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High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification

(atherosclerosis/regression/macrophage)

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ABSTRACT **In this study, we performed oxidative modification of high density lipoprotein (HDL) *in vitro*. The amount of lipid peroxide increased when either HDL2 or HDL3 was incubated with phosphate-buffered saline containing 5 μM $CuSO_4$ for 24 h at 37°C, indicating that both fractions of HDL were oxidatively modified. This modification resulted in denaturation of apolipoprotein AI on SDS/PAGE and increased the negative charge on agarose gel electrophoresis. When incubated with macrophage-derived foam cells, native HDL caused a marked efflux of cholesterol from them, leading to a decrease in the amount of cholesteryl ester in the cells. However, oxidized HDL showed a lessened effect on the decrease of cholesteryl ester in foam cells. These data suggest that oxidative modification of HDL may stimulate development of atherosclerosis by limiting efflux of cholesterol from foam cells.**

In atherosclerotic lesions, there are many foam cells that contain large amounts of cholesteryl ester. Immunocytochemical studies have revealed that these foam cells are derived from monocytes/macrophages and smooth muscle cells (1). Especially important roles of macrophages in the initial stages of atherosclerosis have been recognized (2), and the mechanism of foam cell transformation of macrophages *in vitro* has been studied in detail (3–5). A possible mechanism that is now widely accepted is that monocytes develop into macrophages in subendothelial spaces, where low density lipoprotein (LDL) is considered to be oxidatively modified. Oxidized LDL is then taken up by the macrophages, leading to their foam cell transformation (6, 7). Recently, data have accumulated that indicate oxidized LDL actually exists *in vivo* (8–11).

In 1980, Brown *et al.* (12) reported that, when high density lipoprotein (HDL) was added to the medium of lipid-laden foam cells, significant efflux of cholesterol from the cells occurred. This result strongly suggests that HDL might be involved in the amelioration of the atherosclerotic lesion *in vivo* by way of stimulation of cholesterol efflux from the foam cells. Lipid droplets in foam cells are known to consist largely of cholesteryl esters, and the initial step for the regression of foam cell-rich lesions is hydrolysis of intracellular cholesteryl esters, which is followed by an efflux of free cholesterol. To stimulate efflux of cholesterol from foam cells, HDL should make contact with these cells, which are situated in the subendothelial space, and this is the very place where oxidative modification of LDL is considered to occur. Therefore, it is rational to attempt to determine whether oxidation could also occur in HDL in the subendothelial space and whether this modification may have some important roles in atherogenesis.

In this study, we first ascertained that oxidative modification occurred *in vitro* in HDL2 and HDL3. We next demonstrated that oxidized HDL was less effective on the efflux of cholesterol from the foam cells than nonoxidized or native HDL.

METHODS

Materials. [1-^{14}C]Oleic acid (57.0 mCi/mmol; 1 Ci = 37 GBq) and [9,10-^{3}H(N)]oleic acid (10 Ci/mmol) were purchased from New England Nuclear. Bovine serum albumin (BSA) was obtained from Nacalai Tesque (Kyoto). Radiolabeled oleic acid–BSA complex was prepared as described (13). Fetal calf serum (FCS), obtained from HyClone, was inactivated at 56°C for 30 min before use. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Seiyaku (Tokyo). L-Glutamine and streptomycin/penicillin solutions were from Flow Laboratories and GIBCO, respectively. Plastic Petri dishes were obtained from Falcon and Becton Dickinson. Other chemicals were reagent grade.

Lipoproteins. LDL (ρ = 1.019–1.063), HDL (ρ = 1.085–1.21), HDL2 (ρ = 1.085–1.125), and HDL3 (ρ = 1.125–1.21) were isolated with sequential ultracentrifugation from plasma of healthy human subjects. Acetyl LDL was prepared following a method described previously (14).

Lipoproteins used for oxidative modification were dialyzed against two changes of at least a 500-fold volume of phosphate-buffered saline (PBS, 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl). For oxidative modification, 2.5 mg of lipoprotein was incubated with 1 ml of PBS containing 5 μM $CuSO_4$ at 37°C for 24 h (15). After modification, each lipoprotein was dialyzed against 150 mM NaCl containing 0.24 mM EDTA (pH 7.4) and used for experiments. Lipoproteins not used for oxidative modification were dialyzed against 150 mM NaCl containing 0.24 mM EDTA (pH 7.4).

Preparation of Macrophage Monolayers. Peritoneal cells were harvested from unstimulated female DDY mice by peritoneal lavage with PBS and pooled (16). They were centrifuged and resuspended with DMEM containing 10% (vol/vol) FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml) at a concentration of 3×10^6 cells per ml. Aliquots (1 ml) of this suspension were dispersed onto a plastic Petri dish (35 × 10 mm) and incubated in a CO_2 (5%) incubator at 37°C for 2 h. The cells were then washed twice with 2 ml of DMEM to remove nonadherent cells and used for subsequent experiments.

Preparation of Cholesteryl Ester-Enriched Macrophages. Macrophages obtained from mice were incubated with DMEM containing 10% (vol/vol) FCS and 50 μg of acetyl LDL per ml for 4 h at 37°C to load cholesteryl esters. In some experiments, 20 μg of [^{3}H]oleate (10 mM, 24,000 dpm/nmol)–

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Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; BSA, bovine serum albumin; FCS, fetal calf serum; TBARS, thiobarbituric acid-reactive substances; apo, apolipoprotein.

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BSA complex per ml was added to the incubation mixture to load radiolabeled cholesteryl ester (cholesteryl [^{3}H]oleate) (12). At the end of the incubation, the monolayers were rinsed twice with 2 ml of DMEM containing 0.2% BSA and rinsed once with DMEM. Then the cells supplemented with 1 ml of DMEM (without FCS) were kept at 37°C for 12 h in a CO_2 incubator. The end of this incubation was regarded as the starting point [time (t) = 0] for the further assay of cholesterol efflux.

Assay to Estimate the Decrease of Cellular Cholesteryl Oleate. At t = 0, cells loaded with nonradiolabeled cholesteryl esters were given 1 ml of DMEM containing 50 μg of HDL or oxidized HDL per ml. After the indicated incubation time (12, 24, or 36 h), cells were washed twice with buffer A [150 mM NaCl/50 mM Tris·HCl/0.2% (wt/vol) BSA, pH 7.4] and once with buffer B (150 mM NaCl/50 mM Tris·HCl, pH 7.4). Cellular lipid was then extracted with hexane/isopropyl alcohol, 3:2 (vol/vol). After evaporation of the organic solvent, cellular free cholesterol and total cholesterol were measured with an enzymatic fluorometric method as described (17). Cellular esterified cholesterol was calculated by subtracting free cholesterol from total cholesterol.

To follow the fate of intracellular cholesteryl ester more precisely, we performed another assay using macrophages preloaded with cholesteryl [^{3}H]oleate. At t = 0, cells were incubated with 1 ml of DMEM containing 20 μl of [^{14}C]oleate (10 mM, 7200 dpm/nmol)–BSA complex in the presence of 250 μg of HDL or oxidized HDL per ml for indicated times, as described (12). At the end of the incubation, cells were washed twice with buffer A and washed once with buffer B. Cellular lipid was then extracted with hexane/isopropyl alcohol, 3:2 (vol/vol). After evaporation of the organic solvent, the lipid was resuspended with 100 μl of hexane, and cholesteryl ester was isolated on a thin-layer chromatogram (13). The chromatogram was developed in petrol ether/diethyl ether/acetic acid (90:10:1), and the cholesteryl ester spot was identified with iodine vapor and cut from the plate. The amount of preloaded cholesteryl [^{3}H]oleate and that of newly synthesized cholesteryl [^{14}C]oleate were measured with a liquid scintillation counter. The dose–response effect of HDL and oxidized HDL on foam cells was also investigated using [^{3}H]oleate–BSA complex and [^{14}C]oleate–BSA complex as described above. Changes in the amount of intracellular cholesteryl oleate were followed after 36 h of incubation with several doses of HDL or oxidized HDL.

Other Assays. After the extraction of lipids, cells were dissolved with 1 ml of 0.2 M NaOH, and cellular protein was measured by the method described by Lowry *et al.* (18).

Lipid peroxides were measured in terms of thiobarbituric acid-reactive substances (TBARS) according to the methods of Yokode *et al.* (15) with slight modifications. Lipoproteins (20 μg of protein) were suspended in 0.2 ml of 150 mM NaCl. The suspension was mixed with 0.5 ml of 20% (wt/vol) trichloroacetic acid and 0.5 ml of the reagent [335 mg of 2-thiobarbituric acid in 100 ml of 50% (vol/vol) acetic acid]. The mixture was boiled at 95°C for 60 min. After being cooled, 2.0 ml of 1-butanol was added, shaken vigorously, and centrifuged at 4000 × g for 10 min. The fluorescence of supernatant was measured with excitation at 515 nm and emission at 550 nm. Tetramethoxypropane was used as a standard.

SDS/PAGE and agarose gel electrography were performed as described by Laemmli (19) and Nobel (20), respectively.

RESULTS

Characterization of Oxidized HDL. Fig. 1 shows the amount of TBARS of native HDL and HDL incubated with cupric ion. HDL2 and HDL3 showed significant increase in TBARS after incubation with 5 μM cupric ion for 24 h, indicating that

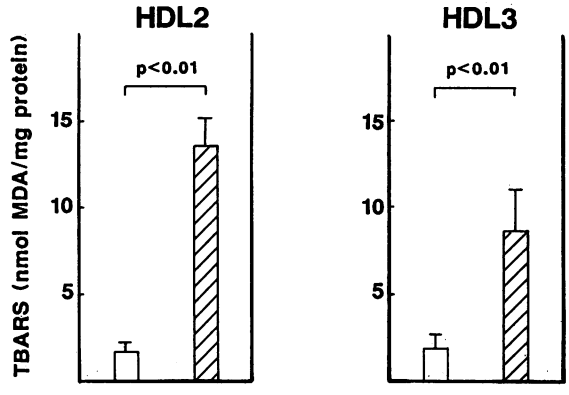

FIG. 1. TBARS in HDL incubated with (5 μM cupric ion, ■) or without (native HDL, □) $CuSO_4$ for 24 h at 37°C. Twenty micrograms of lipoprotein was used to estimate the amount of lipid peroxide. MDA, malondialdehyde. Data represent the mean value ± SD.

cupric ion-catalyzed lipid peroxidation occurred in HDL. We then examined whether lipid peroxidation leads to any modification of HDL particles. We first tested the change of net charge of lipoprotein. On agarose gel electrophoresis, HDL2 and HDL3 increased their negative charge after oxidation compared with their native forms (Fig. 2*A*). We next tested proteolytic degradation of apo AI of oxidized HDL by SDS/PAGE. As shown in Fig. 2*B*, the apo AI (28.3 kDa) band disappeared from oxidized HDL2 and oxidized HDL3.

Influence of Oxidation of HDL on Efflux of Cholesteryl Esters from Foam Cells. Based on the results above, we tested whether there is any difference between native and oxidized HDL concerning the potential to induce cholesterol efflux from foam cells. As shown in Fig. 3*A*, oxidized HDL3 stimulated much less efflux of cholesterol from macrophage-derived foam cells compared with the incubation using native HDL3. Intracellular cholesteryl ester decreased to 17.4% ± 2.4% of the initial amount when incubated with HDL3 for 36 h. However, 59.3% ± 15.9% of the initial amount of cholesteryl ester remained in these cells when incubation was performed with oxidized HDL3 ($P < 0.01$). A similar result was obtained from experiments of incubations with HDL2 and oxidized HDL2 (Fig. 3*B*). The percentages of intracellular cholesteryl ester that remained after 36 h of incubation with HDL2 and oxidized HDL2 were 29.6% ± 3.3% and 68.6% ± 3.2% of the initial amount, respectively ($P < 0.01$). Almost no efflux was stimulated when the incubation was performed with BSA.

To determine the effect of oxidized HDL on cholesteryl ester synthesis and hydrolysis, we used a protocol developed by Brown *et al.* (12). Macrophages were loaded with radio-

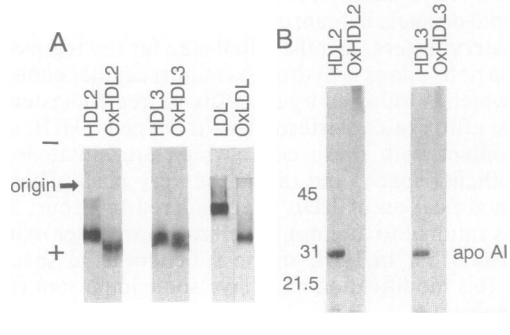

FIG. 2. Agarose gel electrophoresis and SDS/PAGE of lipoproteins. (*A*) Aliquots (3 μg) of lipoproteins were subjected to 1% agarose gel electrophoresis and stained with fat red 7B. Ox, oxidized. (*B*) Aliquots (10 μg) of lipoprotein were loaded on SDS/PAGE (10–20%) and stained with Coomassie brilliant blue. Molecular mass is indicated in kDa. apo, Apolipoprotein.

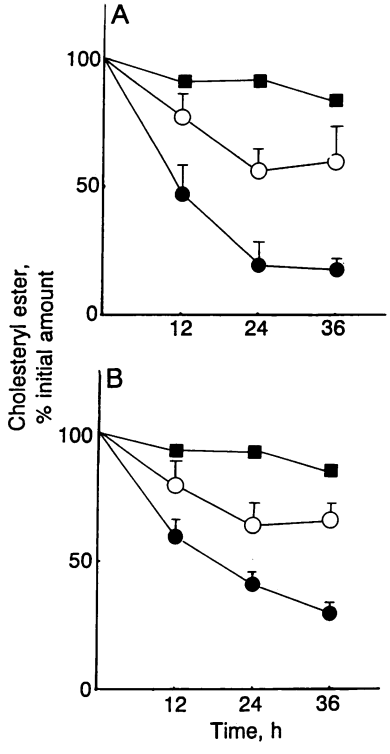

FIG. 3. Change in the amount of cholesteryl ester in macrophage-derived foam cells. Macrophages were loaded with cholesteryl ester by preincubation with 50 μg of acetyl LDL per ml for 4 h. After incubation in DMEM without FCS for 12 h, the experiments were begun by incubation of the cells with DMEM containing 50 μg of HDL3 (●), oxidized HDL3 (○), or BSA (■) per ml (*A*) or 50 μg of HDL2 (●), oxidized HDL2 (○), or BSA (■) per ml (*B*). At the end of each incubation (12, 24, or 36 h), the cells were washed twice with buffer A and washed once with buffer B. Extraction of cellular lipid and measurement of cholesteryl ester were performed as described in the text. Data were reported as % of the initial amount of cholesteryl ester loaded (mean ± SD). The number of experiments was five for incubations with HDL or oxidized HDL and two for BSA. Each assay was performed in duplicate. The original mean value for 100% (t = 0) was 41.3 μg/mg of cellular protein.

labeled cholesteryl esters by incubation with acetyl LDL in the presence of [^{3}H]oleate. The acetyl LDL and [^{3}H]oleate were then removed and the cells were allowed to equilibrate with their newly synthesized cholesteryl ester for 12 h. We then added either HDL2 (Fig. 4*A*) or oxidized HDL2 (Fig. 4*B*) in the presence of [^{14}C]oleate to follow the reesterification of cholesterol. Control cells were incubated with BSA, which neither delivers nor accepts cholesterol (Fig. 4*C*). When the macrophages were incubated with BSA, there was little decline in total cholesteryl esters over 36 h (Fig. 4*C*). However, there was a continual hydrolysis and reesterification of the stored cholesteryl esters, as indicated by the decline in cholesteryl [^{3}H]oleate and the reciprocal increase in cholesteryl [^{14}C]oleate. In the presence of HDL3 (Fig. 4*A*), the prelabeled cholesteryl [^{3}H]oleate was hydrolyzed at a slightly faster rate than in the presence of albumin. However, there was no longer any reesterification with [^{14}C]oleate. Instead of being reesterified, the liberated cholesterol was presumably excreted from the cell (12). Oxidation blunted all of the effects of HDL3 (Fig. 4*B*). The total cholesteryl ester content of the cells declined only slightly. This was attributable to a slight decrease in the rate of cholesteryl ester hydrolysis and a marked increase in cholesterol reesterification. The increased reesterification indicated that the oxidized HDL was no longer able to accept cholesterol from the cells. Similar results were obtained with oxidized HDL2 (Fig. 5 *A* and *B*).

When native HDL2 and HDL3 were compared, HDL3 was more effective than HDL2. After 36 h of incubation, intracellular cholesteryl oleate decreased to 12.4% and 31.6% of the initial amount when the cells were incubated with HDL3 and HDL2, respectively.

Finally, we investigated the dose–response effect of HDL and oxidized HDL using the same system as that in Figs. 4 and 5. For this experiment, total HDL (ρ = 1.085–1.21) or oxidized HDL modified from total HDL was used. As shown in Fig. 6, HDL showed more potential to cause efflux of cholesterol from foam cells than oxidized HDL at any dose. The effect of HDL and oxidized HDL seemed to become saturated at a dose of ≈20 μg/ml.

DISCUSSION

Deposition of macrophage-derived foam cells in the subendothelial space is one of the initial events of atherosclerosis, and oxidized LDL is the likeliest candidate for atherogenic lipoprotein that exists *in vivo*. It is taken up by macrophages and causes their foam cell transformation. Oxidative modification of LDL and incorporation of oxidized LDL by macrophages are considered to take place in the subendothelial space.

Brown *et al.* (12) reported that HDL stimulates efflux of cholesterol from lipid-laden macrophages and decreases the amount of intracellular cholesteryl ester using *in vitro* assay systems. To remove cholesteryl ester from foam cells, HDL should directly make contact with these cells in the subendothelial space, and this is the very place where LDL is considered to be oxidized.

In this report, we questioned whether oxidative modification could occur in HDL using *in vitro* systems. The results in Figs. 1 and 2 clearly show that oxidative modification occurred in HDL as in LDL.

After oxidative modification of HDL, the cholesterol:protein ratio was lowered from 0.52 to 0.37. Oxidized HDL eluted as a slightly larger species on HPLC, but there was no obvious alteration by electron microscopy (data not shown).

Moreover, we demonstrated that oxidized HDL showed a very limited effect on foam cells, which were prepared by incubating macrophages with acetyl LDL, to stimulate efflux of cholesterol compared with native HDL (Figs. 3–5). The contribution of apo AI on efflux of cholesterol from foam cells has been investigated in detail, and its importance has been recognized (21). Brinton *et al.* (22) reported that chemical modification of HDL deprived its effect on cholesterol efflux. Today, oxidative modification of lipoprotein is a matter of interest for many investigators because evidence has accumulated for the existence of oxidized LDL *in vivo*. The limited effect of oxidized HDL to cause efflux of cholesteryl ester could be explained by the denaturation of apo AI on HDL particles after oxidative modification (Fig. 2). Graham and Oram (21) reported that HDL binds to the putative "HDL receptor" (or HDL binding protein) of several kinds of cells, and recently, Barbaras *et al.* (23) purified apo A binding protein from mouse adipose cells. HDL bound to macrophages is not degraded, although there are some conflicts among investigators whether HDL is internalized into macrophages (24, 25). To explain the efflux of cholesterol after binding of HDL to the macrophage, we have to consider some signal transduction system in the cells, which is closely related to the HDL binding. However, our results revealed that even oxidized HDL partially stimulated the efflux of cholesterol, although apo AI underwent denaturation judging from SDS/PAGE. This implies the contribution of some pathway that is independent of apo AI and/or its receptor. Ho *et al.* (26) reported that not only HDL but also

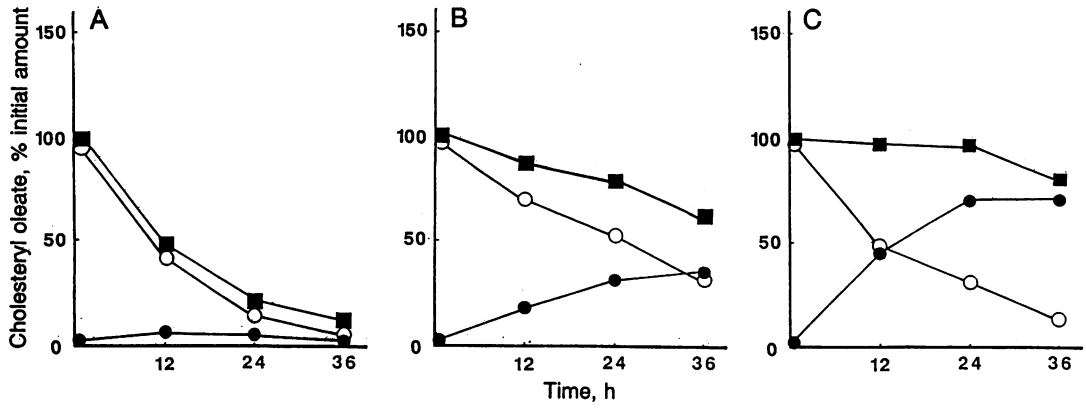

FIG. 4. Change in the amount of cholesteryl ester in macrophage-derived foam cells. Macrophages were loaded with radiolabeled cholesteryl oleate by preincubation with 50 μg of acetyl LDL per ml and 20 μl of [^{3}H]oleate (10 mM)–BSA complex per ml for 4 h. After incubation in DMEM without FCS for 12 h, the experiments were started by incubation of the cells with DMEM containing 250 μg of HDL3 or oxidized HDL3 per ml. During this incubation time, 20 μl of [^{14}C]oleate (10 mM)–BSA complex per ml was added to the medium. At the end of each incubation (12, 24, or 36 h), the cells were washed twice with buffer A and washed once with buffer B. Cellular lipid was extracted with hexane/isopropyl alcohol (3:2) and cholesteryl oleate was isolated on a thin-layer chromatogram. The amounts of cholesteryl [^{3}H]oleate and cholesteryl [^{14}C]oleate were measured with a liquid scintillation counter. ○, Preloaded cholesteryl ester; ●, cholesteryl ester synthesized during incubation with HDL or oxidized HDL; ■, total cholesteryl ester in cells. (*A*) Incubation with HDL3. (*B*) Oxidized HDL3. (*C*) BSA. Data were reported as % of the initial amount of cholesteryl oleate loaded. Each point shows a mean value of two representative assays that were performed in duplicate. The original mean value for 100% (t = 0) was 30.6 nmol/mg of protein.

other proteins, such as casein, thyroglobulin, or even erythrocytes, efficiently stimulate efflux of cholesterol from foam cells.

Recently, Pomerantz and Hajjar (27) reported that cholesteryl ester in smooth muscle cell-derived foam cells is also cleared by incubation of these cells with HDL. Therefore, oxidation of HDL could also cause sustained deposition of lipid in smooth muscle cell-derived foam cells.

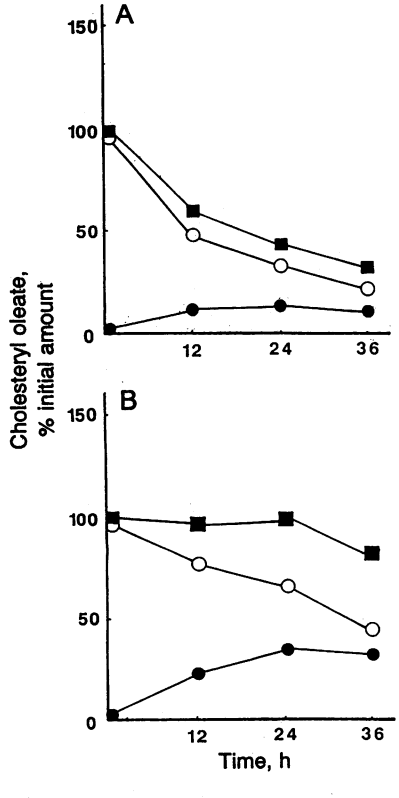

FIG. 5. Change in the amount of cholesteryl ester in macrophage-derived foam cells. The experiments were performed as described in the legend to Fig. 4, except that HDL2 was used in place of HDL3. The symbols are the same as in Fig. 4. (*A*) HDL2. (*B*) Oxidized HDL2. The original mean value for 100% (t = 0) was 30.6 nmol/mg of protein.

The major effect of HDL on foam cells is to abolish reesterification of cholesterol, which depends on the excretion of the free cholesterol from the cells. In the presence of oxidized HDL, reesterification continues, presumably because the cholesterol is no longer excreted. Although oxidized HDL slightly decreased the hydrolysis of stored cholesteryl esters compared with native HDL or BSA, the major reason for cholesteryl ester retention in the cells treated with oxidized HDL is the maintenance of reesterification. The amount of stored cholesteryl esters hydrolyzed does not differ much between the incubations with HDL and those with BSA or control incubations (Figs. 4 and 5).

The dose–response effect of HDL and oxidized HDL on cellular cholesterol efflux revealed that the response curve

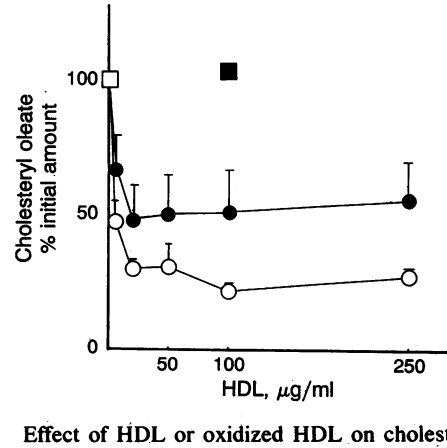

FIG. 6. Effect of HDL or oxidized HDL on cholesterol efflux from foam cells. Macrophages loaded with radiolabeled cholesteryl oleate were prepared as described in the legend to Fig. 4. The experiments of efflux were begun by incubation with DMEM containing several doses of HDL (○), oxidized HDL (●), or 100 μg of BSA per ml (■). During this incubation, 20 μl of [^{3}H]oleate (10 mM)–BSA complex per ml was added to the medium. At the end of 36 h of incubation, the cells were treated as described in the legend to Fig. 4, and intracellular contents of cholesteryl [^{3}H]oleate and cholesteryl [^{14}C]oleate were measured. The total amount of intracellular cholesteryl ester was estimated by adding the amount of these two radiolabeled cholesteryl oleates. The data are reported as percentages of initial amount of intracellular cholesteryl ester, and the original mean value for 100% (t = 0) was 29.7 nmol/mg of cellular protein (□).

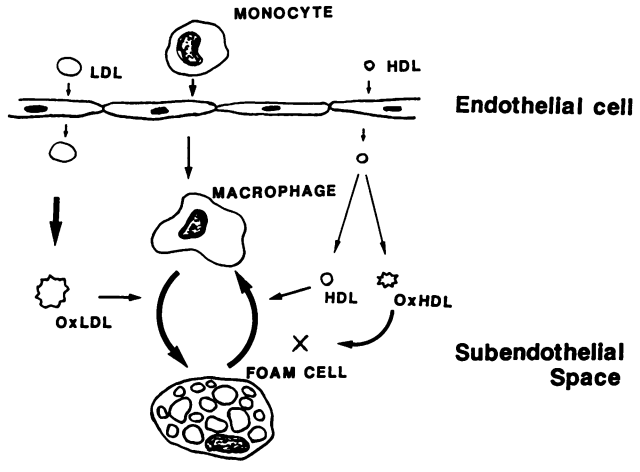

FIG. 7. Proposed scheme of the interaction between macrophages/foam cells and lipoproteins in the subendothelial space with an insight into the oxidative modification of lipoproteins. Ox, oxidized.

reached a plateau when these lipoproteins were added to the incubation medium with a concentration of ≈20 μg/ml (Fig. 6). Oxidized HDL was cytotoxic at 500 μg/ml, and almost all cells detached from dishes at this concentration, although HDL was not cytotoxic at all (data not shown).

Furthermore, we prepared foam cells by incubating macrophages with oxidized LDL, the likeliest candidate for atherogenic lipoprotein *in vivo*, and investigated the effect of HDL and oxidized HDL on these foam cells. The result showed the same tendency as the case of foam cells that were preloaded with cholesterol with acetyl LDL; HDL was very effective for the efflux of cholesterol, whereas oxidized HDL had minimal effect (data not shown).

Fig. 7 shows the possible scheme of what happens in the subendothelial space between lipoproteins and macrophages concerning atherogenesis. The unique mechanism of HDL oxidation would be of importance in the atherosclerotic plaque, since increased evidence has accumulated that lipoproteins are oxidatively modified in the body.

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