteins develop resistance to oxidative modification due to inhibition of cholesteryl ester transfer protein by a monoclonal antibody.** ***J. Lipid Res.*** **2000.** 41: **126–133.**

**Supplementary key words** cholesteryl ester transfer protein • LDL oxidation • cholesteryl ester • triglycerides • fatty acids

Homozygotes for cholesteryl ester transfer protein (CETP) deficiency have been shown to markedly elevate plasma HDL cholesterol (HDL-C) and apoA-I levels, as well as to decrease LDL cholesterol (LDL-C) and apoB levels (1, 2). Antisense oligodeoxynucleotides against CETP targeted to the liver have also been shown to increase HDL-C and decreased LDL-C (3, 4). CETP is thus a plasma glycoprotein that catalyzes the cholesteryl ester (CE) and triglycerides (TG) among lipoproteins, so that it affects the composition of all lipoproteins. However, although there have been many studies concerning the relationship between CETP and HDL remodeling (5–10), only a few studies have focused on the relationship between CETP and LDL (11, 12). Even in such studies, it remains unclear whether the LDL affected with CETP is atherogenic or anti-atherogenic. Recently it has been proposed that oxidation of LDL increases its atherogenecity and that susceptibility of LDL to oxidative modification is one of the most important factors in its atherogenicity (13, 14). Epitopes of oxidized LDL have been found in plasma (15, 16) and atherosclerotic lesion (17) of experi-

Abbreviations: apo, apolipoprotein; TG, triglyceride; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; VLDL, very low density lipoprotein; mAb, monoclonal antibody; HPLC, high performance liquid chromatography; DTNB, dithiobis-dinitrobenzoic acid; LCAT, lecithin:cholesterol acyltransferase; PUF, polyunsaturated fatty acids; SAF, saturated fatty acids; MUF, monounsaturated fatty acids.

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mental animals and humans. An increased susceptibility of LDL to oxidation has also been described in patients with coronary heart disease (18, 19). The susceptibility of LDL to oxidative modification is reduced by anti-oxidants such as vitamin E (20, 21) and increased by adding polyunsaturated fatty acids (PUFs) to the diet (22, 23). PUFs in LDL are more easily oxidized in comparison to saturated fatty acids (SAFs) and/or monounsaturated fatty acids (MUFs) (24, 25). Moreover, LDL enrichment in TG are also more susceptible to oxidation in humans (26). As CETP can change the composition of CE and TG, we hypothesized that CETP may also alter the susceptiblity of LDL to oxidation. In the present study, using the monoclonal antibody (mAb) to CETP in incubated plasma, we examined the effect of the inhibition of CETP on LDL oxidation and the uptake of the oxidized LDL, which was made from the LDL under condition of CETP inhibition, by macrophages.

## METHODS

### Subjects

Healthy human subjects (n = 9) who had fasted for 12 h were used as blood donors (**Table 1**). Blood was collected into chilled tubes (4°C) containing 0.1% EDTA and 0.02% $NaN_3$ (final concentration) at pH 7.4. Plasmas were immediately separated at 4°C by centrifugation and collected.

All authors approve and warrant that informed consent was obtained under the study approved by our institutional review board.

### Monoclonal antibody against human CETP

Monoclonal antibody (mAb) against purified human CETP was prepared as described previously (4). Briefly, human CETP was purified from human plasma according to the purification method of human CETP described by Kato et al. (27). Human CETP was emulsified in an equal volume of Freund's complete adjuvant, then injected into the footpads of Balb/c mice. After the initial injection of human CETP solution, subsequent injections were made 4 times a day over each 5-day period. Popliteal lymph node cells of the mice were obtained on the second day after the final injection. These cells were then fused with mouse myeloma cells using PEG 4000. Media conditioned by the resulting hybridomas were screened for CETP inhibition as described previously (3). Two mAbs capable of neutralizing the activity of human CETP were obtained; JHC1 and JHC2. Becase JHC1 was more effective in inhibiting neutral lipid transfer, this mAb was used in the present study. As nonspecific monoclonal IgG was found to have no effect on CETP activity in human plasma (28), nonspecific monoclonal IgG from mice was used as the control.

### Incubation and assay of CE transfer

Plasmas were incubated at 37°C for 6 h in the absence or presence of the mAb. For the measurement of the mass transfer of CE from HDL to apoB-containing lipoproteins, the samples were also incubated in the presence of 1.5 mm dithio-bis-dinitrobenzoic acid (DTNB) to inhibit LCAT activity with or without the mAb. Aliquots of plasma samples were removed before and at 2, 4, and 6 h of incubation and chilled on ice. VLDL + LDL were then precipitated with 0.1 vol of heparin/$MgCl_2$. At each sampling interval, the masses of free and total cholesterol present in the supernatant were measured, and the amount of CE transferred to apoB-containing lipoproteins was calculated from the difference between the two values.

TABLE 1. Profile of blood donors in the present study

| | Male/Female n = 6/n = 3 | |
|---|---|---|
| Variable | Mean ± SEM | Range |
| Age (yr) | 36.9 ± 2.5 | (25–49) |
| Total cholesterol (mg/dl) | 162.1 ± 8.8 | (125.1–198.1) |
| HDL cholesterol (mg/dl) | 54.5 ± 4.2 | (39.4–72.0) |
| Triglycerides (mg/dl) | 137.8 ± 21.5 | (43.0–236.7) |
| Phospholipids (mg/dl) | 193.5 ± 9.5 | (157.5–252.2) |

### Separation of lipoprotein fraction

To examine the effects of CETP activity on lipid distribution in plasma containing active LCAT in the absence or presence of the mAb, lipoproteins ($d < 1.225$ g/ml) were isolated from the plasma incubated without DTNB by ultracentrifugation and fractionated by size using a 1.6 × 50 cm Superose 6B HPLC column, as described previously (29). After collecting each lipoprotein fraction, the lipid compositions in VLDL, LDL, and HDL from the incubated plasma samples were analyzed. Total cholesterol, free cholesterol, triglycerides, and phosphoplipids were analyzed using enzymatic kits from Wako Chemical, Ltd. (Osaka, Japan). Fatty acid compositions in LDL were determined by gas–liquid chromatography (30).

### Measurement of α-tocopherol

The content of α-tocopherol in the LDL was determined by reverse-phase HPLC according to the method of Barua et al. (31) after first extracting α-tocopherol from the LDL according to Barua and Furr (31).

### LDL oxidation

LDL derived from the plasma incubated without DTNB in the absence or presence of the mAb were dialyzed for 24 h against PBS before oxidation, to remove the EDTA. Oxidation of LDL was carried out in a shaking water bath at 37°C under air. LDL (100 μg of protein per milliliter) were incubated at 37°C with freshly prepared $CuSO_4$ (10 μmol/L). LDL oxidation was terminated by refrigeration at 4°C and addition of 0.1 mmol/L EDTA to the $CuSO_4$ system. LDL oxidation was determined by measuring the amount of TBARS (32) over 2 h of incubation and by continuous monitoring of the formation of conjugated dienes by measuring the increase in absorbance at 234 nm (33).

### Cellular uptake of oxidized LDL by J774.1 cells

J774.1 cells were obtained from the RIKEN GENE BANK (Tsukuba, Japan). The cells were grown and maintained in RPMI-1640 containing 5% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), and glutamine (2 mmol/L). When the cultured cells appeared subconfluent, the cells were incubated with RPMI-1640 containing 5% LPDS without FBS in the presence or absence of oxidized LDL (50 μg of protein per ml), which had been oxidized over incubation for 2 h at 37°C with $CuSO_4$ (10 μmol/L), in order to measure cellular uptake of oxidized LDL by J774.1 cells. The oxidized LDL was derived from the LDL fractionated from the plasma incubated without DTNB in the absence or presence of the mAb. After incubation for 24 h, the cells were washed three times with PBS and then lipid-extracted in hexane–isopropanol 3:2 (vol/vol). The hexane–isopropanol extracts were evaporated and analyzed to determine the total and free cholesterol using determiner L TC and determiner L FC (Kyowa Medix, Tokyo, Japan).

### Statistical analysis

A paired *t* test (two-tailed) was used to determine statistical significance. Differences were considered to be statistically significant at a value of $P < 0.05$. All data were expressed as mean ± SEM.

## RESULTS

### Effect of the mAb on mass transfer of CE in plasma

To evaluate the effect of the mAb on net mass transfer of CE in the absence of CE formation, plasma was incubated at 37°C with the LCAT inhibitor, DTNB, in the presence and absence of the mAb. The incubation of plasma caused a CE transfer from HDL to apoB-containing lipoproteins during the 6-h incubation. In contrast, when CETP was inhibited by the addition of the mAb, the CE transfer was almost completely suppressed (**Fig. 1**).

### Effect of the mAb in plasma containing active LCAT

To determine the effects of CETP activity on lipid distribution in plasma containing active LCAT, plasma was incubated without DTNB for 6 h at 4°C (open bar) or at 37°C without (hatched bar) or with (solid bar) the mAb; the symbols above the bar indicate significant differences among the samples (**Fig. 2**). When CETP was active, there were net increases of CE mass in VLDL, LDL, and HDL, reflecting the combined activities of LCAT and CETP (Fig. 2A). When CETP was inhibited and only LCAT was active, CE accumulated only in HDL but not in VLDL and LDL. These results indicate that CETP transferred CE from HDL to VLDL and/or LDL, as in the LCAT inhibited state. There was no significant difference in total CE between the plasma incubated with and without the mAb (Fig. 2A), indicating that CETP had no effect on the plasma cholesteryl esterification rate in the incubated plasma. Transfer of triglycerides from VLDL to LDL and HDL was seen when CETP and LCAT were active, and this transfer was abolished by inhibition of CETP (Fig. 2B). After incubation of plasma at 37°C, the amount of free cholesterol declined in all lipoprotein classes (Fig. 2C). The amount of phospholipids did not change significantly in any lipoprotein classes (Fig. 2D). The protein concentrations also did not change significantly in any lipoprotein classes (data not shown). The percentage of fatty acid composition of LDL did not differ significantly among the unincubated plasma, the plasma incubated with the mAb, and that incubated without the mAb (data not shown). The amount of α-tocopherol in the LDL was not significantly different between the LDL fractionated from the plasma incubated with (6.05 ± 0.97 μg/mg protein) and without mAb (6.19 ± 1.00 μg/mg protein). The size of each lipoprotein was also evaluated by fractionating the lipoproteins by size using a 1.6 × 50 cm Superose 6B HPLC. When CETP and LCAT were active, the peak center of the LDL and HDL was shifted significantly into a lower density region (**Fig. 3, middle**) indicating that the LDL and/or HDL became buoyant particles. The extent of the shift of the LDL peak into a lower density region was abolished by CETP inhibition (Fig. 3, bottom), and the estimated LDL size was not significantly different from that in the unincubated plasma. The retention times of the peak center of the LDL in the unincubated plasma, in the plasma incubated without, and in the plasma incubated with mAb were 20.46 ± 0.11, 20.16 ± 0.11, and 20.48 ± 0.13 min, respectively.

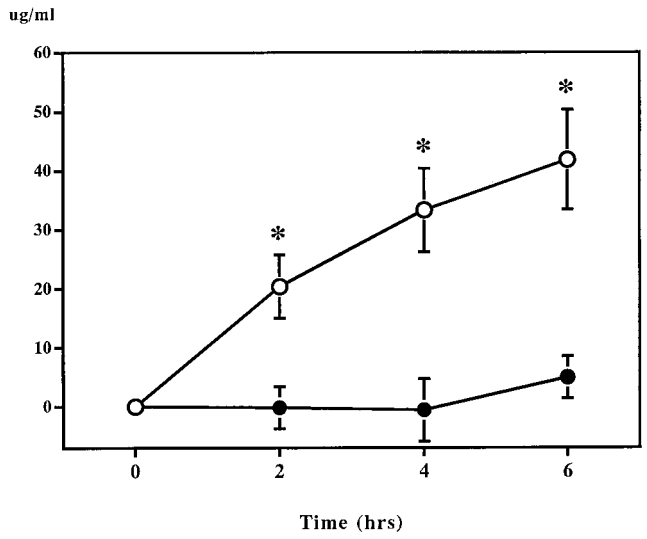


**Fig. 1.** Cholesteryl ester transfer from HDL to apoB-containing lipoproteins in plasma samples containing the LCAT inhibitor DTNB. The plasma samples were incubated in the presence (closed circle) and absence (open circle) of monoclonal antibody, and the amount of cholesteryl ester (mg/ml) transferred from HDL to apoB-containing lipoproteins was calculated. Statistically significant differences between the samples are shown (* $P < 0.01$), using paired *t* test (n = 9 in each experiment).

### LDL oxidative state in the incubated plasma

Determination of the extent of LDL oxidation by measuring the formation of TBARS after LDL incubation for 2 h at 37°C with $CuSO_4$ (10 μmol/L) revealed that the LDL from the plasma with CETP inhibition resulted in a significant reduction in copper ion-induced oxidation compared with the LDL from the plasma without CETP inhibition (**Fig. 4A**). The LDL fractionated from the plasma incubated with mAb also showed a significant delay in the onset of LDL oxidation, as measured by continuous monitoring of the formation of conjugated dienes, compared with the LDL fractionated from the plasma incubated without mAb (Fig. 4B). In addition, the extent of LDL oxidation measured by both methods was not significantly different between the LDL fractionated from the plasma incubated with mAb and the LDL fractionated from the unincubated plasma (Fig. 4 A and B).

### Uptake of CE of oxidized LDL by J774.1 cells

The increase in cholesteryl ester in J774.1 cells by the oxidized LDL that was made from the LDL fractionated from the plasma incubated with mAb was significantly lower than that by the LDL fractionated from the plasma incubated without the mAb and similar to that by the unincubated LDL (**Fig. 5**). The amount of the uptake of cellular CE of the oxidized LDL that was made from the LDL fractionated from the plasma incubated with the mAb and the LDL from the unincubated plasma was about 60% of that of the oxidized LDL that was made from the LDL fractionated from the plasma incubated without the mAb. These results indicate that the uptake of the particles of

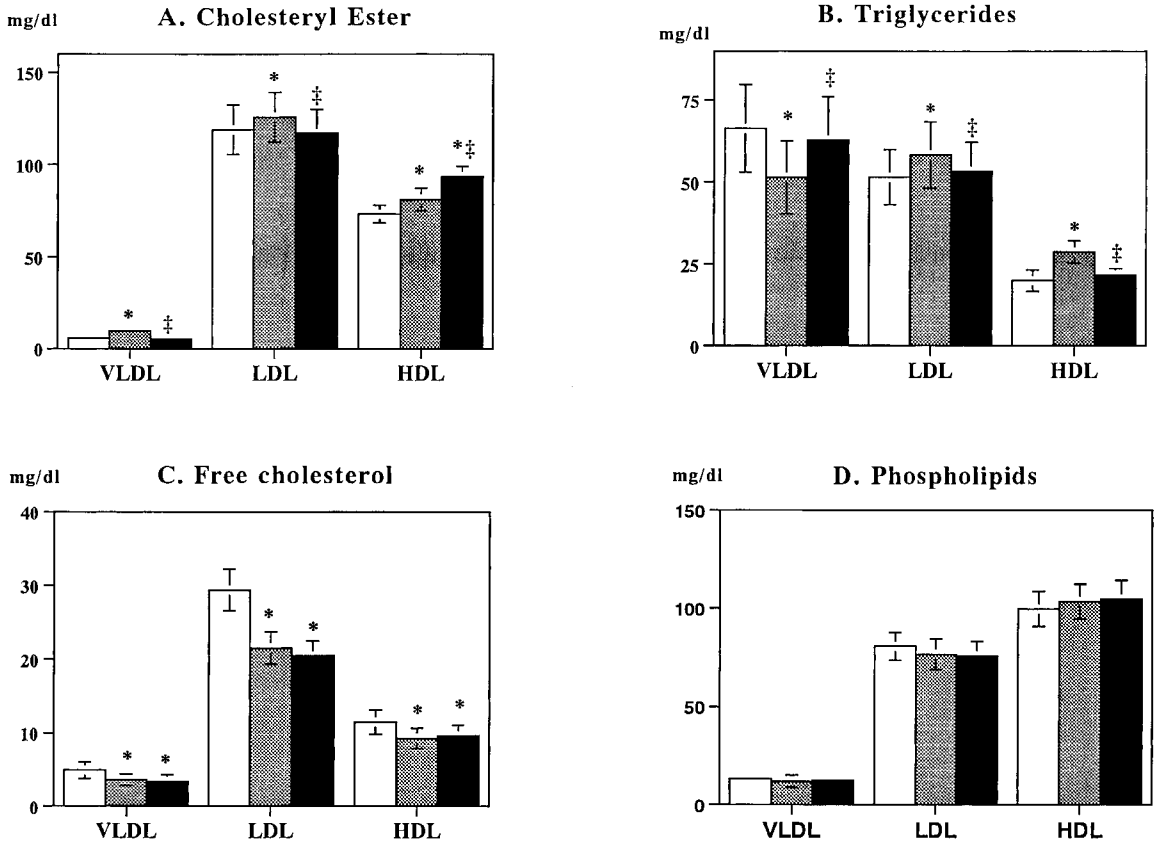


**Fig. 2.** Lipid profile of VLDL, LDL, and HDL from plasma incubated 6 h with and without CETP activity. Plasma samples were incubated for 6 h at 4°C (open bar) and at 37°C without (hatched bar) and with monoclonal antibody (solid bar), while LCAT was active during incubation. Statistically significant differences between samples at 4°C (*) or at 37°C without monoclonal antibody (*) are shown ($P < 0.05$), using paired *t* test (n = 9 in each experiment).

oxidized LDL itself was also reduced when the LDL fractionated from the plasma incubated with the mAb was oxidized. (The percentage of the original CE in LDL fractionated from the plasma incubated with the mAb and the unincubated plasma was about 93% of that in LDL fractionated from the plasma incubated without the mAb as described in Fig. 2A).

## DISCUSSION

The present study investigated whether inhibition of CETP by the mAb in plasma altered LDL susceptiblity to oxidation in the incubated plasma. In the incubated plasma, although the plasma total cholesterol and triglycerides did not change with CETP, only the distribution of these neutral lipids in the lipoproteins did change. Although inhibition of CETP did not change the percentage of the fatty acid composition of LDL, it reduced the amount of CE and TG in LDL without changing phospholipid levels. As a result, the total amount of fatty acids in CE and TG of LDL was also thought to be reduced by the mAb-induced inhibition of CETP in the incubated plasma. We therefore speculated that inhibition of CETP altered LDL such that they were poor in CE, TG, and fatty acids, and thereby altered their resistance to oxidation. In fact, the compositions of CE, TG, and PL of the unincubated LDL and the CETP-inhibited LDL were similar (Fig. 2), and therefore their oxidizability was also similar (Fig. 4). Diets rich in n–6 PUFs (34–37) and n–3 PUFs (23) have been shown to increase the susceptibility of LDL to oxidative modification. LDL rich in TG have also been shown to be more susceptible to oxidation in normolipidemic and dyslipidemic patients (26). Moreover, PUFs in LDL have been shown to be more easily oxidized in comparison to saturated fatty acids and/or monounsaturated fatty acids (24, 25). CE contains one fatty acid (FA) per molecule, PL two FAs per molecule, and TG three FAs per molecule. In the present study, because the total amount of fatty acids in LDL was thought to be reduced, the total PUFs in LDL were also thought to be reduced by CETP inhibition. As the amount of CE and TG in LDL and the total PUFs in LDL were reduced by CETP inhibition, the LDL were thought to be resistant to oxidative modification. In fact, the oxidized LDL that were made from the LDL fractionated from the plasma incubated with the mAb accumulated fewer in macrophages (Fig. 5). However, other mechanisms such as change in physical properties of LDL may also relate to the change of the LDL oxidizability, though the present study could not clarify them. Elevated

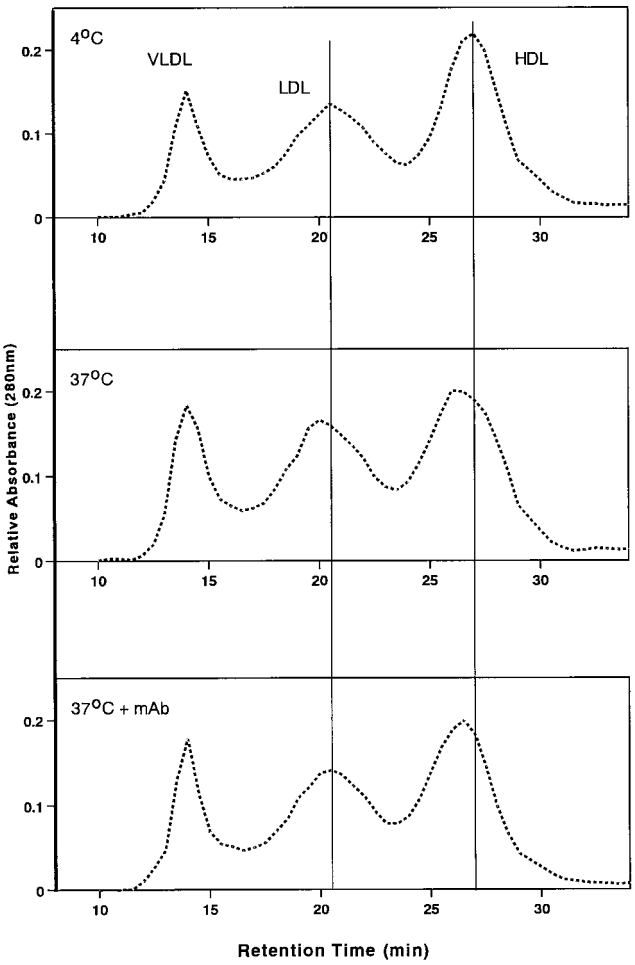


**Fig. 3.** HPLC chromatograms in lipoproteins (d < 1.225). Top, lipoproteins from the unincubated plasma. Middle, lipoproteins from the plasma incubated without mAb. Bottom, lipoproteins from the plasma incubated with mAb.

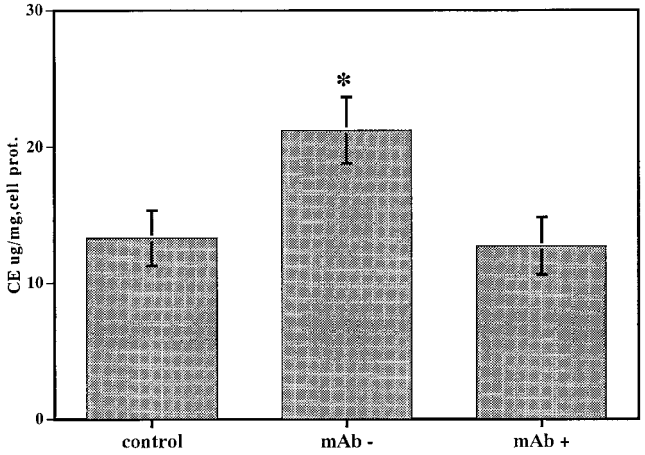


**Fig. 5.** Effect of the inhibition of CETP on the accumulation of CE in J774.1 cells in response to oxidized LDL. Oxidized LDL was made from the LDL derived from the plasma incubated for 6 h at 4°C (control), at 37°C without (mAb −) or with monoclonal antibody (mAb +) by incubating for 2 h at 37°C with freshly prepared $CuSO_4$ (10 μmol/L). Statistically significant differences between the samples are shown (* $P < 0.05$, vs control), using paired *t* test (n = 9 in each experiment).

serum LDL cholesterol levels are known to be an important risk factor in atherogenesis (38, 39). Susceptibility of LDL to oxidative modification is one of the most important factors in its atherogenicity (13, 14). LDL hypercholesterolemia has been associated with accumulation of oxidized LDL in the coronary arteries of miniature pigs (40). Oxidized LDL are rapidly taken up by macrophages via scavenger receptors, a process that transforms them into foam cells, which are essential components of fatty steaks and fibrofatty plaques (41). LDL undergoes oxidative modification when incubated in vitro with endothelial

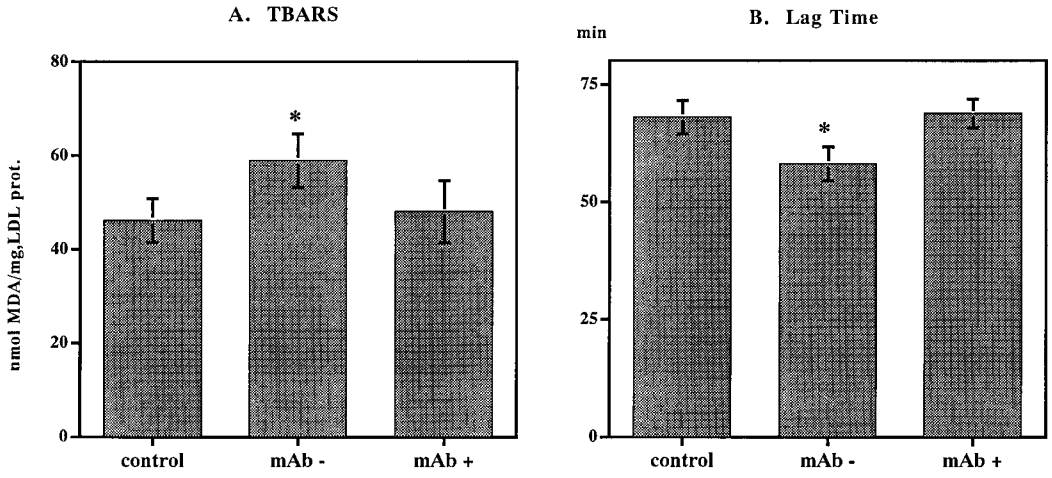


**Fig. 4.** LDL (100 μg of protein per milliliter) derived from the plasma incubated for 6 h at 4°C (control), at 37°C without (mAb−) or with monoclonal antibody (mAb +) was incubated for 2 h at 37°C with freshly prepared $CuSO_4$ (10 μmol/L). LDL oxidation was determined by measuring the amount of TBARS (A) and by measuring the lag phase in the formation of conjugated dienes by continuous monitoring of the increase in absorbance at 234 nm (B). Statistically significant differences between the samples are shown (* $P < 0.05$, vs control), using paired t test (n = 9 in each experiment).

cells, smooth muscle cells or macrophages, or when exposed to $Cu^{2+}$ (13, 14). And, in fact, in the present study, inhibition of CETP in plasma conferred LDL resistance to oxidative modification when LDL were exposed to $Cu^{2+}$. Higher dietary antioxidant levels have also been proposed to be associated with reduced risk of cardiovascular disease, possibly through the protection of LDL from oxidative modification (42, 43). In the present study, however, the levels of the common antioxidant, α-tocopherol, in the LDL were not significantly different between the LDL fractionated from the plasma incubated with and that incubated without mAb. It is known that small dense LDL are more susceptible to oxidation than large buoyant LDL (44). In the present study, although LDL appeared as large buoyant particles when CETP and LCAT were active, the size of LDL under CETP inhibition was not significantly different from that in the unincubated plasma. The susceptibility of LDL to oxidative modification was not thought to be related to the size of LDL in the present study. Antisense oligodeoxynucleotides against CETP targeted to liver have been shown to decrease both total and LDL cholesterol, and to increase level of LDL receptor mRNA in liver (3, 4). In homozygotes for CETP deficiency, the catabolic rate of LDL also increased (45).

In homozygotes for CETP deficiency, LDL cholesterol and plasma apoB levels decreased, while LDL triglycerides increased (1, 2). Inhibition of CETP by TP2 in incubated plasma, however, revealed that LDL had a reduced composition of triglycerides with suppression of transfer of TG from VLDL to LDL though the fatty acid composition of LDL was not examined (11). It is thus speculated that factors other than the original function of CETP, such as the metabolism in the liver, may act for the lipoprotein remodeling in CETP deficiency. If inhibition of CETP by some drugs occurs in circulating plasma and does not affect the metabolism of the lipoproteins in organs such as the liver, LDL might be altered such that they were poor in CE, TG, and fatty acids and resistant to oxidation, as in the present study. Recently, however, it has been proposed that genetic alterations in CETP levels lead to secondary changes in plasma LCAT reaction, possibly due to remodeling of HDL by CETP acting in concert with the in vivo factor (46). Thus, even if a drug that inhibits CETP in circulating plasma is developed, the LDL remodeling may not differ from that seen in the present study. However, the present study has for the first time shown the possibility that CETP inhibition in plasma may alter LDL to become anti-atherogenic. The exact role of CETP in the development of atherosclerosis has been controversial (2,4, 47–53). Zhong et al. (52) reported that the increased risk of coronary heart disease in Japanese-American men with CETP gene mutation was largely present in individuals with HDL cholesterol 40–60 mg/dl, which was in the normal range. In a study by Hirano et al. (53), coronary heart disease (CHD) patients in Omagari, Japan could be classified into two different groups, one including patients without CETP deficiency who had lower HDL cholesterol levels, and the other patients with CETP deficiency who had higher HDL cholesterol levels. Moreover, Kuivenhoven et al. (54) have shown that there is a significant relationship between variation at the CETP gene locus and the progression of coronary atherosclerosis that is independent of plasma HDL cholesterol levels. Thus, although increased levels of HDL cholesterol are an independent negative factor for the development of CHD (55), there are clearly factors other than HDL which help to define the relationship between the development of atherosclerosis and CETP.

The present results indicate that it is important to examine the atherogenicity of LDL when CETP is studied. In conclusion, we have shown that CETP acts not only for the remodeling of HDL, but also for the remodeling of LDL, and that inhibition of CETP in plasma can change the LDL resistance to oxidative modification, which is thought to be anti-atherogenic. In addition, it should be mentioned that whole lipoproteins and/or whole plasma should be considered when examining the relationship between the development of atherosclerosis and CETP.

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