Foam cell formation containing lipid droplets enriched with free cholesterol by hyperlipidemic serum

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Abstract **A monoclonal antibody, ASH1a/256C (256C), which binds to atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbit (WHHL) aorta in vivo, recognizes complex structures of phosphatidylcholine mixed with neutral lipids. In the present study, a cell culture system is described in which foam cells express 256C-positive lipid droplets. J774.1 macrophages were incubated in the presence of a small volume of WHHL serum for 24 h to produce foam cells, which were then incubated without the WHHL serum for 3 days. Oil red O-positive lipid droplets appeared on day 1, and were present in the cells during the whole incubation period. The lipid droplets in the cells were positively immunostained with antibody 256C on day 4, although they were negative on day 1. Expression of the antigenic lipid droplets was also induced by the addition of acetylated LDL or sera from patients with hyperlipidemia. When foam cells were induced by the addition of WHHL serum, cellular content of cholesteryl ester was greatly increased but then decreased to near basal levels by day 4. Concomitantly, cellular free cholesterol increased during the culture period, indicating that the cholesteryl ester changes to free cholesterol by day 4. The lipid droplets in the foam cells on day 4 were positively stained with filipin, a fluorescent probe for free cholesterol, as well as with 256C antibody, indicating that free cholesterol is enriched in antigenic lipid droplets. These observations suggest that hydrolysis and rearrangement of cellular cholesterol take place in foam cells to form complex structures of phosphatidylcholine and free cholesterol in lipid droplets.—** Mori, M., H. Itabe, Y. Higashi, Y. Fujimoto, M. Shiomi, M. Yoshizumi, Y. Ouchi, and T. Takano. **Foam cell formation containing lipid droplets enriched with free cholesterol by hyperlipidemic serum.** ***J. Lipid Res.*** **2001.** 42: **1771–1781.**

Supplementary key words atherosclerosis • lipoproteins • macrophages • monoclonal antibody • phosphatidylcholine • WHHL rabbit

Intracellular accumulation of lipid droplets is a remarkable feature of foam cells in atherosclerosis (1–3). The initial steps of foam cell formation have been extensively studied [see reviews in refs. (3) and (4)]. Scavenger receptors expressed by macrophages bind and take up modified LDL but not native LDL. Modified lipoproteins are taken up extensively by macrophages, because the recycling systems of scavenger receptors are not downregulated. Components in modified lipoproteins including cholesteryl ester (CE) are hydrolyzed in lysosomes (3). The resulting free cholesterol (FC) is transferred to the endoplasmic reticulum and then re-esterified to CE, which accumulates in the cytosol to form intracellular lipid droplets (3). The enzyme that re-esterifies cholesterol on endoplasmic reticulum is ACAT, the involvement of which in foam cell formation has been demonstrated by using ACAT inhibitors and by generating gene-targeted mice (5, 6).

Atherosclerosis is a chronic pathological condition, and can take decades to develop severe atheromatous lesions in humans (7). Foam cells appear even in early stages of atherosclerosis, and the accumulation of large numbers of foam cells is often observed in advanced lesions. Because macrophages are a differentiated cell type derived from monocytes, foam cells should therefore have a defined lifetime and they could be replaced by other foam cells during the development of the atherosclerotic lesion. It is, however, poorly understood what happens in macrophages after they change into foam cells. Lipid droplets in foam cells regress when cholesterol acceptors such as apolipoprotein A-I or HDL are present in sufficient quantities, thus suggesting that lipid droplets are not stable stores of excess amount of lipids but, rather, are metabolically active.

In our previous work a unique anti-atheroma monoclonal antibody, ASH1a/256C (256C), was isolated and

Abbreviations: AcLDL, acetylated low density lipoprotein; CE, cholesteryl ester; FC, free cholesterol; MPM, murine peritoneal macrophage; OxLDL, oxidized low density lipoprotein; OxPC, oxidized phosphatidylcholine; PC, phosphatidylcholine; TRITC, tetramethyl rhodamine isothiocyanate; WHHL, Watanabe heritable hyperlipidemic rabbit.

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characterized (8). This antibody was shown to bind to atheromatous lesions when radiolabeled antibody was injected intravenously into Watanabe heritable hyperlipidemic (WHHL) rabbits. Using immunohistochemical staining, this antibody detected lipid-rich and foam cell-rich regions in atherosclerotic lesions. The epitope was identified to be phosphatidylcholine (PC) molecules containing polyunsaturated fatty acyl chains; however, oxidized PC (OxPC) was not antigenic. A unique characteristics of this antibody is that its binding to PC tested by ELISA is accelerated when neutral lipids are mixed with PC, suggesting that 256C preferentially recognizes PC molecules in complexes with neutral lipids. These observations lead us to believe that certain forms of complex structures consisting of PC with neutral lipids are generated in atherosclerotic lesions.

In the present study, we found that 256C antigen is generated in macrophage-derived foam cells only when lipid-laden foam cells were, in addition, cultured for more than 3 days. The appearance of 256C antigen was closely related to increased amounts of cellular FC, and the distribution of intracellular FC clearly corresponded with that of 256C antigen. We therefore propose that unique PC-FC complex structures may be generated in lipid droplets through cellular metabolic actions.

MATERIALS AND METHODS

Monoclonal antibodies

Monoclonal antibodies were raised using homogenates of human atheroma as immunogen, and 256C and FOH1a/DLH3 (DLH3) were prepared from murine ascites as described previously (8–10). The epitopes of these antibodies were characterized previously; 256C recognizes PC and the binding was enhanced in the presence of cholesterol, and DLH3 recognizes OxPC but not native PC.

Induction and culture of foam cells

Murine macrophage cell line J774.1 was grown in log phase ($1–8 \times 10^5$ cells/ml), using RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 7% (v/v) FCS, potassium penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37°C in an atmosphere of 95% air-5% CO_2. Cultures were free of mycoplasma as demonstrated by routine screening.

J774.1 cells were inoculated on culture slides [Falcon 54114 (Becton Dickinson Labware, Lincoln Park, NJ); 1×10^5 cells/well] and cultured for 1 day. After removing culture medium, the cells were washed with RPMI 1640 medium (serum free) twice, and then incubated for 24 h with RPMI 1640 medium (serum free) containing lipopolysaccharide (LPS, 10 μg/ml; Difco, Detroit, MI). The cells pretreated with LPS were well differentiated and expressed macrophage-like characteristics (11). The cells were then incubated for 24 h with RPMI 1640 containing 0.2% FCS supplemented with a small volume of WHHL serum (final concentration, 40 μg of cholesterol per ml). After washing with RPMI 1640 (serum free) twice, the culture medium was replaced with RPMI 1640 containing 7% FCS (medium A). Foam cells were maintained at 37°C, and the medium was changed twice a week.

BALB/c mice (6-week-old females) were intraperitoneally injected with 1 ml of 3% thioglycolate 4 days before being killed. Peritoneal exudate cells were recovered with RPMI 1640 medium without serum, and the cells were washed twice by centrifugation (220 *g* for 10 min at 4°C). These cell were inoculated on culture slides (1×10^5 cells/well) and washed with RPMI 1640 medium once before WHHL serum treatment and the remaining cells were used as murine peritoneal macrophages (MPM).

Separation of human plasma lipoproteins

Human lipoproteins were separated from human plasma, using a sodium bromide stepwise density gradient centrifugation as described previously (12). LDL and HDL fractions were dialyzed against PBS containing 0.25 mM EDTA to remove sodium bromide. Oxidized LDL (OxLDL) was prepared as described previously (13). Briefly, after removing EDTA by passage through a 10DG desalting column (Bio-Rad, Hercules, CA), LDL (2 mg/ml) was incubated with 50 μM $CuSO_4$ at 37°C for 24 h. The reaction was stopped by the addition of EDTA. Acetylated LDL (AcLDL) was prepared according to the method of Basu et al. (14). Briefly, to the LDL fraction (1 mg/ml) mixed with an equal volume of saturated sodium acetate, 1 μl of acetic anhydride per mg of LDL was added and incubated under continuous mixing for 90 min at 0°C. The samples were dialyzed against PBS containing 0.25 mM EDTA at 4°C.

Staining procedures for foam cells

Cells in culture slides were fixed with 10% formaldehyde in PBS for 10 min followed by washing twice with PBS. The cells were pretreated with 60% 2-propanol for 2 min and then stained with 0.2% oil red O (Sigma) in 60% 2-propanol for 10 min. The slides were washed with 2-propanol and then with PBS. Finally, the cells were treated with Mayer's hematoxylin (Wako Pure Chemicals, Osaka, Japan) for 5 min to stain nuclei.

For immunostaining, fixed cells were incubated with blocking buffer [PBS containing 1% (v/v) goat serum (Chemicon, Temecula, CA) and 1% (w/v) BSA (Sigma)] for 1 h at room temperature followed by incubation for 1 day at 4°C with 256C antibody diluted with dilution buffer (PBS containing 0.1% goat serum and 0.1% BSA). After washing five times with PBS followed by incubation with the blocking buffer for 40 min at room temperature, the cells were then incubated for 1 day at 4°C with biotin-conjugated goat anti-mouse Ig(G+M) antibody (BioSource, Camarillo, CA) diluted with the dilution buffer. After the washing and blocking steps were performed again, the cells were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-labeled streptavidin (Zymed, South San Francisco, CA) or tetramethyl rhodamine isothiocyanate (TRITC)-labeled avidin (EY Laboratories, San Mateo, CA) diluted with the dilution buffer (15, 16). After extensive washing with PBS, the antigen-antibody complex was visualized with an Optiphot fluorescence microscope (Nikon, Tokyo, Japan) with excitation at 460–490 nm (for FITC; emission at 515 nm) or 520–550 nm (for TRITC; emission at 580 nm).

Filipin complex (Sigma) was dissolved in DMSO (1 mg/ml) to make a stock solution. For detection of FC, using filipin, cells in culture were treated with filipin at a final concentration of 1.33 μg/ml for 1 h at 37°C. After extensive washing with PBS, the cells were fixed, and filipin-FC complexes were visualized by fluorescence microscopy with excitation at 330–385 nm (emission at 420 nm) (17, 18).

For double staining experiments, the cells were stained with oil red O and hematoxylin before immunostaining with 256C. Cells were fixed with 0.2% oil red O in 35% 2-propanol for 30 min followed by staining with hematoxylin. The cells were then blocked with the blocking solution and treated with 256C, biotin-conjugated secondary antibody, and FITC-conjugated streptavidin as described above. After extensive washing with PBS, the antigen-antibody complexes were visualized by fluorescence microscope with excitation at 460–490 nm (for both FITC and oil red O).

Preparation of CE liquid crystals

CE particles in liquid crystal structure were prepared as described previously (19). Briefly, 600 nmol of cholesteryl oleate (Sigma) was suspended in 1.5 ml of sterilized water at 80°C, and then sonicated for 3 min with a probe-type ultrasound cell homogenizer (Branson, Danbury, CT). The suspension was chilled on ice, and BSA was added to make a final concentration of 0.01%. After centrifugation at 180 *g* for 10 min, white-colored suspension was recovered as CE liquid crystals.

Measurement of cellular cholesterol and TG

The amounts of total cholesterol and FC were measured by a cholesterol oxidase method (20, 21) using a Cholestase-V kit (Nissui) and FC-Test Wako kit (Wako Pure Chemicals). The amount of TG was measured with a Nescoat TG kit (Azwell, Nagoya, Japan) (22).

RESULTS

Appearance of 256C antigen in macrophage-derived foam cells

Murine macrophage cell line J774.1 was pretreated with LPS for 1 day before addition of lipoproteins in the current studies. This pretreatment allowed the cells to become well differentiated and express macrophage-like characteristics (11). When J744.1 macrophages were incubated for 24 h with medium containing serum from WHHL rabbits, they were strongly stained with oil red O, indicating a massive accumulation of neutral lipids in the cells (**Fig. 1A**). Subsequently the culture medium was replaced by RPMI medium containing 7% FCS but not WHHL serum (medium A), and cultured for three more days (Fig. 1C; at day 4). The morphology of the foam cells changed slightly, with the lipid droplets becoming smaller and distributing over peripheral regions of the cells. However, the major difference found in these foam cells at days 1 and 4 was the appearance of antigens for the 256C antibody. Figure 1D shows that lipid droplets in the foam cells at day 4 are stained with 256C antibody, whereas those in the foam cells at day 1 are negative (Fig. 1B), suggesting that nascent lipid droplets themselves are not antigenic but that they become antigenic after 3 days of culture. The formation of lipid droplets and appearance of the antigen were not observed when the cells were incubated without the addition of WHHL serum (Fig. 1G and H) or incubated with normal rabbit serum (under the same final cholesterol concentration; data not shown).

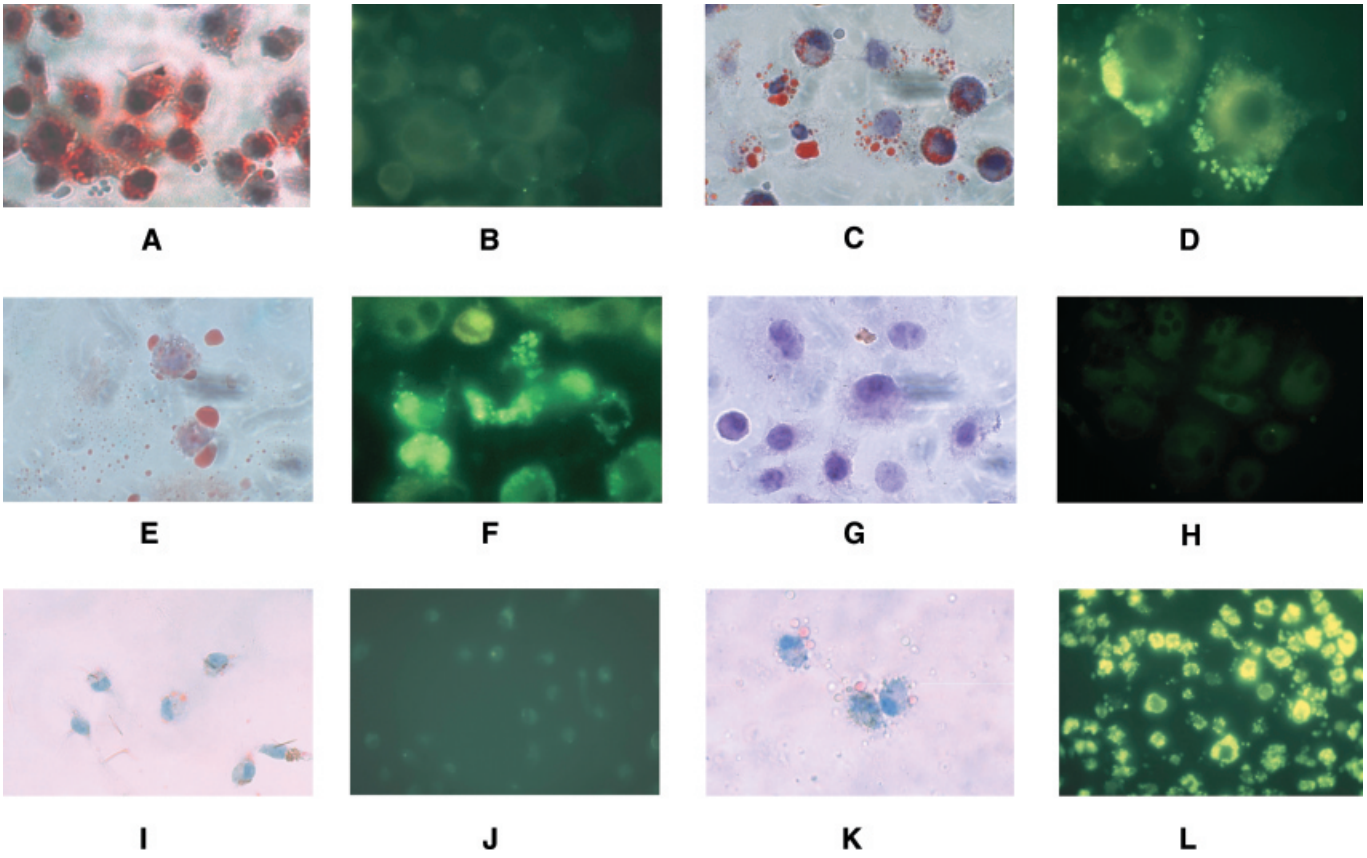

Fig. 1. Expression of antigen against 256C in J774.1 macrophages (A–H) and MPM (I–L). J774.1 cells were pretreated with LPS (10 μg/ml) for 24 h followed by incubation with WHHL serum (final cholesterol concentration, 40 μg/ml) for 24 h (A and B), and then cell culture was continued with RPMI medium containing 7% FCS but without WHHL serum (medium A) for 3 days (C and D) or for 6 days (E and F). MPM were incubated with WHHL serum for 24 h (I and J), and then cultured with medium A for 3 days (K and L). Cells were fixed with neutral formalin and stained either with oil red O and hematoxylin (A, C, E, G, I, and K; original magnification, ×400) or with 256C antibody immunohistochemically (B, D, F, and H: original magnification, ×1,000; J and L: original magnification, ×400). As a control, cells were incubated without addition of WHHL serum for 24 h and further cultured for 3 days (G and H).

The appearance of 256C antigen was observed when MPM was incubated with WHHL serum under the same conditions. Oil red O-positive droplets were formed on day 1 but they were not stained with the antibody (Fig. 1I and J); however, a large number of 256C-positive staining cells appeared after the extended culture for 3 days (Fig. 1K).

Under these cell culture conditions, the foam cells could be cultured for at least 14 days. During culture, however, several foam cells were found to be ruptured. On day 7, lipid droplets appeared without cell nuclei together with lipid-laden foam cells (Fig. 1E). The lipid droplets seemed to be associated with cellular matrix proteins. When the cells were cultured without addition of WHHL rabbit serum, lipid droplets without cell nuclei could not be detected during the culture procedure (data not shown).

To determine why WHHL serum is capable of inducing 256C antigen expression in foam cells, we investigated whether there was a particular type of lipoprotein that mimicks WHHL serum. The major lipoprotein classes were isolated from human plasma by sequential density ultracentrifugation, and AcLDL and copper-induced OxLDL were prepared. J774.1 macrophages were pretreated with LPS for 1 day, and then the cells were incubated with the human lipoproteins and the modified LDL for 24 h, after which the cell culture was continued for 3 days in medium A. Addition of AcLDL as well as WHHL serum to the cells generated foam cells by day 4 (**Fig. 2A** and **B**). Foam cells were also strongly induced by addition of VLDL (data not shown). Oil red O-positive cells were also observed with the addition of OxLDL, but the change was much less pronounced. LDL and HDL did not cause any foam cell formation under the experimental conditions used. The appearance of 256C antigen was induced by addition of AcLDL as well as WHHL serum to the cells (Fig. 2B and D), whereas the other lipoproteins failed to induce antigen expression by day 4 (Fig. 2E–H). Among these native or modified lipoproteins, AcLDL and WHHL serum were able to induce 256C antigen by day 4, whereas virtually no staining occurred in MPM incubated with LDL. MPM incubated with OxLDL or VLDL showed much fainter and diffuse staining with 256C antibody (data not shown).

We have demonstrated, using monoclonal antibody DLH3, which recognizes OxPC generated in OxLDL (9, 10), that OxPC-apolipoprotein B complex is accumulated in foam cells in human atherosclerotic lesions and in J774.1 macrophages incubated with copper-induced OxLDL (13). Although 256C does recognize PC containing polyunsaturated fatty acyl groups but does not bind to OxPC (8), it is of interest whether lipid peroxidation is involved in the expression of 256C antigen in foam cells. After macrophages were treated with either WHHL serum or OxLDL, or without addition of any lipoprotein, the cells on day 4 were immunostained either with 256C or DLH3. The foam cells induced by addition of WHHL serum were positive against 256C antibody (**Fig. 3A**), whereas 256C failed to stain OxLDL-induced foam cells (Fig. 3B). Under the same experimental conditions, DLH3 antibody strongly stained the macrophages after incubation with OxLDL for 1 day (data not shown) and were still positive on day 4 (Fig. 3E), whereas DLH3 stained foam cells induced by WHHL serum slightly (Fig. 3D). The cells incubated without lipoprotein were negative for either 256C or DLH3 antibody (Fig. 3C and F).

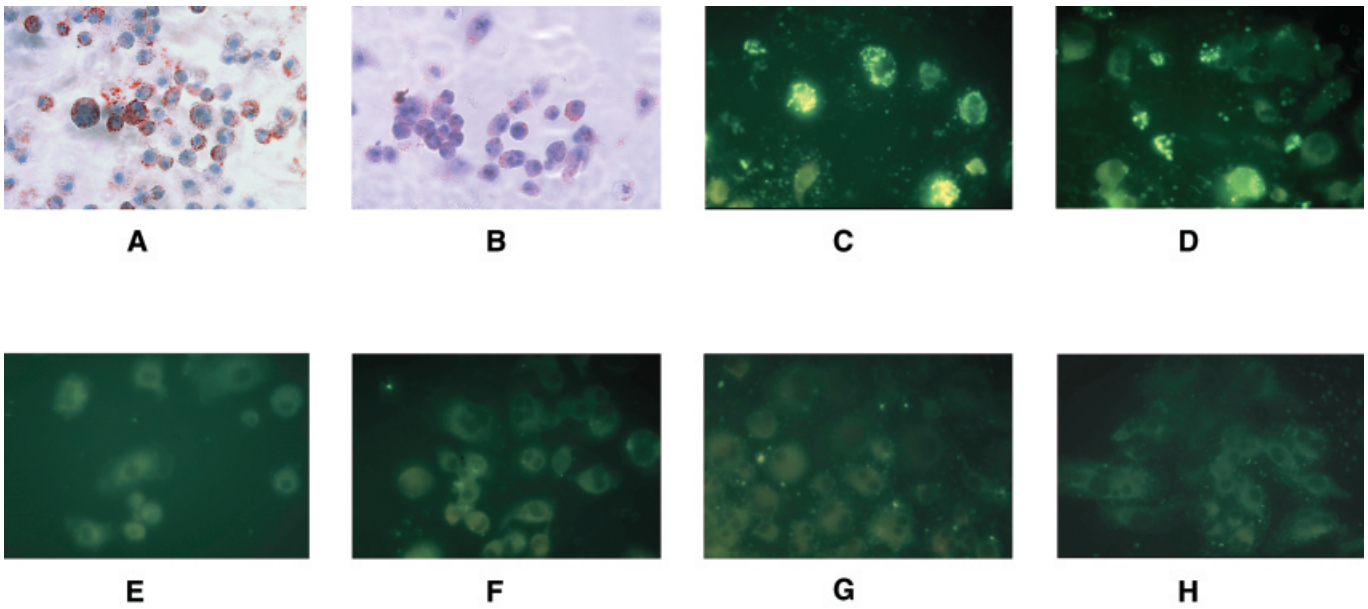

Fig. 2. The effect of various lipoproteins on 256C antigen expression. VLDL, LDL, and HDL fractions were separated from human plasma, and modified LDL were prepared as described under Materials and Methods. J774.1 macrophages pretreated with LPS were incubated with or without these lipoprotein fractions for 24 h, and then further cultured for 3 days with medium A. Cells were stained either with oil red O and hematoxylin (A and B; original magnification, ×400) or with 256C antibody immunohistochemically (C–H; original magnification, ×1,000). The cells were treated with WHHL serum (final cholesterol concentration, 50 μg/ml; A and C), AcLDL (50 μg/ml; B and D), LDL (50 μg/ml; E), OxLDL (50 μg/ml; F), or VLDL (50 μg/ml; G), or were not treated (H).

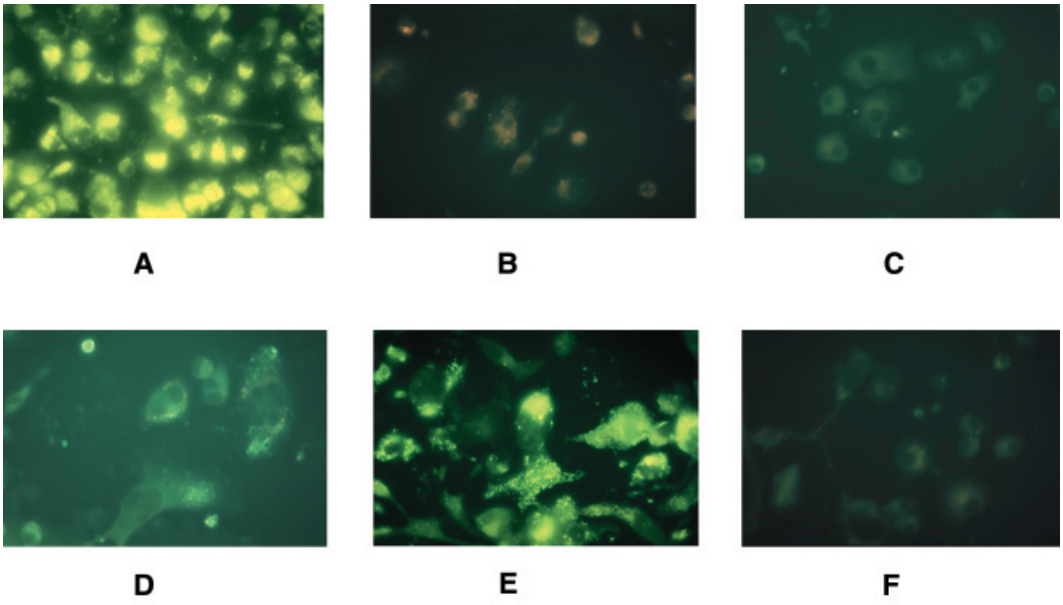

Fig. 3. The expression of 256C antigen is independent of oxidized lipids. J774.1 macrophages pretreated with LPS were incubated with WHHL serum (final cholesterol concentration, 30 μg/ml; A and D) or OxLDL (100 μg/ml; B and E), or without addition of lipoproteins (C and F) for 24 h, and then further cultured for 3 days with medium A. Cells were immunostained with either 256C (A–C) or DLH3 (D–F). Original magnification, ×400.

Table 1 summarizes the effects of various types of lipoprotein on induction of morphological changes and antigen expression in J774.1 macrophages. The 256C-positive lipid droplets appeared only when AcLDL or WHHL serum was added to macrophages; both AcLDL and WHHL serum were strong inducers of foam cell formation. It is suggested that the expression of 256C antigen is associated with foam cell formation but is independent of lipid peroxidation, because expression of DLH3 antigen did not correlate with that of 256C antigen. The same results were observed when MPM were used (see above).

It is interesting to ask whether human serum under certain pathological conditions can induce 256C antigen in foam cells, just like WHHL serum. To test this possibility, small volumes of plasma obtained from hyperlipidemic patients were added to J774.1 macrophages. Foam cells were formed in most of the cases (18 of 21) and 256C antigen was induced in 4 cases. **Figure 4** shows the typical features of foam cells derived from J774.1 macrophages after treatment of human plasma from a hyperlipidemic patient. Just as in the experiments using WHHL serum, lipid droplets are clearly stained by 256C antibody. No 256C antigen was observed when cells were treated with plasma from healthy subjects (data not shown). These results suggest that the induction of 256C-positive foam cells is not a specific property of WHHL serum but may be a common property of sera from patients with certain pathological conditions including hyperlipidemia.

TABLE 1. Expression of antigen in J774.1 macrophages after incubation with various human plasma lipoproteins

Lipoprotein	Foam Cell Formation	Expression of DLH3 Antigen	Expression of 256C Antigen
LDL(100 μg/ml)	–	–	–
AcLDL (100 μg/ml)	++	–	++
OxLDL (100 μg/ml)	+	++	–
HDL_3 (100 μg/ml)	–	–	–
HDL_2 (100 μg/ml)	–	–	–
VLDL (100 μg/ml)	++	–	–
WHHL plasma (40 μg cholesterol/ml)	++	+	++

256C recognizes lipid droplets

As shown in Fig. 4, lipid droplets in foam cells are stained primarily by 256C antibody. We subsequently performed double staining experiments to further investigate the intracellular localization of the antigen and neutral lipids. Macrophages were treated with WHHL serum for 24 h and cultured in medium A for 3 days, and then the cells were immunostained with 256C, followed by staining with hematoxylin and oil red O. The same foam cells were observed by phase-contrast light microscopy (**Fig. 5A**) and by fluorescence microscopy (Fig. 5B). Lipid droplets, faintly stained in pink with oil red O, were localized to peripheral regions of the foam cells. The green fluorescent spots, stained with 256C antibody, closely overlapped with the oil red O-positive droplets. Because oil red O shows red fluorescence under excitation at the same wavelength as FITC, colocalization of oil red O-positive red stain and FITC-positive green stain was clearly observed in some spots (Fig. 5B). This result suggests that antigen recognized by 256C antibody is specifically located in the lipid droplets in foam cells. The similar colocalization between

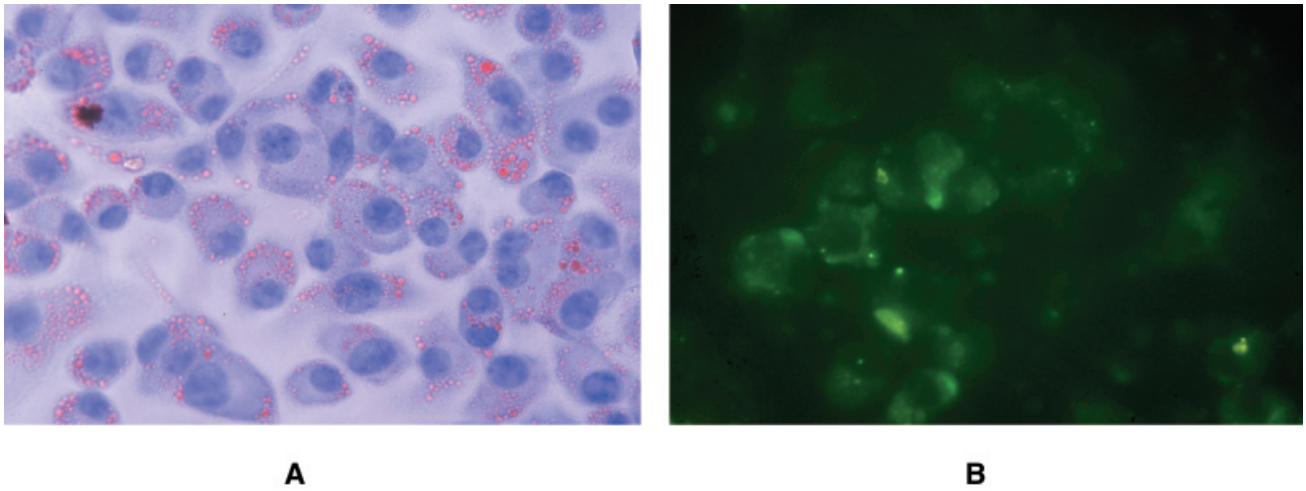

Fig. 4. Expression of 256C antigen induced by hyperlipidemic patients. J774.1 macrophages pretreated with LPS were incubated with plasma from hyperlipidemic patients (final cholesterol concentration, 50 μg/ml). Cells were fixed with neutral formalin and stained either with oil red O and hematoxylin (A) or 256C antibody (B). Original magnification, ×400.

lipid droplets and 256C antigens was also observed in the case of MPM (data not shown).

Lipid metabolism in foam cells

Foam cell formation was not sufficient to express 256C antigen; prolonged incubation of the foam cells was required. To understand the biochemical changes in cellular lipid droplets that are related to the expression of 256C antigen, the amounts of neutral lipid in the cells during 4 days of culture were measured. J774.1 macrophages were pretreated with LPS for 24 h without serum to allow the cells to become differentiated. The cells were then incubated with various human lipoproteins, modified lipoproteins, or WHHL serum for 1 day, followed by additional incubation without lipoproteins for up to 3 days. The cells did not grow and they remained in good condition throughout the culture schedule, so that the changes in cell numbers and amount of cell protein were less than 15%. After cells were treated with WHHL serum for 24 h, cellular contents of CE and TG increased extensively, and the amounts of FC increased back to basal levels (~6 μg per 2×10^6 cells). After the medium was replaced

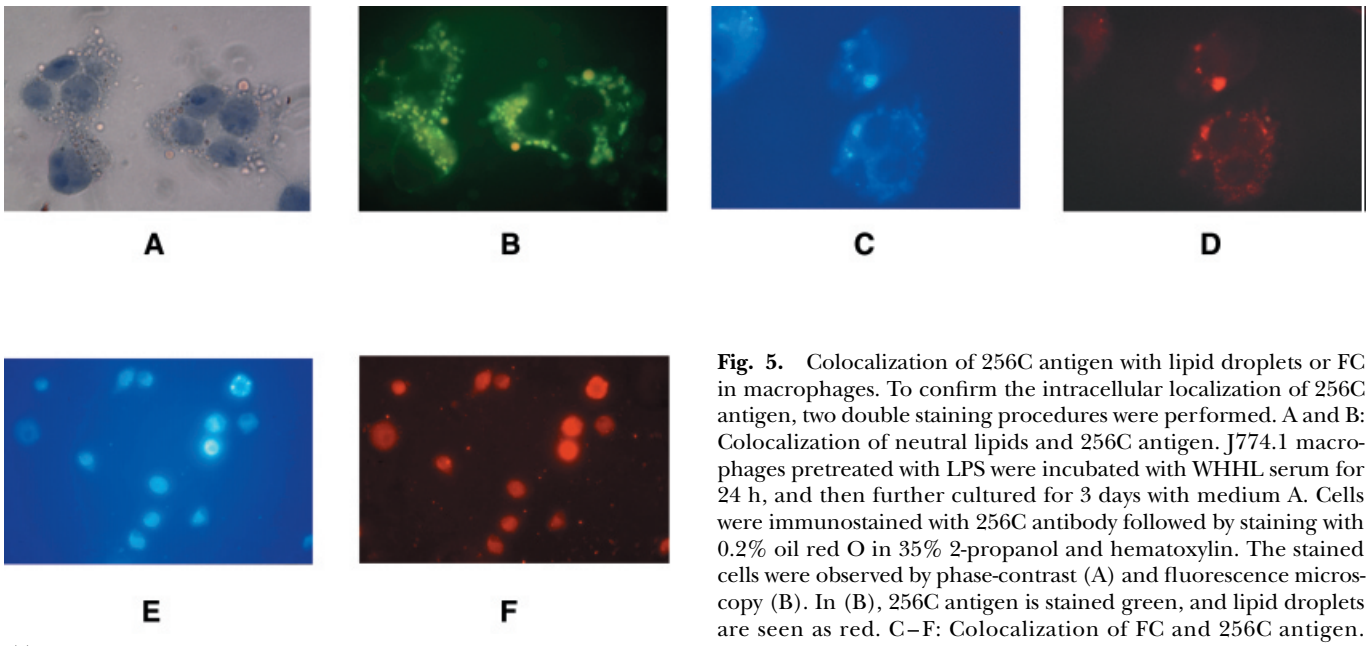

Fig. 5. Colocalization of 256C antigen with lipid droplets or FC in macrophages. To confirm the intracellular localization of 256C antigen, two double staining procedures were performed. A and B: Colocalization of neutral lipids and 256C antigen. J774.1 macrophages pretreated with LPS were incubated with WHHL serum for 24 h, and then further cultured for 3 days with medium A. Cells were immunostained with 256C antibody followed by staining with 0.2% oil red O in 35% 2-propanol and hematoxylin. The stained cells were observed by phase-contrast (A) and fluorescence microscopy (B). In (B), 256C antigen is stained green, and lipid droplets are seen as red. C–F: Colocalization of FC and 256C antigen. J774.1 macrophages pretreated with LPS were incubated with WHHL serum for 24 h, and then further cultured for 6 days with medium A (C and D). MPM was incubated with WHHL serum for 24 h, and then further cultured for 3 days (E and F). Cells were stained with filipin (1.33 μg/ml) for 1 h before fixation (C and E), and then the cells were immunostained with 256C and developed with TRITC-conjugated avidin and biotin-labeled secondary antibody (D and F). Blue spots in (C) and (E) show filipin-positive materials and red spots in (D) and (F) show 256C-positive materials. Original magnification: (C and D) ×1,000; (E and F) ×400.

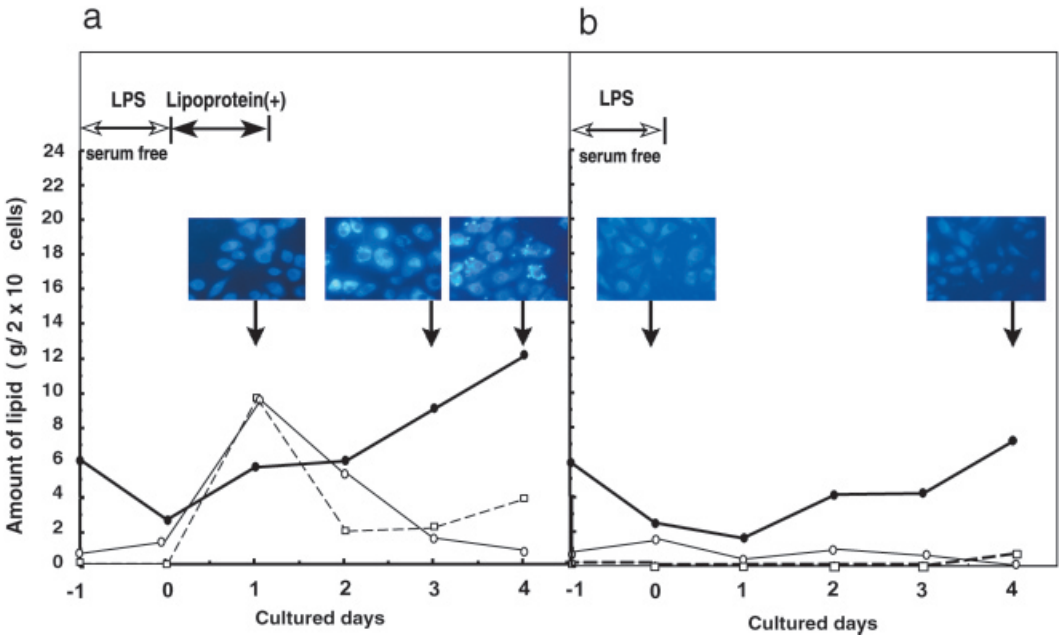

Fig. 6. Intracellular lipid profiles during foam cell culture. J774.1 macrophages pretreated with LPS (2×10^6 cells) were incubated with (A) or without (B) WHHL serum (final cholesterol concentration, 30 μg/ml) for 24 h, and then further cultured for up to 3 days with medium A. The amounts of CE (open circles), FC (closed circles), and TG (open squares) in total cell lysates were measured as described in Materials and Methods. Inset figures show cells stained with filipin (1.33 μg/ml) for 1 h before fixation on the day indicated by arrows. Filipin-positive FC-enriched droplets were observed on days 3 and 4 when cells were treated with WHHL serum (A, inset).

with medium A containing 7% FCS, CE and TG contents were reduced to nearly basal levels; however, the amounts of FC increased during the incubation (**Fig. 6A**). Because total cholesterol content (sum of FC and CE) did not change during the 5-day culture, the increased FC should be due to hydrolysis of CE. FC in the cells treated with WHHL serum was visualized with filipin as a fluorescent probe. Filipin-positive droplets were clearly observed in the cells on days 3 and 4, whereas staining with filipin was slight on days 0 and 1 (as shown in Fig. 6, inset). When the cells were cultured without addition of WHHL serum, the amount of FC returned to basal levels by day 4 and the lev-

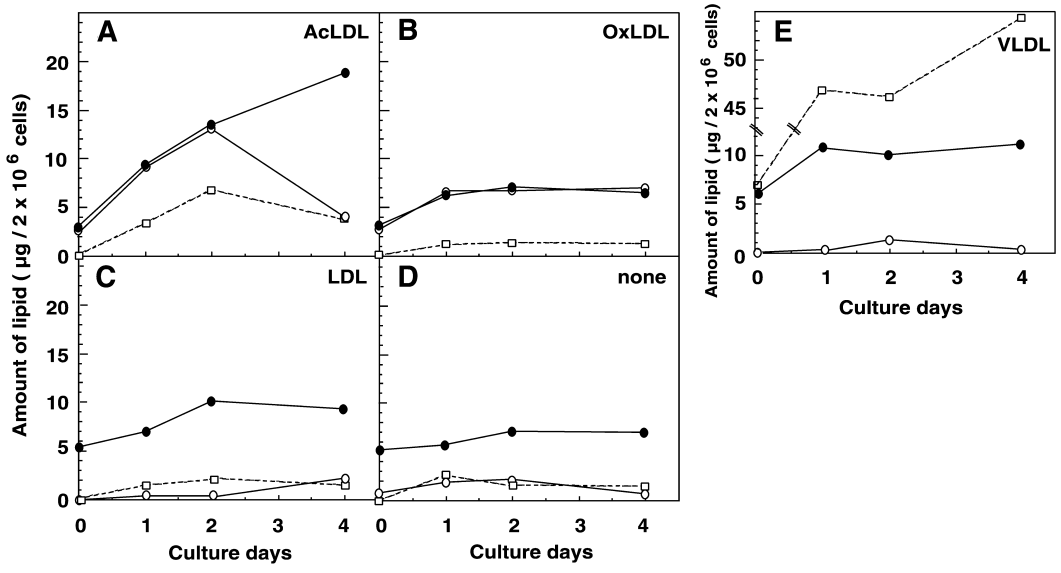

Fig. 7. Intracellular lipid profiles after incubation with various lipoproteins. J774.1 macrophages pretreated with LPS (2×10^6 cells) were incubated with various human lipoproteins (50 μg/ml) for 24 h and further cultured for up to 3 days. The amounts of CE (open circles), FC (closed circles), and TG (open squares) in total cell lysates were measured. Cells were treated with either AcLDL (A), OxLDL (B), LDL (C), nothing (D), or VLDL (E).

els of CE and TG were not significantly altered (Fig. 6B). Filipin staining of the cells without WHHL serum treatment on day 4 was faint and did not change from that on day 0 (Fig. 6B, inset)

Sustained increases in cellular FC beyond basal levels were observed after incubation of the cells with AcLDL (**Fig. 7A**), but cellular FC during the culture was only slightly increased by treatment of cells with other types of lipoproteins (Fig. 7B–E). Treatment of cells with OxLDL induced slight increases in CE as well as FC; however, metabolic transformation of CE to FC did not seem to occur during culture (Fig. 7B). TG content was significantly increased by treatment with VLDL, but FC levels changed moderately (Fig. 7E). These results suggest that expression of 256C antigen is associated with an increase in cellular FC content.

Relationship between FC-rich droplets and 256C antigen

To determine whether FC is directly involved in 256C antigen formation, the cells treated with WHHL serum were doubly stained with 256C antibody and filipin. As shown in Fig. 5, WHHL serum-induced foam cells were stained with filipin for 1 h before fixation, and then the cells were immunostained with 256C antibody. It is clear that the droplets stained positively with both filipin (Fig. 5C) and the antibody (Fig. 5D), and the distributions of these positive spots corresponded with each other. These results suggest that the 256C-positive lipid droplets are enriched with FC. The colocalization between filipin-positive structures and 256C antigens was again observed in MPM (Fig. 5E and F).

We have previously determined that smooth muscle cells as well as macrophages take up lipid droplets composed of cholesteryl oleate to form foam cells (19, 23). Cholesteryl oleate can form two physicochemical structures, a gel state and a liquid crystalline state. Above the transition temperature (42°C) liquid crystal is formed, in which cholesteryl oleate molecules align properly but still are allowed to move without breaking intermolecular interactions. Cellular droplets forming liquid crystals are clearly observed under polarized light. When the composition of lipid droplets changes, liquid crystal structure is destroyed, so that it no longer remains visible under polarized light.

We utilized this property of liquid crystallization to demonstrate the involvement of FC in the expression of 256C antigen in macrophages. J774.1 macrophages were incubated with cholesteryl oleate liquid crystals for 24 h,

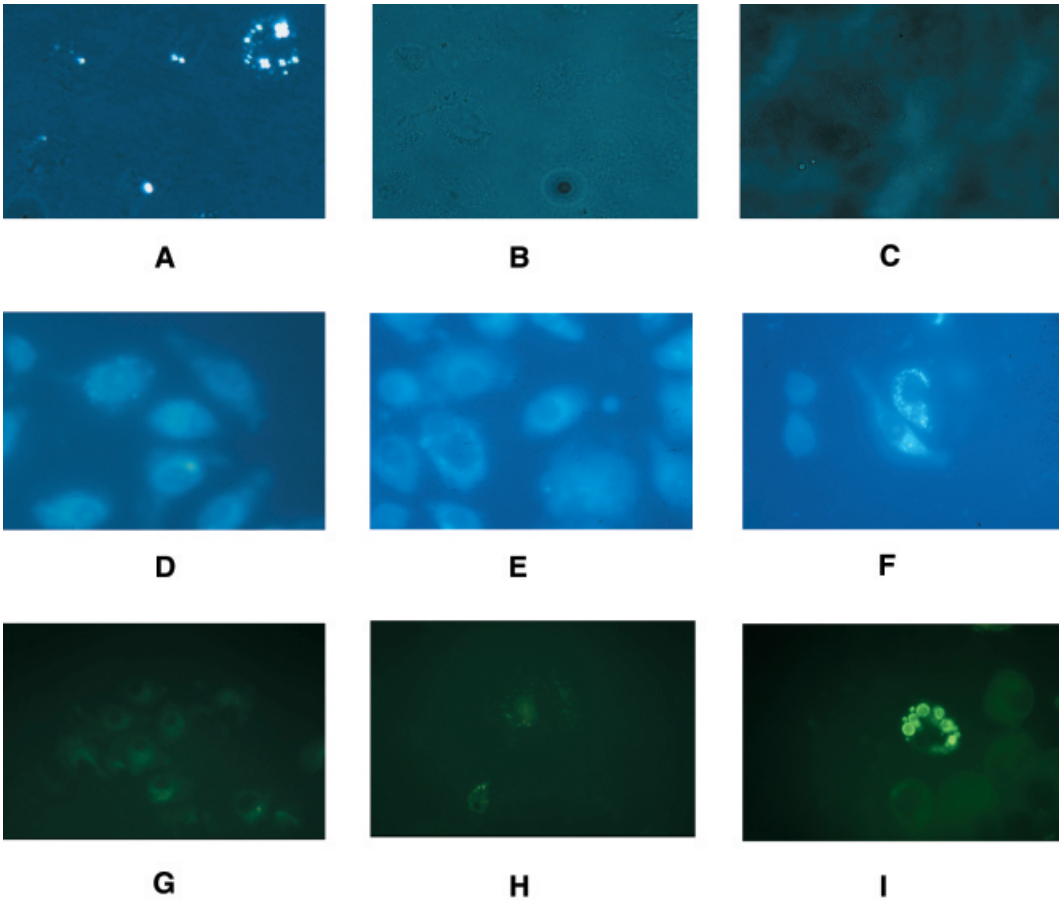

Fig. 8. Hydrolysis of cholesteryl oleate liquid crystals in cultured foam cells leads to expression of 256C antigen. Cholesteryl oleate liquid crystals were prepared as described in Materials and Methods. J774.1 macrophages pretreated with LPS were incubated with liquid crystals (final cholesteryl oleate concentration, 42 nmol/ml) for 24 h (A, D, and G), and then further cultured for either 1 day (B, E, and H) or 3 days (C, F, and I) with medium A. Cells were observed by polarized microscopy (A–C), stained with filipin (D–F), or immunostained with 256C antibody (G–I). Original magnification, ×400.

and then the cells were further incubated for 3 days in the absence of cholesteryl oleate. After incubation with cholesteryl oleate, bright circles with black crosses were observed under polarized light, indicating the presence of liquid crystals in the cells (**Fig. 8A**). Under the same conditions, cells were negative for either filipin or 256C antibody (Fig. 8D and G). The liquid crystals disappeared by day 2 (Fig. 8B and C). Filipin staining of these cells shows that there were no FC-rich particles in macrophages observed on day 2, although the fluorescence intensity is slightly increased (Fig. 8E), and that FC-enriched particles appeared on day 4 (Fig. 8F). As corresponding to the filipin staining, 256C-positive droplets were expressed in the cells on day 4 (Fig. 8I), whereas a few positive dots were seen on days 1 and 2 (Fig. 8G and H). These results suggest that PC-FC complexes were formed after CE in lipid particles are hydrolyzed in macrophages.

DISCUSSION

The appearance of foam cells that accumulate lipid droplets intracellularly is a remarkable feature of atherosclerotic lesions (1–3). The mechanisms of foam cell formation have been extensively studied for more than a decade. More than 10 scavenger receptors have been identified and cloned and their pathological properties have been investigated (24). The presence of OxLDL, the putative ligand for scavenger receptors, in human circulating plasma has been demonstrated (12, 25–27). These studies strongly suggest the importance of foam cell formation in atherogenesis in vivo. However, the fate of foam cells after lipid droplet formation is still poorly understood. Because atherosclerosis is considered to be a chronic condition requiring decades to develop (4), understanding the fate of foam cells could be of great importance in the elucidation of the precise pathophysiology of the disease.

In the current study, we established a cell culture system that enables us to maintain foam cells for at least 14 days. Using this system, we found that the antigenic lipid droplets recognized by monoclonal antibody 256C could be generated in foam cells. This antibody binds to atherosclerotic plaques of WHHL rabbits in vivo and stains immunohistochemically foam cell-enriched lesions (8). We propose that these cell culture conditions provide a useful model system to investigate the behavior of foam cells as well as the cellular basis of atherosclerosis.

Among a series of monoclonal antibodies against atherosclerotic lesions established in our previous studies, 256C recognizes PC molecules containing polyunsaturated fatty acyl chains as its epitope (8). It should be noted that binding of this antibody to PC is greatly enhanced by the addition of neutral lipids such as FC (8). The antibody bound effectively to PC mixed with FC when it was coated onto microtiter wells for ELISA, but it did not bind with liposomes containing PC and FC or native LDL, suggesting that 256C antibody preferably recognizes PC molecules present in certain unusual structures as antigen. The possibility that phospholipid molecules in certain structures can be recognized by antibodies has been shown by Aguilar et al. (28). This group obtained a novel monoclonal antibody that recognized phospholipids in hexagonal II, a nonbilayer structure with an inverted cylindrical shape. Because PC is a common component present ubiquitously in animal cells, the 256C antigen present in atherosclerotic lesions is expected to be a unique complex structure containing PC and FC.

We found in this study that lipid droplets that were immunologically reactive with 256C antibody appeared in foam cells under certain conditions. This observation supports the possibility that 256C antigen is one of the unique features of atherosclerotic lesions. Expression of 256C antigen was induced by treatment of cells with human plasma from patients with hyperlipidemia as well as WHHL serum. Addition of normal rabbit serum to cells, however, did not induce 256C antigen expression, even when a large volume of normal rabbit serum was used to achieve the same cholesterol concentrations in the culture medium. Thus it is assumed that certain yet unidentified materials present under hyperlipidemic conditions might be responsible for induction of 256C antigen in foam cells.

It is interesting that WHHL serum, but not serum from normal rabbits, is capable of inducing 256C antigen. We found that only AcLDL induced 256C antigen in foam cells on addition to macrophages, but other lipoproteins as well as OxLDL did not mimic WHHL serum. AcLDL is a well-known ligand for scavenger receptors but it is thought to be a nonphysiological model of modified LDL (3, 4). The actual modified LDL present in vivo has not been clarified yet; however, OxLDL is a presumed candidate for it, and strong evidence of the presence of OxLDL in human plasma has been reported (12, 25–27). Under our experimental conditions, WHHL-induced foam cells were immunologically positive against DLH3 antibody, which recognizes OxPC present in OxLDL (21, 22), suggesting a contribution of OxLDL present in WHHL serum to foam cell formation. Nevertheless, oxidative modification of lipid is unlikely to be directly involved in expression of 256C antigen, because OxLDL failed to induce 256C antigen and AcLDL-induced foam cells were negative for DLH3.

It is noteworthy that 256C antigen appeared only after 2 days of incubation with fresh medium. We found that over 3 days of culture hydrolysis of CE to FC proceeded when foam cells were induced by treatment with WHHL serum or AcLDL. Our double staining experiments demonstrated that 256C antigen colocalized with oil red O-positive lipid droplets and that FC-enriched lipid droplets detected by filipin were 256C positive. These results strongly suggest that 256C antigens found in foam cells are lipid droplets enriched with FC and that 256C antigens are formed because of metabolic modification of CE to FC. It is noted that VLDL fractions failed to induce 256C antigen expression, although activity inducing foam cell formation was prominent. The neutral lipids accumulated in VLDL-induced foam cells are largely TG, and the metabolic transformation of CE to FC was not observed in these foam cells.

Current thinking suggests that after modified lipopro-

teins are taken up by macrophage scavenger receptors CE is hydrolyzed in lysosomes by acid CE hydrolase to yield FC, which is then re-esterified to CE by ACAT on endoplasmic reticulum (3, 4). However, our results from the present study show that FC content increases after CE-rich lipid droplets are formed. The accumulation of intracellular FC was prominent when foam cells were induced by addition of AcLDL or WHHL serum followed by a further 3-day incubation. There are a few earlier studies reporting intracellular accumulation of FC in atherosclerotic lesions; for example, Shio, Haley, and Fowler (29) investigated biochemical features of lipids accumulated in foam cells in cholesterol-fed rabbits. They observed the accumulation in foam cells of FC that was often in the form of lipid globules, membranous whorls, or crystals. Accumulation of FC in the plasma membrane fraction isolated from smooth muscle cells in cholesterol-fed rabbits has also been reported (30). Tabas et al. (31) reported in the earlier study that FC accumulated when J774 cells were incubated with AcLDL in the presence of ACAT inhibitor. Under our experimental conditions, AcLDL-induced foam cells were cultured in the presence of 7% FCS from day 2 to day 4; this condition may allow efflux of cholesterol from the cells and concomitant hydrolysis of CE to FC. Cytosolic FC pools, which are equilibrated with CE by the action of ACAT and neutral CE hydrolase, are hypothesized to be present in macrophages as well as hepatic cells (32, 33). We speculate that delayed accumulation of intracellular FC is one of the metabolic features of foam cells, although CE is accumulated to form lipid droplets at the early response.

It is possible that at least some 256C-positive lipid droplets, which are enriched with FC, are associated with lysosomes. Tangirala et al. (34) reported that FC-enriched lysosomal fractions were separated after incubation of J774.1 macrophages with sonicated cholesteryl oleate droplets for 24 h. Under their experimental conditions, 30% of FC formed during the incubation was recovered in lysosomal fractions. In our preliminary experiments, the lysosomal marker enzyme β-hexosaminidase was present in cholesterol-rich and less dense fractions from foam cells induced with WHHL serum on day 4 (data not shown). It was reported that at least part of the excess cellular cholesterol is associated with lysosomes in atherosclerotic lesions in cholesterol-fed rabbits (29). Lupu, Danaricu, and Simionescu (35), after extensive observation of aortic lesions from cholesterol-fed rabbits, reported that there were lipid droplets in a liquid crystal state in foam cells in early lesions and that multilamellar structures, which may be enriched with FC, were accumulated in lysosomes in advanced stages of the lesions. It is possible that FC-enriched droplets are associated with lysosomes; however, the presence of FC in lysosomes should not be sufficient for the appearance of 256C antigen. Previous articles reported that when cholesteryl oleate liquid crystals were incubated with macrophages, CE-containing phagosomes fused to lysosome within 2 h and that most of the CE was hydrolyzed within 24 h (36, 37). In the present study, 256C antigen did not appear within 2 days after the addition of liquid crystals (Fig. 8). We would like to point out that the mere presence of a large amount of FC does not seem to be enough to induce the appearance of 256C antigen, because macrophages incubated with PC-FC liposomes were 256C negative on day 1 but had become 256C positive by day 4 (data not shown). Accumulation of intracellular FC may not be sufficient for the expression of 256C antigen; rather, it is likely that lipids underwent rearrangement during the following culture period to form antigenic complex structures.

It is interesting that delayed hydrolysis of CE in lipid droplets was observed only when macrophages were treated with AcLDL, WHHL serum, or patient plasma to induce foam cells (Fig. 2 and Table 1), and the induction of delayed CE hydrolysis could be directly responsible for expression of 256C antigen. It is thought that CE in cytosolic lipid droplets are hydrolyzed by neutral CE hydrolase (31, 32, 38). An additional possibility to be considered is that AcLDL and modified lipoproteins in WHHL serum may modulate neutral CE hydrolase activity, because an endogenous inhibitor protein for neutral CE hydrolase has been found in macrophages (39).

Another intriguing finding in this study is that some foam cells induced by treatment with WHHL serum rupture after 7 days of culture (Fig. 1C). There were a number of lipid droplets remaining on culture slides while nuclei and membranous structures were absent. We speculated that one possible route for the accumulation of extracellular lipids in atherosclerotic lesions may be foam cell rupture and subsequent scattering of cytosolic lipid droplets (23, 40). Chao et al. (41) showed that there were two types of cholesterol-containing particles in the extracellular space in atherosclerotic lesions: one type of particle was enriched with CE and the other type was enriched with FC. It has been reported that increases in intracellular FC could be toxic to foam cells, which can then lead to cell death (31, 42). The mechanism leading to cell death is not known; however, increased cellular FC may damage the membrane organization of foam cells. We are currently studying the mechanisms of this foam cell rupture.

In conclusion, the current study suggests that cellular lipids in foam cells are metabolized during culture to generate modified lipid droplets containing PC-FC complex structures. This could be a novel feature of atherosclerotic lesions, and our experimental conditions provide a useful cell culture system to investigate long-term characterization of macrophage-derived foam cells.

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