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Cross-linking of apolipoproteins is involved in a loss of the ligand activity of high density lipoprotein upon Cu^{2+}-mediated oxidation

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A recent study demonstrated that Cu^{2+}-mediated oxidation of high density lipoprotein (HDL) resulted in a loss of the capacity to reduce cholesterol from macrophage foam cells [(1991) Proc. Natl. Acad. Sci. USA 88, 6457–6461] In the present study we characterized the physicochemical properties of oxidized HDL and correlated them with the ligand activity toward the HDL receptor. Among them, the cross-linking of apolipoproteins and an increase in lipid peroxides were characteristic and closely similar to those of tetranitromethane-treated HDL, an abortive ligand for the HDL receptor. Cellular experiments with murine peritoneal macrophages revealed that both the cellular binding activity of HDL and its capacity to enhance cholesterol efflux from macrophage foam cells were markedly reduced upon oxidation These results suggest that cross-linking of HDL apolipoproteins is involved in the loss of the ligand activity of oxidized HDL.

High density lipoprotein; Apolipoprotein; Chemical modification; Cholesterol efflux; Oxidation

1. INTRODUCTION

The early lesions of atherosclerotic plaques are characterized by macrophage-derived foam cells [1]. Recent immunohistochemical studies demonstrated the presence of chemically modified low density lipoprotein (LDL) in atherosclerotic plaques in vivo [2], and oxidized LDL isolated in situ from human lesions could induce cholesteryl ester (CE)-accumulation in human monocyte macrophages in vitro [3], suggesting that an oxidative mechanism may be operative in the development of atherosclerosis [4]. In contrast to oxidized LDL, no evidence is available for the existence of oxidatively modified high density lipoprotein (HDL) in vivo. However, it was recently reported that Cu^{2+}-mediated oxidation of HDL reduced its capacity to enhance cholesterol efflux from macrophage foam cells [5]. Although studies are needed to demonstrate oxidized HDL in vivo, it would be interesting to know how oxidative modification of HDL could affect the capacity of cholesterol efflux.

Although the HDL receptor is being characterized [6–8], the most crucial issue is whether the receptor, or the specific ligand binding is functionally correlated with the cholesterol efflux phenomenum [9–11]. To answer this, chemically modified HDLs with no or a reduced ligand activity for the HDL receptor would be desirable. Among several modifications tested, treatment with tetranitromethane (TNM) [12,13] and chemical cross-linkers [14,15] successfully led to a loss of the ligand activity. Our previous experiments using rat macrophage foam cells showed that the capacity of HDL to reduce cellular CE was markedly weakened upon treatment with TNM or a chemical cross-linker, dithiobissuccinimidyl propionate, suggesting a functional link of the specific binding to the HDL-mediated cholesterol efflux [11].

During the course of our study on this issue we encountered the above mentioned report that the cholesterol efflux capacity of HDL was significantly reduced upon Cu^{2+}-mediated oxidation [5]. Since previous studies indicate that the cross-linking of HDL apolipoproteins is important in the loss of its ligand activity [12–15], we reasoned that similar cross-linking of apolipoproteins might also be involved in Cu^{2+}-mediated oxidation of HDL. The present study was undertaken to characterize the physicochemical features of oxidized HDL and correlate them with the ligand activity of HDL. The results support our contention.

2. MATERIALS AND METHODS

2.1. *Lipoprotein modification*

Human LDL (d = 1.019–1.063) and HDL (d = 1.063–1.21) were isolated by sequential ultracentrifugation and traces of apolipoprotein B and E were removed from HDL by a heparin–agarose column [16] After extensive dialysis against PBS to remove EDTA, HDL (0.1 mg/ml) was incubated for 24 h at 37°C with 5 μM $CuSO_4$. The reaction was terminated both by 1 mM EDTA and cooling, followed by dialysis

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Abbreviations: HDL, high density lipoprotein; TNM, tetranitromethane; TNM-HDL, TNM-treated HDL; LDL, low density lipoprotein; acetyl-LDL, acetylated LDL; TBARS, thiobarbituric acid reactive substances; CE, cholesteryl esters.

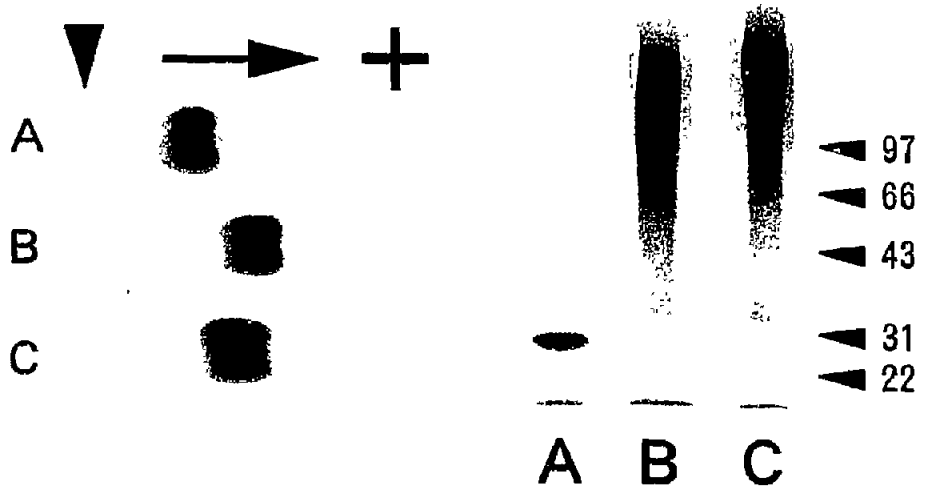

Fig. 1. Agarose (left) and SDS-polyacrylamide gel (right) electrophoresis of oxidized HDL. (Left panel) HDL (A), oxidized HDL (B) and TNM-HDL (C) (5 μg) were electrophoresed on an agarose gel The arrowhead shows the origin and the arrow shows the direction. (Right panel) HDL (A), oxidized HDL (B) and TNM-HDL (C) (10 μg) were electrophoresed on a gradient (4–20%) SDS-polyacrylamide gel. Molecular mass (arrowheads) is indicated in kDa.

against 0.15 M NaCl and 1 mM EDTA (pH 7.4) [17]. To prepare TNM-HDL. 1.5 mg/ml of HDL was treated with 3 mM TNM [13]. Acetyl-LDL was prepared as described [16] Thiobarbituric acid reactive substances (TBARS) levels of modified HDLs were measured [17]. Iodination of HDL by ^{125}I was performed as described [15].

2.2. *Cellular assays*

Peritoneal macrophages were collected from non-stimulated female DDY mice (25–30 g) and suspended in Dulbecco's modified Eagle's medium containing 3% bovine serum albumin, streptomycin (0 1 mg/ml) and penicillin (100 U/ml) (buffer A) [15]. For uptake studies, cells (1×10^6) in suspension were incubated at 37°C for 90 min in 0.1 ml of buffer A with 5 μg/ml of [^{125}I]HDL in the absence or presence of the unlabeled HDL or modified HDLs to be tested. The cells were washed and the cell-associated radioactivity was determined [15]. The cholesterol efflux from macrophages was assayed as described previously [11]. Peritoneal cells (3×10^6) were dispersed to each culture dish and macrophage monolayers thus obtained were used for the study. Cells were first converted to foam cells by incubating for 12 h with 50 μg/ml acetyl-LDL. After a 6-h equilibration with buffer A, cells were further incubated for 24 h with 250 μg/ml of HDL, oxidized HDL or TNM-HDL, and cellular lipids were extracted as described [11,15]. The lipid extracts were determined both for CE and total cholesterol mass levels by a modification [11] of the enzymic fluorometric method of Heider and Boyett [18]. The cells after lipid extraction were dissolved in 0.1 M NaOH to determine cell proteins [11].

3. RESULTS

The mobility of oxidized HDL significantly increased upon agarose gel electrophoresis (Fig. 1, left). A parallel run of TNM-HDL also showed an almost similar increase in electrophoretic mobility, suggesting that an increase in net negative charge is a common feature among oxidized HDL and TNM-HDL. Upon SDS-PAGE, unmodified HDL exhibited a major apolipoprotein A-I band, with minor apolipoprotein A-II and apolipoprotein C bands (Fig. 1, right). When HDL was treated with Cu^{2+} or TNM, the apolipoprotein A-I bands disappeared but broad bands of high molecular weight emerged, suggesting that intermolecular cross-linking occurred to HDL apolipoproteins (Fig. 1, right). TBARS levels of oxidized HDL and TNM-HDL similarly increased 4.5-fold and 6.4-fold higher than that of unmodified HDL, respectively (Fig. 2), suggesting that free radical intermediates may play an important role in the modification of HDL with TNM and Cu^{2+} as well. Thus, these results taken together indicate that TNM-HDL and oxidized HDL share common physicochemical properties.

The ligand activity of oxidized HDL was determined by its effect on the cell association of [^{125}I]HDL to peritoneal macrophages. Excess unlabeled HDL competitively inhibited the association of [^{125}I]HDL to these

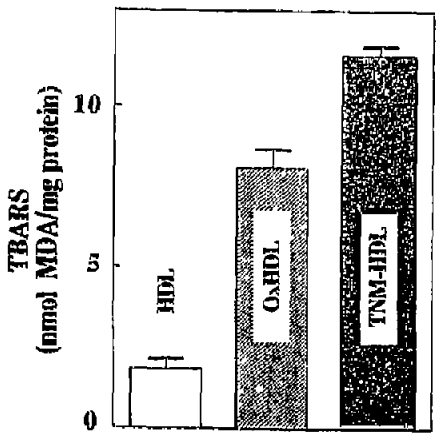

Fig. 2. TBARS level of oxidized HDL and TNM-HDL. The amounts of lipid peroxides were determined with 0.5 mg of each lipoprotein. Data represent the mean value ± S.D. ($n = 3$).

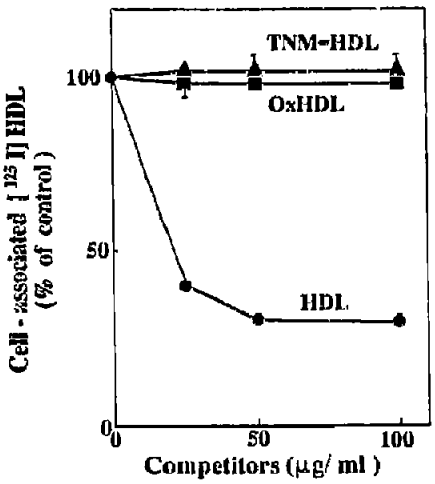

Fig. 3. Effect of oxidized HDL on cellular association of [^{125}I]HDL. Mouse macrophages (1×10^6 cells) were incubated at 37°C for 90 min with 5.0 μg/ml of [^{125}I]HDL with or without the indicated concentrations of unlabeled HDL, oxidized HDL or TNM-HDL, and the cell-associated radioactivity was determined. The 100% value was 10.9 nmol/10^6 cells. The deviation was less than 5%.

cells (>70%), whereas TNM-HDL had no effect on it, suggesting that the binding capacity of HDL might be abolished by treatment with TNM (Fig. 3). Oxidized HDL also did not exhibit an inhibitory effect on the cell association of [^{125}I]HDL. Thus, it is evident that Cu^{2+}-mediated oxidation of HDL leads to reduction of the ligand activity for the HDL receptor, as was observed with TNM-HDL [12,13].

Fig. 4 shows the effect of oxidized HDL on macrophage foam cells. Cells were first converted to foam cells with acetyl-LDL and then incubated with oxidized HDL. The CE level of foam cells was reduced by 47% by unmodified HDL, whereas the reducing effects of oxidized HDL and TNM-HDL were less than 5% (Fig. 4A). On total cholesterol mass (free cholesterol plus CE), HDL also showed a significant reducing effect (by 42%), whereas both oxidized HDL and TNM-HDL had much weaker effects (by less than 10%), indicating that the capacity of HDL to enhance net cholesterol efflux from macrophage foam cells was markedly diminished when modified with Cu^{2+} or TNM. This result is consistent with previous reports on oxidized HDL [5] and TNM-HDL [11]. When non-loaded macrophages were incubated with 250 μg/ml oxidized HDL or TNM-HDL, an increase in cellular CE levels was less than 10 nmol/mg cell protein (data not shown), indicating that these modified HDLs per se were unable to induce CE accumulation. Since the ligand activity for the HDL receptor was abolished both with oxidized HDL and TNM-HDL (Fig. 3), it was suggested that the specific binding of HDL might be functionally correlated with the capacity of cholesterol efflux from macrophage foam cells.

4. DISCUSSION

The present study demonstrated that the significant loss of the ligand activity of HDL towards the HDL receptor upon Cu^{2+}-mediated oxidation is due to cross-linking of HDL apolipoproteins, particularly of apoA-I. This notion was supported by previous reports that cross-linking of apolipoproteins observed with oxidized HDL was extremely similar to TNM-HDL, an abortive ligand for the HDL receptor [12,13]. The capacity of HDL to promote cholesterol efflux from macrophage foam cells was significantly reduced upon oxidation, a similar behavior to HDL when modified with TNM (Fig. 4). Thus, the finding that both oxidized HDL and TNM-HDL share these physicochemical and biological properties is taken as strong evidence for the presence of a functional link between the HDL receptor and cholesterol efflux from macrophage foam cells.

Nagano et al. observed that a 24-h incubation of 2.5

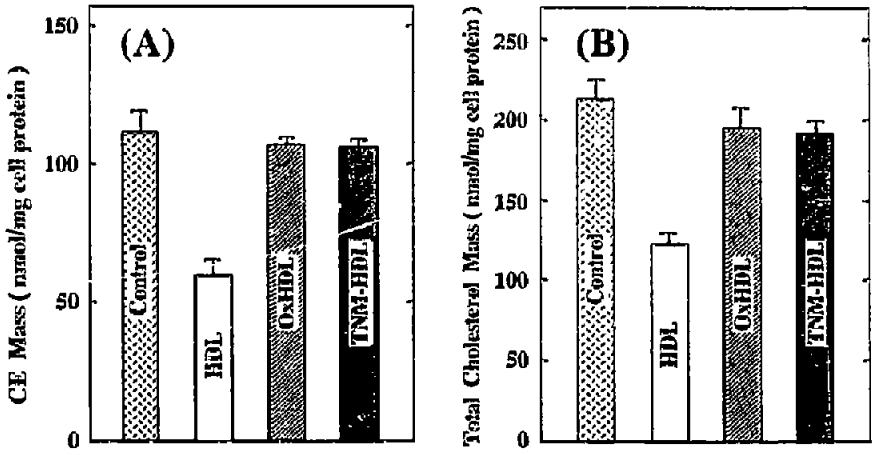

Fig. 4. Cholesterol efflux by oxidized HDL from macrophage foam cells. Mouse macrophages (3×10^5 cells) were incubated for 12 h with 50 μg/ml acetyl-LDL. After a 6-h equilibration in buffer A, cells were further incubated for 24 h with 250 μg/ml of HDL, oxidized HDL or TNM-HDL. Cellular CE mass (A) and total cholesterol mass (B) were determined.

mg/ml of HDL with 5 μM of Cu^{2+} resulted in the total disappearance of apoA-I bands [5]. In the present study, apoA-I bands indeed disappeared but cross-linking products of high molecular weight became visible, whereas the complete fragmentation of HDL apolipoproteins could not be observed (see Fig. 1). Thus, it is likely that the ligand inactivation of HDL by Cu^{2+}-mediated oxidation could not simply be ascribed to fragmentation per se, but largely to cross-linking of apolipoproteins. The finding that HDL did not undergo complete fragmentation with $CuSO_4$ suggests that HDL might be more resistant to oxidative modification than LDL. In this context, we have previously reported that HDL particles containing apoA-I but not apoA-II (LpA-I) had a strong inhibitory effect on the Cu^{2+}-mediated oxidation of LDL, suggesting the possibility that HDL might inhibit generation of atherogenic lipoproteins in vivo [17].

It is well known that oxidized LDL is recognized and endocytosed by the macrophage scavenger receptor [2]. If this was the case with oxidized HDL, it could supply, rather than remove, cholesterol from foam cells, so that a reducing effect on CE could be weakened. However, when non-loaded macrophages were incubated for 24 h with 250 μg/ml of oxidized HDL, the increase in the cellular CE level was less than 10 nmol/mg cell protein (data not shown), which would largely be explained by the non-specific cell association of oxidized HDL. Thus, it is unlikely that oxidized HDL could induce a significant CE accumulation in macrophages. This notion would be supported by the observation by Parthasarathy et al. [19] that oxidized HDL did not apparently behave as a ligand for the scavenger receptor. Thus, the loss of the capacity of oxidized HDL to reduce cellular CE might be attributable to the reduction in cholesterol efflux capacity rather than to an increase in CE accumulating capacity. Although TNM-HDL was reported to be recognized by the scavenger receptor of rat liver endothelial cells [20], its contribution to CE accumulation in macrophages was also negligible [11].

During preparation of this manuscript, the HDL binding protein or a putative HDL receptor was cloned [21]. Further characterization of this protein would provide a functional insight into the mechanism for the HDL receptor-mediated cholesterol efflux from cells.

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