Journal of Cellular Biochemistry 86:258–267 (2002)

Contribution of HDL-Apolipoproteins to the Inhibition of Low Density Lipoprotein Oxidation and Lipid Accumulation in Macrophages

Kae-Yuan Lin,[1] Yuh-Lien Chen,[2] Chun-Che Shih,[3] Ju-Pin Pan,[4] Woan-Eng Chan,[1] and An-Na Chiang[1]*

[1]Institute of Biochemistry, National Yang-Ming University, Taipei 112, Taiwan, Republic of China
[2]Institute of Anatomy and Cell Biology, National Yang-Ming University, Taipei 112, Taiwan, Republic of China
[3]Division of Cardiovascular Surgery, Taipei Veterans General Hospital-Taipei, Taipei 112, Taiwan, Republic of China
[4]Division of Cardiology, Department of Internal Medicine, Veterans General Hospital-Taipei, Taipei 112, Taiwan, Republic of China

Abstract High-density lipoprotein (HDL) is known as a protective factor against atherosclerosis. However, whether HDL-apolipoproteins (apo-HDL) contribute to the protection in arterial cells remains unclear. The localization patterns of human apolipoproteins in atherosclerotic arteries were determined using immunohistochemical examination. The results indicate that several apolipoproteins are retained in component cells of the coronary artery walls. To elucidate the possible roles of apo-HDL in the protection of atherosclerotic lesion formation, we investigated the effects of apo-HDL on the formation of conjugated diene (CD) in a cell-free system and thiobarbituric acid-reactive substances (TBARS) in the medium of a macrophage-mediated LDL oxidation system. The results showed that apo-HDL significantly exerted an inhibitory effect on LDL lipid oxidation in vitro. In addition, apo-HDL decreased cholesterol influx but enhanced cholesterol efflux from J774 macrophages in a dose-dependent manner. These results are consistent with the notion that there is reduced intracellular lipid accumulation in apo-HDL treated macrophages. These data provide a direct evidence for apo-HDL in protecting LDL from oxidative modification and in reducing the accumulation of cholesterol and lipid droplets by J774 macrophages. J. Cell. Biochem. 86: 258–267, 2002.

Key words: atherosclerosis; oxidative modification; cholesterol influx; cholesterol efflux; lipid accumulation

Plasma lipoproteins are composed of lipids (triacylglycerols, cholesterol, and phospholipids) and one or more protein components named apolipoproteins. Clinical interest in lipoproteins arises primarily from their association with coronary heart disease (CHD). Among all the major lipoproteins, high-density lipoprotein (HDL) has been considered as a potentially protective factor against atherosclerosis [Barter and Rye, 1996; Calabresi and Franceschini, 1997]. One of the purposes of the present study was to elucidate the contribution of HDL-apolipoproteins (apo-HDL) to the protective role of HDL in atherosclerosis.

Oxidation of low-density lipoprotein (LDL) has been shown to render the lipoprotein more atherogenic [Steinberg, 1997]. Recent studies have suggested that HDL has the potential to limit oxidative modification of LDL [Mackness and Durrington, 1995; Navab et al., 2000a; Navab et al., 2000b]. However, it has not been determined whether apo-HDL potentiates the oxidizability of LDL. We hypothesize that apo-HDL has the ability to pass into the vessel wall and exerts its physiological function within the cardiovascular environment. In the present study, the localization of apolipoprotein AI

Grant sponsor: VGH-NYMU Joint Research Program; Grant number: VGHYM 86-S4-16; Grant sponsor: Tsou's Foundation; Grant sponsor: National Science Council of Taiwan, ROC (to Dr. Chiang); Grant number: NSC 89-2320-B-010-145-M26. E-mail: anchia@ym.edu.tw

*Correspondence to: An-Na Chiang, Institute of Biochemistry, National Yang-Ming University, Taipei 112, Taiwan, Republic of China. E-mail: anchia@ym.edu.tw

Received 4 February 2002; Accepted 8 April 2002
DOI 10.1002/jcb.10210

(apoAI), apolipoprotein B-100 (apoB-100), apolipoprotein E (apoE), and apolipoprotein J (apoJ) was examined in atherosclerotic coronary arteries by immunohistochemistry. The observation prompted us to study the possible role of apolipoproteins in the pathogenesis of atherosclerosis. In human plasma, apoAI is the major component of HDL [Segrest et al., 2000] and apoB-100 is the key structural protein of very low-density lipoprotein (VLDL) and LDL [Davis, 1999; Segrest et al., 2001]. ApoE is a constituent of all lipoproteins except LDL [Davignon et al., 1999] and apoJ is one of the protein components of HDL [de Silva et al., 1990]. We assessed the effect of apolipoproteins in HDL (apo-HDL), LDL (apo-LDL), and very low-density lipoprotein (apo-VLDL) on oxidative resistance of LDL by assays of conjugated diene (CD) and thiobarbituric acid-reactive substances (TBARS) [Kostner et al., 1997; Tesoriere et al., 1998]. CD is one of the intermediate products formed during the peroxidation of polyunsaturated fatty acids in LDL lipids [Kleinveld et al., 1992]. CD formation can be measured by continuously monitoring the change of optical absorbance at 234 nm. TBARS are measured as an indication of aldehydic breakdown products of oxidized LDL [Gutteridge and Halliwell, 1990]. The extent of TBARS formation can be detected by colorimetry or fluorometry. Both CD and TBARS are important indexes of LDL susceptibility to lipid peroxidation.

Macrophages play a central role in atherosclerosis as they represent the cellular site of cholesterol accumulation in the arterial wall [Stary et al., 1995; Plenz and Robenek, 1998]. The accumulation of cholesteryl ester-rich foam cells results from the uptake of oxidized LDL (ox-LDL). Ox-LDL is taken up by macrophages via the scavenger receptor, which is not regulated by the cellular cholesterol content and can thus lead to atherosclerosis [Quinn et al., 1987; Steinberg, 1997; Yamada et al., 1998]. When macrophage foam cells are exposed to certain acceptors for cholesterol in the acellular environment, cholesterol is removed from cells and intracellular cholesterol is decreased accordingly [Eckardstein, 1996; Kritharides et al., 1998]. Plasma HDL or reconstituted HDL particles have been known as potent cholesterol acceptors [Rothblat et al., 1999]. Apolipoprotein-mediated cholesterol efflux appears to involve specific cellular events distinct from mechanisms involved in efflux of cell cholesterol by the apolipoprotein-depleted acceptors [Mendez, 1997]. If apolipoproteins are present in atheromatous plaques of human coronary arteries, it would be important to investigate the role of apolipoproteins in cholesterol and lipid transport in macrophages. The impact of apo-HDL in a macrophage system was assessed in this study. The results revealed that apo-HDL not only attenuated the oxidation of LDL, but also reversed cholesterol transport and prevented the accumulation of lipids in ox-LDL loaded J774 macrophage cells.

MATERIALS AND METHODS

Immunohistochemistry

Coronary arteries were obtained from 22 patients undergoing surgery for cardiac transplantation or severe atherosclerosis. All patients were given an informed consent to participate in studies approved by the Institutional Review Board at the Veterans General Hospital-Taipei. Nineteen human coronary arteries were collected from patients with severe atherosclerosis and three arterial specimens from donors in the surgery of cardiac transplantation were obtained as control. Immediately after surgery, tissues were rinsed with ice-cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde solution, and paraffin-embedded. Tissues were serially sectioned at 5 μm intervals and the tissue sections were deparaffinized, rehydrated, and washed with PBS. Endogenous peroxidase activity was eliminated by incubation with 3% H_2O_2. Sections were then incubated with PBS containing 5 mg/ml bovine serum albumin (BSA) to block non-specific binding.

For immunohistochemical detection of apolipoproteins, tissue sections were incubated with primary antibodies (anti-apoAI, anti-apoB-100, anti-apoE, and anti-apoJ) in PBS containing 1% BSA, followed by incubation with a horseradish peroxidase-conjugated second antibody. Positive reaction was visualized by using 3,3′-diaminobenzidine (DAB) as a chromogen (Vector). Specific signals recognized by the primary antibody are brown. Sections were counterstained with hematoxylin and visualized by light microscope. As a negative control, the primary antiserum was replaced by normal rabbit immunoglobulins. For the identification of macrophages and smooth muscle cells (SMCs), tissue sections were incubated with mouse monoclonal antibodies

against human macrophage (DAKO, Japan) and SMCα-actin (Sigma), respectively. These two sections were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Sigma) and observed by fluorescence microscopy.

Isolation of Apolipoproteins

VLDL (1.006–1.019 g/ml), LDL (1.019–1.063 g/ml), and HDL (1.063–1.21 g/ml) were isolated from human plasma of fasted normolipidemic volunteers using sequential ultracentrifugation [Chiang et al., 1999] and de-lipidated by cold methanol:ether (3:1). The apolipoproteins were dialyzed against PBS and the content of apolipoproteins was determined by the method of Bradford [Bradford, 1976] with BSA as a standard protein.

Cell Culture

J774 murine macrophages were obtained from the American Type Culture Collection (Rockville). Macrophages were plated in 16-mm-diameter wells at 4×10^5 cells/well in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were fed every 3 days and were used for experiments within 7 days of plating. Before the experiment, macrophages were incubated with lipoprotein-deficient serum (LPDS) for 24 h, followed by removal of the medium and further incubation of the cells with pre-dialyzed LDL and copper sulfate in the absence or presence of apo-HDL at final concentrations ranging from 50 to 1500 μg/ml for 24 h at 37°C.

LDL Oxidation

LDL isolated in the presence of EDTA was dialyzed against PBS overnight and stored at 4°C before experiments. Increasing amounts of apo-VLDL, apo-LDL, or apo-HDL were added to the LDL, and LDL oxidation was undertaken by adding 5 μM $CuSO_4$ in PBS at 37°C. CD formation during copper-catalyzed LDL (50 μg/ml) oxidation in a cell-free system was determined by monitoring the increase in absorbance at 234 nm according to the technique previously described [Lin et al., 2001]. Analyses of CD from each group were performed simultaneously using a programmable multi-cuvette holder.

To examine the effect of apolipoproteins on macrophage-mediated LDL oxidation, cells (J774 macrophages, 4×10^5/16-mm dish) were incubated with LDL (100 μg/ml) in lipoprotein-deficient RPMI-1640 medium with or without apolipoproteins, supplemented with 5 μM $CuSO_4$ for 24 h at 37°C. The extent of LDL oxidation was determined directly in the medium using the TBARS assay [Gutteridge and Halliwell, 1990]. At the end of the incubation period, 1 mM EDTA and 20 μM butylated hydroxytoluene were added to terminate LDL oxidation. The media and cells were immediately separated by centrifugation at 600*g* for 10 min at 4°C. The content of TBARS was measured in the cell-free culture supernatant. Freshly diluted 1,1,3,3-tetramethoxypropane, which yields malondialdehyde (MDA), was used as the standard. Macrophage-mediated oxidation of LDL was calculated by subtraction of the TBARS value in the absence of cells (control) from that obtained in the presence of macrophages.

Changes in LDL charge were evaluated by electrophoretic analysis as previously described [Lin et al., 2001] using 1% agarose gel in Tris-barbital buffer (pH 8.6). LDL samples from cultured media were loaded at 10 μg of protein/well and run at 60 V for 4 h. Bands were visualized using Fat Red 7B. The effect of apo-HDL on LDL modification was examined by measuring the change in the electrophoretic mobility of LDL.

Determination of Cholesterol Influx and Cholesterol Efflux

Labeling of ox-LDL with [^{3}H]cholesteryl linoleate ([^{3}H]CE) was performed by a method described by Mazière et al. [1996]. [^{3}H]CE (25 μCi) was evaporated to dryness under nitrogen and resuspended in dimethylsulfoxide. The [^{3}H]CE solution was progressively mixed with 250 μg of ox-LDL in a total volume of 500 μl, and the mixture was incubated at 37°C for 3 h. The labeled ox-LDL was separated from the free [^{3}H]CE through a Sephadex G-50 column. The specific activity was about 50 dpm/ng protein.

Macrophages containing [^{3}H]cholesterol were prepared by exposing cells to RPMI-1640 supplemented with 10% LPDS, 10 μg protein/ml of labeled ox-LDL, 1.2 mg/ml BSA, and various concentrations of apo-HDL (50–1500 μg/ml). After incubation for 24 h, the medium was removed and cells were rinsed with cold PBS several times. Cells were dissolved in 1 ml of 0.2 N NaOH and the amount of [^{3}H]cholesterol incorporation was determined by counting the radioactivity. Apo-HDL-mediated cholesterol

influx was calculated as the ratio of the radioactivity in cell lysate/the radioactivity in the medium and in cells. Cholesterol efflux from cells after incubation with [^{3}H]CE-labeled ox-LDL was measured after washing the cells in ice-cold PBS three times. Cells were incubated with RPMI-1640 medium in the absence or presence of 50–1500 μg/ml apoHDL at 37°C for 24 h. After the addition of 0.2 N NaOH, the mixture was shaken for 15 min at room temperature to digest cells. Cellular and medium [^{3}H]cholesterol were determined by counting the radioactivity. Apo-HDL-mediated cholesterol efflux was calculated as the ratio of the radioactivity in the medium/the radioactivity in the medium and in cells [Maor and Aviram, 1994].

Intracellular Lipid Accumulation in Macrophages

Macrophages were prepared on glass coverslips contained in 6-well plates. After incubation with RPMI-1640 medium containing 10% LPDS, 1.2 mg/ml BSA, and ox-LDL (50 μg/ml) in the absence or presence of 500 μg/ml apo-HDL for 24 h, the coverslips were washed with cold 1 × PBS for three times. The coverslips were then fixed with 6% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 30 min and stained with oil red *O*-solution (2% w/v) in isopropanol. The stained cells were examined by an inverted microscope.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was performed by the unpaired Student's *t*-test. A value of $P < 0.05$ was regarded as significant.

RESULTS

To investigate the significance of apolipoproteins in the pathogenesis of atherosclerosis, we examined the distribution of apolipoproteins in atherosclerotic arterial walls. The immunostaining patterns of apoAI, B-100, E, and J were different in vascular areas containing atheroma. ApoAI was observed mainly in the intimal area, especially in the shoulder region of atheroma. The positivity of apoAI in the stroma of the media was weak (Fig. 1A). ApoB-100 was also distributed mainly in the intima and the extent of apoB-100 staining was more extensive than that of apoA-I (Fig. 1B). The distribution of apoE was very similar to that of apoAI but the extent of apoE deposits was more extensive in the intimal stroma adjacent to the lesions and at the intima–media boundaries (Fig. 1C). The extent of apoJ was less extensive but more sporadic than that of other apolipoproteins in the coronary artery wall with atheroma (Fig. 1D). In addition, positive immunostaining for human macrophages was observed both in the thickened intima and media using immunofluorescence

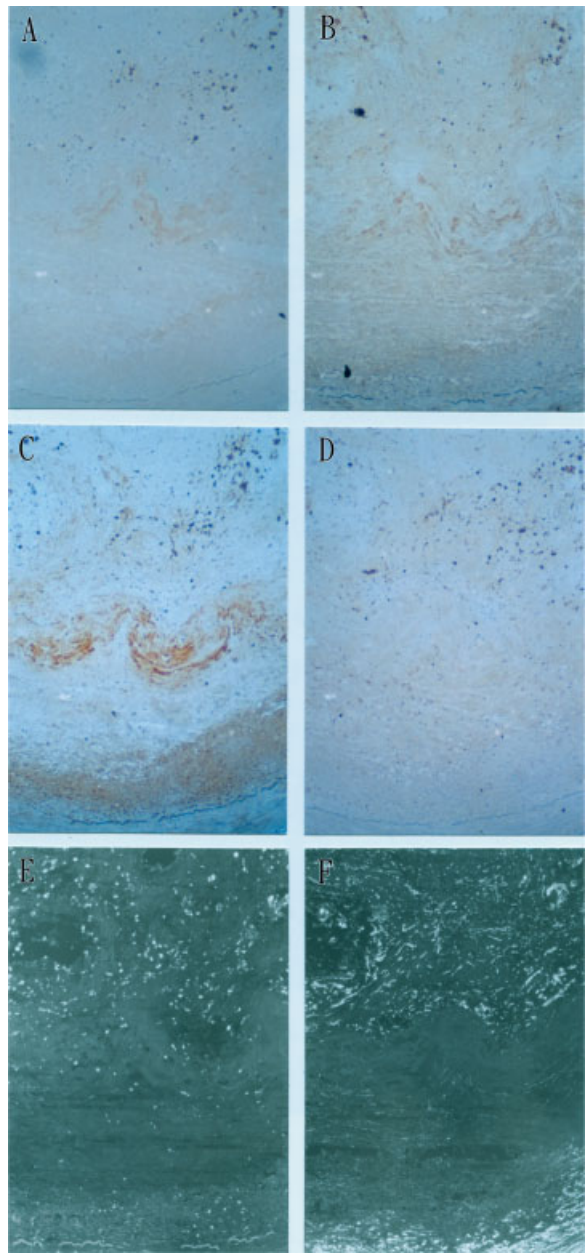

Fig. 1. Immunohistochemical localization of apoAI (**A**), apoB-100 (**B**), apoE (**C**), apoJ (**D**), macrophages (**E**), and smooth muscle cells (**F**) were conducted in serial sections from a human coronary artery with atheroma as described in Materials and Methods. Magnification, × 100.

techniques (Fig. 1E). The marker for SMC α-actin was positively immunoreactive in SMCs in the media of coronary artery with atherosclerotic lesions (Fig. 1F). Results of immunohistochemistry indicate that apolipoproteins, macrophages, and SMC α-actin were widely scattered in the atherosclerotic arterial walls.

Apo-VLDL, apo-LDL, and apo-HDL were isolated from human plasma and were used to determine their effects on the susceptibility of LDL oxidation by photometric determination of CD at 234 nm (Fig. 2). The initial and the maximal amounts of CD formed were not different among groups. However, addition of 50–1000 μg/ml apo-HDL significantly decreased the rate of diene formation but apo-LDL and apo-VLDL had little effect. The lag phase duration of CD formation, which represents the resistance of LDL to lipid peroxidation, was prolonged from 64 to 68, 104, and 136 min, after treatment of 50, 200, and 1000 μg/ml apo-HDL, respectively. The presence of apo-VLDL or apo-LDL at different concentrations (50, 100, and 200 μg/ml) did not exert any significant effect on lag time. Apo-VLDL and apo-LDL were insoluble when concentration of the proteins was over 200 μg/ml.

We next examined the effect of apo-HDL on the lipid peroxidation by the TBARS assay (Fig. 3). Incubation of macrophages with increased concentrations of apo-HDL in the presence

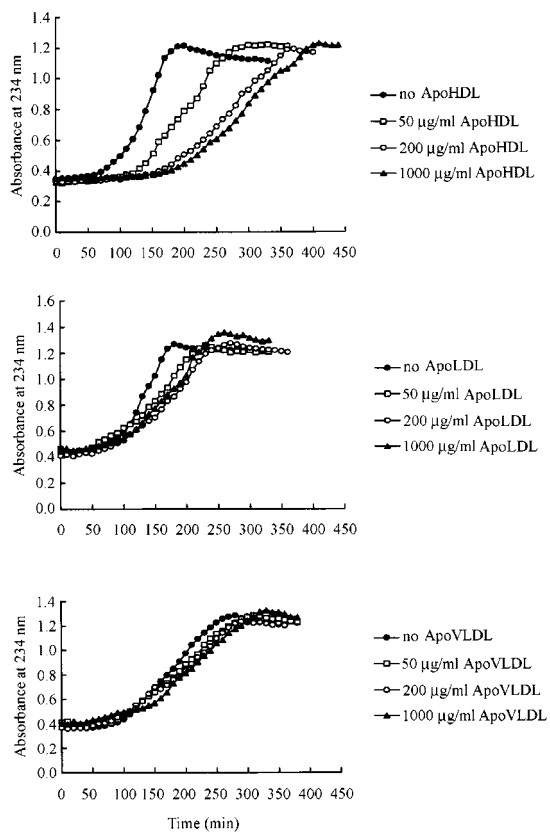

Fig. 2. Effects of HDL-apolipoproteins (apo-HDL), LDL-apolipoproteins (apo-LDL), and VLDL-apolipoproteins (apo-VLDL) on the generation of conjugated dienes (CD) in LDL. The oxidation of LDL (50 μg protein/ml) was initiated by the addition of 5 μM copper. Apo-HDL, apo-LDL, and apo-VLDL were added to LDL to final concentrations as shown. CD generation was monitored by measuring the increase in absorbance at 234 nm. Values shown are from a representative experiment.

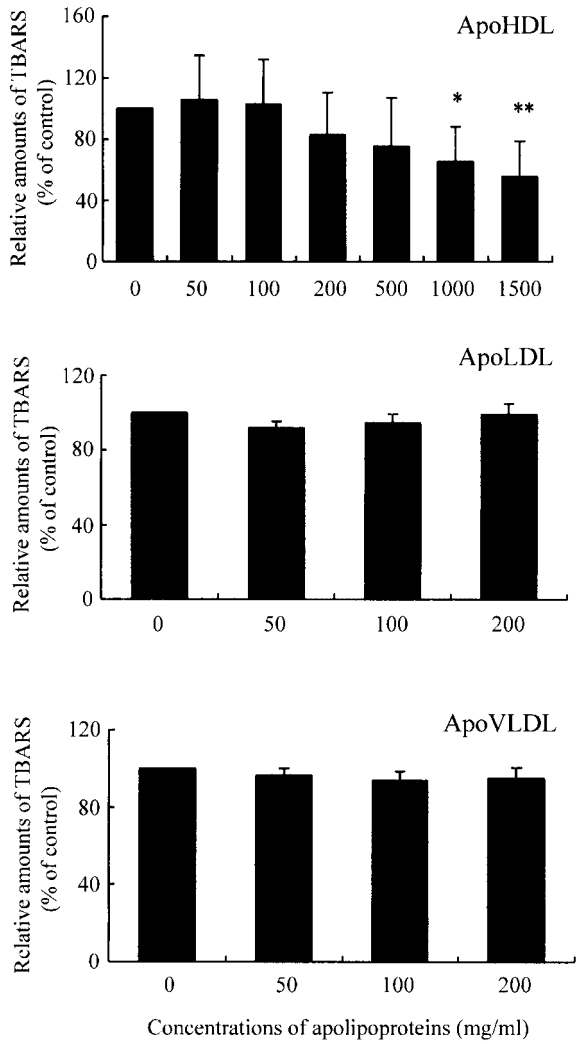

Fig. 3. Effects of apo-HDL, apo-LDL, and apo-VLDL on the formation of thiobarbituric acid-reactive substance in the medium of J774 macrophage culture. LDL (100 μg protein/ml) was incubated with J774 cells in the absence (control) or presence of various concentrations of apolipoproteins. Each bar represents the mean ± SD of twelve determinations from four experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to the result of the control group.

of copper (5 μM) resulted in an up to 56.8 and 70.1% reduction in TBARS formation at apo-HDL concentrations of 1000 and 1500 μg/ml, respectively. The reduction of TBARS was in a concentration-dependent process. No significant change was observed on treatment with apo-LDL or apo-VLDL. An additional experiment was conducted to investigate whether apo-HDL inhibited LDL oxidation induced by 2,2′-azobis-2′-amidino propane hydrochloride (AAPH), a generator of aqueous free radicals. Consistent with the above-mentioned results, apo-HDL potently suppressed the formation of CD and TBARS in LDL exposed to AAPH (data not shown).

In another set of experiments, we explored the effect of apo-HDL on LDL oxidizability by determination of the relative electrophoretic mobility (REM). As shown in Figure 4, incubation of macrophages with LDL (100 μg protein/ml) and 5 μM $CuSO_4$ for 24 h, REM of ox-LDL was significantly enhanced compared to macrophage-mediated oxidized LDL in the absence of copper. By addition of 50–1500 μg/ml apo-HDL, the REM was significantly reduced in a dose-dependent manner, suggesting that the modification of LDL particles was decreased with increasing amount of apo-HDL.

Macrophages were incubated with ox-LDL that had been labeled with [^{3}H]cholesteryl linoleate in the presence or absence of apo-HDL to evaluate the effect of apo-HDL on cholesterol transport. As shown in Figure 5A, the [^{3}H]CE incorporation was significantly decreased in cells treated with apoHDL over 200 μg/ml when compared to apo-HDL-untreated cells. The relationship between apo-HDL-treatment and cholesterol influx was negatively correlated in a dose-dependent manner. When macrophages

Fig. 4. Effects of apo-HDL on changes in electrophoretic mobility of LDL under oxidative stress. LDL (100 μg protein/ml) plus 5 μM copper in the absence or presence of various concentrations of apo-HDL were incubated with J774 macrophage for 24 h at 37°C. Cell cultures containing 10 μg of LDL protein were applied on a 1% agarose gel and stained with Fat red 7B. **Lane 1**, macrophage-mediated oxidized LDL; **lane 2**, macrophage-mediated oxidized LDL in the presence of copper; **lane 3**, with 50 μg/ml apo-HDL during macrophage- and copper-induced LDL oxidation; **lane 4**, with 200 μg/ml apo-HDL during LDL oxidation; **lane 5**, with 500 μg/ml apo-HDL during oxidation; **lane 6**, with 1000 μg/ml apo-HDL during LDL oxidation. The arrow on the left indicates the position of electrophoretic origin.

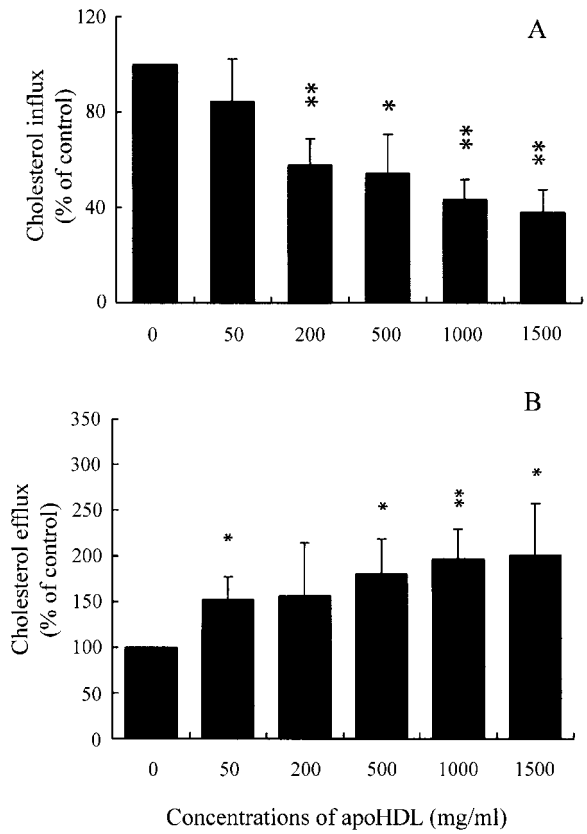

Fig. 5. Effects of apo-HDL on cholesterol influx (**A**) and cholesterol efflux (**B**) from ox-LDL loaded macrophages. After a 24 h starvation, macrophages were treated with BSA and labeled ox-LDL (10 μg/ml LDL protein) at 37°C for another 24 h. The cholesterol influx content was calculated by the ratio of the radioactivity in cell lysate/the radioactivity in the medium and in cells. The cholesterol efflux content was calculated by the ratio of the radioactivity in cell medium/the radioactivity in the medium and in cells. Results are expressed in percentage of the control group with no apo-HDL treatment. Data are the mean ± SD of 12 incubations from six separate experiments. Statistical analyses are: $^*P < 0.05$, $^{**}P < 0.01$, significantly different from control.

were treated with 10 μg/ml [^{3}H]CE-labeled ox-LDL for 24 h and the labeled ox-LDL was removed by washing with PBS for three times, cholesterol efflux could then be evaluated in the culture medium by counting the radioactivity. To investigate whether apo-HDL was able to affect cholesterol efflux, macrophages were incubated in 10% LPDS RPMI-1640 medium for additional 24 h in the absence or presence of various concentrations of apo-HDL. Lipid-free apo-HDL was shown to promote cholesterol efflux in a dose-dependent manner (Fig. 5B). Co-incubation of macrophages with 1500 μg/ml apo-HDL caused a twofold increase in cholesterol efflux compared to control cells.

The influence of apo-HDL on cellular lipid accumulation was examined microscopically after staining of macrophages with oil-red *O* that could detect deposits of neutral lipids. In cells exposed to native LDL, few lipid droplets were present (Fig. 6A). Oil red *O* positive staining droplets were greatly increased when macrophages were cultured in 50 μg/ml ox-LDL for 24 h (Fig. 6B). This accumulation of lipid droplets was remarkably suppressed by incubating cells with 1000 μg/ml apo-HDL (Fig. 6C), suggesting that apo-HDL contributes to the reduction of foam cell formation.

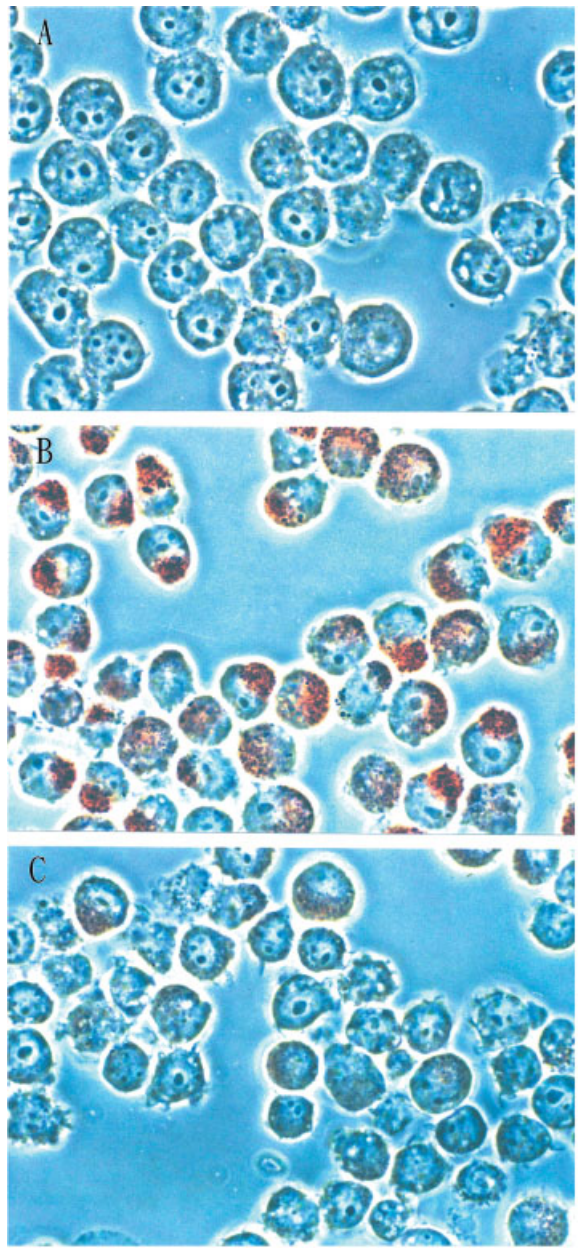

Fig. 6. Inhibition of the accumulation of oil red *O*-staining lipid droplets by apo-HDL in J774 macrophages. Monolayers of macrophages prepared on glass coverslips were cultured with RPMI-1640 medium (**A**) or medium containing 50 μg protein/ml ox-LDL (**B**) in the presence of 1000 μg/ml apo-HDL (**C**). Magnification: A–C, × 1000.

DISCUSSION

Oxidation of LDL is now considered to be of prime importance in initiating the atherogenic process [Ross, 1993; Steinberg, 1997]. Thus, reduction of LDL oxidation is an essential target to prevent atherosclerosis. The resistance of LDL to oxidative modification depends on intrinsic as well as extrinsic factors [Frei and Gaziano, 1993]. HDL has been shown to have the potential to limit oxidative modification of LDL whether induced by transition metals or by tissue cells [Parthasarathy et al., 1990; Mackness et al., 1993a; Watson et al., 1995]. However, it is still unproved whether apo-HDL plays the significant role in retarding the oxidative process [Mackness et al., 1993b]. If apo-HDL could be very helpful in reducing LDL oxidation, it would represent another important mechanism for the prevention of atherosclerosis in vivo.

Lipoproteins normally flux into and out of the arterial wall by crossing the endothelium by what appears to be a non-receptor-mediated pathway [Kaartinen et al., 1995; Hurt-Camejo et al., 1997; Camejo et al., 1998]. Selective retention of lipoproteins probably determines the concentration and perhaps susceptibility to the oxidative modification of LDL in the arterial wall [Ross, 1993; Tertov and Orekhov, 1997]. In the study of immunohistochemistry, immunostaining with antibodies for various apolipoproteins revealed that both LDL-apolipoprotein (apoB-100) and HDL-apolipoproteins (apoAI,

ApoE, and apoJ) were retained in atherosclerotic arteries. Several apolipoproteins have been reported to play a role in the protection of LDL against oxidation [Ohta et al., 1989; Kunitake et al., 1992; Qin et al., 1998]. This raises the possibility that the protein components from HDL residing in the arterial wall may counteract the damaging consequences of LDL oxidation.

In the present study, initiation of LDL oxidation was carried out either in a cell-free system or in a macrophage-mediated LDL oxidation system. Lipid peroxidation of the polyunsaturated fatty acids esterified to the phospholipids or cholesteryl esters proceeds via the formation of CD and results in the formation of a wide variety of aldehydes, ketones, hydroxylipids, and TBARS [Pinchuk et al., 1998]. Aldehydes generated during the oxidative modification of LDL form adducts to the epsilon amino groups of lysine in the apoB moiety of LDL, leading to a net increase in negative charge as shown by electrophoresis [Requena et al., 1996]. Formation of CD and TBARS, and changes in electrophoretic mobility were measured to evaluate the efficiency of resistance to LDL oxidation after ex vivo treatment of apo-HDL.

Paraoxonase, lecithin-cholesterol acyltransferase (LCAT), transferrin, and ceruloplasmin isolated from HDL were also shown to inhibit the oxidation of LDL [Mackness et al., 1993; Atanasiu et al., 1998; Huang et al., 1998; Vohl et al., 1999]. Most of these proteins may be lost during lipoprotein isolation by sequential ultracentrifugation [Kunitake and Kane, 1982]. However, we have found that the proteins retained in HDL after sequential ultracentrifugation still inhibited Cu^{++}-induced oxidation of LDL. It has been shown that HDL is capable of protecting LDL against lipid peroxidation by acting as a reservoir for lipid peroxides and therefore breaking the chain of lipid peroxide propagation in LDL [Parthasarathy et al., 1990; Bowry et al., 1992]. Whether apo-HDL contributes to the metabolism of lipid peroxides may be a promising area for further study. Results of inhibition of AAPH-induced LDL oxidation by apo-HDL (data not shown) indicate that apo-HDL may exert its antioxidant activities through free-radical-scavenging as well as metal ion-chelating.

Another prominent feature of atherosclerosis is cholesterol accumulation, particularly cholesteryl ester, in arterial macrophage foam cells [Stary et al., 1995; Plenz and Robenek, 1998]. It has been reported that HDL might exert its protective effect against CHD by reversing cholesterol transport from cells to extracellular acceptors [Eckardstein, 1996; Rothblat et al., 1999]. Our observation indicates that apo-HDL can reduce the influx of cholesterol by macrophages. It is possible that apo-HDL may interfere with the endocytosis of ox-LDL by macrophages through changing the affinity between ox-LDL protein moiety and scavenger receptors [Sakai et al., 1996]. In addition, the increase of apoHDL-mediated cholesterol efflux implies that apo-HDL may facilitate the translocation of cholesterol from intracellular pools to the plasma membrane, and subsequently to the extracellular space, probably via signal transduction processes [Deeg et al., 1997; Sakr et al., 1999]. Recent studies have demonstrated that an ATP-binding cassette (ABC) transporter called ABCA1 mediates the transfer of cellular cholesterol and phospholipids to lipid-poor apolipoproteins [Borst et al., 2000; Wang et al., 2000; Remaley et al., 2001; Santamarina-Fojo et al., 2001]. This protein therefore represents the first and rate-controlling step in the reverse cholesterol transport pathway. In situ hybridization studies showed that ABCA1 is highly expressed in macrophages [Lawn et al., 2001]. We assume that lipid-poor apo-HDL could participate in the removal of excess cholesterol from macrophage-derived foam cells through ABCA1, although cholesterol efflux from macrophages may be more than a simple mechanism. Since cholesterol efflux by macrophages is essential to inhibit progression of atherosclerosis, removal of cholesterol from macrophages by apo-HDL is therefore important to interfere with the atherogenesis pathway.

Macrophage foam cells are the predominant lipid-accumulating cell type in atherosclerotic plaques [Quinn et al., 1987; Plenz and Robenek, 1998; Glass and Witztum, 2001]. The lipid droplets in macrophage foam cells are exclusively triglycerides. To assess the effect of apo-HDL on lipid accumulation in macrophages, we examined the development of macrophage foam cell phenotype by oil red *O*-staining. The present study clearly indicates a potent inhibitory effect of apoHDL on the development of macrophage foam cells via decreasing lipid accumulation.

Overall, the present study suggests a protective role of apo-HDL in atherosclerosis. The protective action is associated with the inhibition of

LDL oxidation as well as the reverse effect of cholesterol transport.

ACKNOWLEDGMENTS

This research was supported by Fund of VGH-NYMU Joint Research Program (VGHYM 86-S4-16), Tsou's Foundation and a grant NSC 89-2320-B-010-145-M26 (to Dr. Chiang) from the National Science Council of Taiwan, ROC.

REFERENCES

Atanasiu RL, Stea D, Mateescu MA, Vergely C, Dalloz F, Briot F, Maupoil V, Nadeau R, Rochette L. 1998. Direct evidence of ceruloplasmin antioxidant properties. Mol Cell Biochem 189:127–135.

Barter PJ, Rye KA. 1996. High density lipoproteins and coronary heart disease. Atherosclerosis 121:1–12.

Borst P, Zelcer N, van Helvoort A. 2000. ABC transporters in lipid transport. Biochim Biophys Acta 1486:128–144.

Bowry VW, Stanley KK, Stocker R. 1992. High density lipoprotein is the major carrier of lipid hydroperoxides in human blood plasma from fasting donors. Proc Natl Acad Sci USA 89:10316–10320.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quanties of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Calabresi L, Franceschini G. 1997. High density lipoprotein and coronary heart disease: Insights from mutations leading to low high density lipoprotein. Curr Opin Lipidol 8:219–224.

Camejo G, Hurt-Camejo E, Wiklund O, Bondjers G. 1998. Association of apoB lipoproteins with arterial proteoglycans: Pathological significance and molecular basis. Atherosclerosis 139:205–222.

Chiang AN, Chang CP, Chou YC, Huang KY, Hu HH. 1999. Differential distribution of apolipoprotein E in young and aged spontaneously hypertensive and stroke-prone rats. J Hypertens 17:793–800.

Davignon J, Cohn JS, Mabile L, Bernier L. 1999. Apolipoprotein E and atherosclerosis: insight from animal and human studies. Clin Clim Acta 286:115–143.

Davis RA. 1999. Cell and molecular biology of the assembly and secretion of apolipoprotein B-containing lipoproteins by the liver. Biochim Biophys Acta 1440:1–31.

de Silva HV, Harmony LAK, Stuart WD, Gil CM, Robbins J. 1990. Apolipoprotein J: Structure and tissue distribution. Biochemistry 29:5380–5389.

Deeg MA, Bowen RF, Oram JF, Bierman EL. 1997. High density lipoproteins stimulate mitogen-activated protein kinases in human skin fibroblasts. Arterioscler Thromb Vasc Biol 17:1667–1674.

Eckardstein A. 1996. Cholesterol efflux from macrophages and other cells. Curr Opin Lipidol 7:308–319.

Frei B, Gaziano JM. 1993. Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation. J Lipid Res 34: 2135–2145.

Glass CK, Witztum JL. 2001. Atherosclerosis: The road ahead. Cell 104:503–516.

Gutteridge JMC, Halliwell B. 1990. The measurement and mechanism of lipid peroxidation in biological systems. Trends Biochem Sci 15:129–135.

Huang JM, Huang ZX, Zhu W. 1998. Mechanism of high-density lipoprotein subfractions inhibiting copper-catalyzed oxidation of low-density lipoprotein. Clin Biochem 31:537–543.

Hurt-Camejo E, Olsson U, Wiklund O, Bondjers G, Camejo G. 1997. Cellular consequences of the association of apoB lipoproteins with proteoglycans. Potential contribution to atherogenesis. Arterioscler Thromb Vasc Biol 17: 1011–1017.

Kaartinen M, Penttilä A, Kovanen PT. 1995. Extracellular mast cell granules carry apolipoprotein B-100-containing lipoproteins into phagocytes in human arterial intima. Functional coupling of exocytosis and phagocytosis in neighboring cells. Arterioscler Thromb Vasc Biol 15: 2047–2054.

Kleinveld HA, Hak-Lemmers HLM, Stalenhoef AFH, Demacker PNM. 1992. Improved measurement of low-density lipoprotein susceptibility to copper-induced oxidation: Application of a short procedure for isolating low-density lipoprotein. Clin Chem 38:2066–2072.

Kostner K, Hornykewycz S, Yang P, Neunteufl T, Glogar D, Weidinger F, Maurer G, Huber K. 1997. Is oxidative stress causally linked to unstable angina pectoris? A study in 100 CAD patients and matched controls. Cardiovasc Res 36:330–336.

Kritharides L, Christian A, Stoudt G, Morel D, Rothblat GH. 1998. Cholesterol metabolism and efflux in human THP-1 macrophages. Arterioscler Thromb Vasc Biol 18: 1589–1599.

Kunitake ST, Kane JP. 1982. Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. J Lipid Res 23:936–940.

Kunitake ST, Jarvis MR, Hamilton RL, Kane JP. 1992. Binding of transition metals by apolipoprotein A-I-containing plasma lipoproteins: Inhibition of oxidation of low density lipoproteins. Proc Natl Acad Sci USA 89: 6993–6997.

Lawn R, Wade DP, Couse TL, Wilcox JN. 2001. Localization of human ATP-binding cassette transporter 1 (ABC1) in normal and atherosclerotic tissues. Arterioscler Thromb Vasc Biol 21:378–385.

Lin KY, Pan JP, Yang DL, Huang KT, Chang MS, Ding PYA, Chiang AN. 2001. Evidence for inhibition of low density lipoprotein oxidation and cholesterol accumulation by apolipoprotein H (β2-glycoprotein I). Life Sci 69: 707–719.

Mackness MI, Durrington PN. 1995. HDL, its enzymes and its potential to influence lipid peroxidation. Atherosclerosis 115:243–253.

Mackness MI, Arrol S, Abbott C, Durrington PN. 1993a. Protection of low density lipoprotein against oxidative modification by high density lipoprotein associated paraoxonase. Atherosclerosis 104:129–135.

Mackness MI, Abbott C, Arrol S, Durrington PN. 1993b. The role of high-density lipoprotein and lipid-soluble antioxidant vitamins in inhibiting low-density lipoprotein oxidation. Biochem J 294:829–834.

Maor I, Aviram M. 1994. Oxidized low density lipoprotein leads to macrophage accumulation of unesterified cholesterol as a result of lysosomal trapping of the lipoprotein hydrolyzed cholesteryl ester. J Lipid Res 35:803–819.

Mazière C, Barbu V, Auclair M, Mazière J. 1996. Interleukin I stimulates cholesterol esterification and cholesterol deposition in J774 monocytes-macrophages. Biochim Biophy Acta 1300:30–34.

Mendez AJ. 1997. Cholesterol efflux mediated by apolipoproteins is an active cellular process distinct from efflux mediated by passive diffusion. J lipid Res 38:1807–1821.

Navab M, Hama SY, Cooke J, Anantharamaiah GM, Chaddha M, Jin L, Subbanagounder G, Faull KF, Reddy ST, Miller NE, Fogelman AM. 2000a. Normal HDL inhibits three steps in the formation of mildly oxidized LDL—step 1. J Lipid Res 41:1481–1494.

Navab M, Hama SY, Anantharamaiah GM, Hassan K, Hough GP, Watson AD, Reddy ST, Sevanian A, Fonarow GC, Fogelman AM. 2000b. Normal HDL inhibits three steps in the formation of mildly oxidized LDL—step 2 and 3. J Lipid Res 41:1495–1508.

Ohta T, Takata K, Horiuchi S, Morino Y, Matsuda I. 1989. Protective effect of lipoproteins containing apoprotein A-I on Cu^{2+}-catalyzed oxidation of human low density lipoprotein. FEBS Lett 257:435–438.

Parthasarathy S, Barnett J, Fong LG. 1990. High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein. Biochim Biophys Acta 1044:275–283.

Pinchuk I, Schnitzer E, Lichtenberg D. 1998. Kinetic analysis of copper-induced peroxidation of LDL. Biochim Biophys Acta 1389:155–172.

Plenz G, Robenek H. 1998. Monocytes/macrophages in atherosclerosis. Eur Cytokine Netw 9:701–703.

Qin X, Swertfeger DK, Zheng S, Hui DY, Tso P. 1998. Apolipoprotein AIV: A potent endogenous inhibitor of lipid oxidation. Am J Physiol 274:H1836–H1840.

Quinn MT, Parthasarathy S, Fong LG, Steinberg D. 1987. Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. Proc Natl Acad Sci USA 84:2995–2998.

Remaley AT, Stonik JA, Demosky SJ, Neufeld EB, Bocharov AV, Vishnyakova TG, Eggerman TL, Patterson AP, Duverger NJ, Santamarina S, Brewer HB, Jr. 2001. Apolipoprotein specificity for lipid efflux by the human ABCA1 transporter. Biochem Biophys Res Commun 280: 818–823.

Requena JR, Fu MX, Ahmed MU, Jenkins AJ, Lyons TJ, Thorpe SR. 1996. Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions. Nephrol Dial Transpl 11(Suppl 5):48–53.

Ross R. 1993. The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature 362:801–809.

Rothblat GH, de la Llera-Moya M, Atger V, Kellner-Weibel G, Williams DL, Phillips MC. 1999. Cell cholesterol efflux: Integration of old and new observations provides new insights. J Lipid Res 40:781–796.

Sakai M, Miyazaki M, Hakamata H, Suginohara Y, Sakamoto YI, Morikawa W, Kobori S, Shichiri M, Horiuchi S. 1996. Reconstituted high density lipoprotein reduces the capacity of oxidatively modified low density lipoprotein to accumulate cholesteryl esters in mouse peritoneal macrophages. Atherosclerosis 119:191–202.

Sakr SW, Stoudt GW, Williams D, Phillips MC, Rothblat GH. 1999. Induction of cellular cholesterol efflux to lipid-free apolipoprotein AI by cAMP. Biochim Biophys Acta 1438:85–98.

Santamarina-Fojo S, Remaley AT, Neufeld EB, Bryan Brewe H, Jr. 2001. Regulation and intracellular trafficking of the ABCA1 transporter. J Lipid Res 42:1339–1345.

Segrest JP, Harvey SC, Zannis V. 2000. Detailed molecular model of apolipoprotein A-I on the surface of high-density lipoproteins and its functional implications. Trends Cardiovasc Med 10:246–252.

Segrest JP, Jones MK, De Loof H, Dashti N. 2001. Structure of apolipoprotein B-100 in low density lipoprotein. J Lipid Res 42:1346–1367.

Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. 1995. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Arterioscler Thromb Vasc Biol 15: 1512–1531.

Steinberg D. 1997. Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 272:20963–20966.

Tertov VV, Orekhov AN. 1997. Metabolism of native and naturally occurring multiple modified low density lipoprotein in smooth muscle cells of human aortic intima. Exp Mol Pathol 64:127–134.

Tesoriere L, D'Arpa D, Maggio A, Giaccone V, Pedone E, Livrea MA. 1998. Oxidation resistance of LDL is correlated with vitamin E status in β-thalassemia intermedia. Atherosclerosis 137:429–435.

Vohl MC, Neville TA, Kumarathasan R, Braschi S, Sparks DL. 1999. A novel lecithin-cholesterol acyltransferase antioxidant activity prevents the formation of oxidized lipids during lipoprotein oxidation. Biochemistry 38: 5976–5981.

Wang N, Silver DL, Costet P, Tall AR. 2000. Specific binding of apoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. J Biol Chem 275:33053–33058.

Watson AD, Navab M, Hama SY, Sevanian A, Prescott SM, Stafforini DM, McIntyre TM, La Du BN, Fogelman AM, Berliner JA. 1995. Effect of platelet-activating factor acetylhydrolase on formation and action of minimally oxidized LDL. J Clin Invest 95:774–782.

Yamada Y, Doi T, Hamakubo T, Kodama T. 1998. Scavenger receptor family proteins: Roles for atherosclerosis, host defense and disorders of the central nervous system. Cell Mol Life Sci 54:628–640.