Monoclonal antibody detection of plasma membrane cholesterol microdomains responsive to cholesterol trafficking

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Abstract The hypothesis of lipid domains in cellular plasma membranes is well established. However, direct visualization of the domains has been difficult. Here we report direct visualization of plasma membrane cholesterol microdomains modulated by agents that affect cholesterol trafficking to and from the plasma membrane. The cholesterol microdomains were visualized with a monoclonal antibody that specifically detects ordered cholesterol arrays. These unique cholesterol microdomains were induced on macrophages and fibroblasts when they were enriched with cholesterol in the presence of an ACAT inhibitor, to block esterification of excess cellular cholesterol. Induction of the plasma membrane cholesterol microdomains could be blocked by agents that inhibit trafficking of cholesterol to the plasma membrane and by cholesterol acceptors that remove cholesterol from the plasma membrane. In addition, plasma membrane cholesterol microdomains did not develop in mutant Niemann-Pick type C fibroblasts, consistent with the defect in cholesterol trafficking reported for these cells. The induction of plasma membrane cholesterol microdomains on inhibition of ACAT helps explain how ACAT inhibition promotes cholesterol efflux from cells in the presence of cholesterol acceptors such as HDL. The anti-cholesterol monoclonal antibody also detected extracellular cholesterol-containing particles that accumulated most prominently during cholesterol enrichment of less differentiated human monocyte-macrophages. For the first time, cholesterol microdomains have been visualized that function in cholesterol trafficking to and from the plasma membrane.—Kruth, H. S., I. Ifrim, J. Chang, L. Addadi, D. Perl-Treves, and W-Y. Zhang. **Monoclonal antibody detection of plasma membrane cholesterol microdomains responsive to cholesterol trafficking. *J. Lipid Res.* 2001.** 42: **1492–1500**.

Supplementary key words acyl-CoA:cholesterol acyltransferase • cholesterol efflux • macrophages • atherosclerosis • HDL • progesterone • ketoconazole • cyclodextrin • apoA-I • apoE • Niemann-Pick type C

Studies of the cellular trafficking of cholesterol derived from the metabolism of LDL show that after hydrolysis of LDL cholesteryl ester in lysosomes, most LDL-derived cholesterol trafficks to the plasma membrane (1–3). This cholesterol is believed to mix with the bulk cholesterol in the plasma membrane (4). Excess plasma membrane cholesterol then enters the cytoplasm, where the cholesterol is re-esterified by ACAT and stored in cellular lipid droplets (**Fig. 1**).

How cells and macrophages in particular eliminate excess cholesterol has been of great interest, and is important to the study of macrophage function with respect to cholesterol deposition in developing atherosclerotic plaques. Previous studies have shown a role for amphipathic apolipoproteins in this process (5). Amphipathic apolipoproteins such as apolipoprotein A-I (apoA-I) and apoE are carried by HDL in the blood, and for apoE, can also be produced by macrophages. Amphipathic apolipoproteins can mediate cholesterol efflux from many cell types including macrophages. In this regard, inhibition of ACAT promotes cholesterol efflux from cholesterol-enriched macrophages during incubation with amphipathic apolipoproteins (6–9). This may occur in part because blocking cholesterol esterification in macrophages with an ACAT inhibitor causes more cholesterol to appear in the plasma membrane (10, 11). The plasma membrane is one location from where cholesterol can efflux from cells. Kinetic studies suggest that cholesterol does not efflux randomly from the cell plasma membrane, but rather that cholesterol exits the plasma membrane from specific pools (12).

Here we show monoclonal antibody (MAb) detection of a plasma membrane pool of cholesterol that responds to agents that modulate cholesterol trafficking within human monocyte-derived macrophages. Labeling of macrophage plasma membrane cholesterol was carried out by indirect

Abbreviations: AcLDL, acetylated LDL; apo, apolipoprotein; DPBS, Dulbecco's phosphate-buffered saline; MAb, monoclonal antibody; NPC, Niemann-Pick type C.

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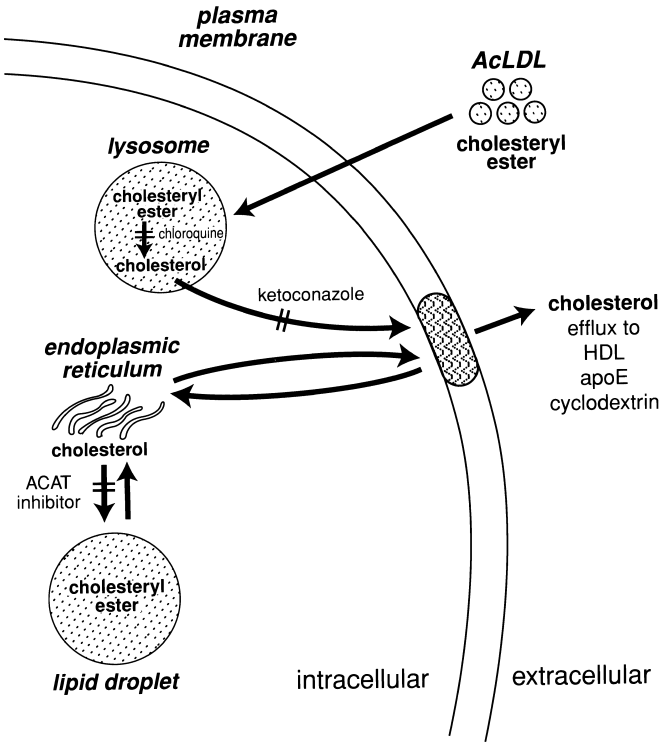

Fig. 1. Schema of cholesterol trafficking in monocyte-macrophages.

immunofluorescence using MAb 58B1 characterized previously (13). This antibody was selected by using an assay based on recognition of cholesterol monohydrate crystal surfaces. The antibody interacts specifically also with monolayers of cholesterol deposited at the air-water interface but does not recognize individual cholesterol molecules (13, 14). It thus reacts cooperatively with several cholesterol molecules (approximately 12; L. Addadi unpublished results) assembled in structured domains. Evidence of the existence of anti-cholesterol antibodies is controversial (15, 16). Our findings substantiate the existence of antibodies that specifically recognize organized arrays of cholesterol (17). With such an antibody we show that inhibition of ACAT may facilitate cholesterol efflux by inducing cholesterol-rich microdomains in the plasma membrane.

MATERIALS AND METHODS

Materials

Human HDL, LDL, apoA-I, lipoprotein-deficient serum, and fetal bovine serum were obtained from PerImmune (Rockville, MD); cholesterol oxidase was obtained from Boehringer Mannheim (Indianapolis, IN); bovine pancreas trypsin (3,000 U/mg) was from Life Technologies (Rockville, MD); cholesterol (C8667), progesterone, ketoconazole, chloroquine, 2-hydroxypropyl-β-cyclodextrin, and fatty acid-free BSA for rinsing cells before fixation were obtained from Sigma (St. Louis, MO); Triton X-100 was obtained from Research Organics (Cleveland, OH); methanol-free paraformaldehyde was obtained from Polysciences (Warrington, PA); ethanol was obtained from Burdick & Jackson (division of Baxter Healthcare, Muskegon, MI); mouse anti-cholesterol MAb 58B1 in ascites was obtained from L. Addadi (Weizmann Institute of Science, Rehovot, Israel) and purified with a Unisyn (Hopkinton, MA) Ig pure antibody purification kit; mouse anti-*Clavibacter michiganense* MAb (clone 9A1) in ascites was obtained from Agdia (Elkhart, IN) and was purified as described above; biotinylated goat anti-mouse IgM (BA 2020) and fluorescein-avidin (A2011) were obtained from Vector Laboratories (Burlingame, CA); BSA (ARO1) for antibody dilution was obtained from Chemicon International (Temecula, CA); Glycergel mounting medium was obtained from Dako (Carpinteria, CA); plastic chamber slides were obtained from Lab-Tek (Nalge Nunc, Naperville, IL); recombinant human apoE and S58-035 ACAT inhibitor were gifts from Bio-Technology General (Iselin, NJ) and Sandoz (Basel, Switzerland), respectively; normal human dermal fibroblasts were obtained from Clonetics (Walkersville, MD), and mutant Niemann-Pick type C (NPC) dermal fibroblasts (GM00110B and GM03123A) were obtained from the Coriell Institute for Medical Research (Camden, NJ).

Labeling of cholesterol

Human monocyte-derived macrophage cultures were established as previously described (7). Four, 7-, or 14-day-old macrophage cultures (grown on two- or four-well plastic chamber slides) were incubated for 2 days with either microcrystalline cholesterol (50 μg/ml) (18) or acetylated LDL (AcLDL) (50 μg/ml) (19) and the indicated concentration of drugs or cholesterol acceptors in RPMI 1640 medium. Normal and NPC mutant human dermal fibroblast cultures (two different donors) were established by seeding fibroblasts at a density of $2.5 \times 10^3/cm^2$ into two-well plastic chamber slides in DMEM plus 10% fetal bovine serum. After 1 week of incubation and replenishing medium every other day, cultures were rinsed with DMEM, fed with DMEM plus 10% human lipoprotein-deficient serum, and incubated for 3 days to upregulate LDL receptor expression in the fibroblasts. Fibroblast cultures were then incubated for 3 days without or with LDL (50 μg/ml) in the absence or presence of the ACAT inhibitor S58-035 (4 μg/ml).

After incubations, cell cultures were rinsed three times in Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (DPBS) containing 0.35% BSA and then three times (5 min each) in DPBS without BSA. All procedures were carried out at room temperature and all subsequent rinses were performed three times in DPBS. Cultures were fixed for 1 h with 4% paraformaldehyde in DPBS, rinsed, incubated for 1 h with purified mouse anti-cholesterol MAb 58B1 [IgM(κ); 5 μg/ml] or an irrelevant purified mouse anti-*C. michiganense* MAb [IgM(κ)]. After cultures were rinsed with DPBS, they were incubated for 30 min with biotinylated goat anti-mouse IgM (50 μg/ml). All antibodies were in DPBS containing 0.1% BSA. After rinsing, cultures were incubated for 30 min with fluorescein-avidin (20 μg/ml) in 0.1 M sodium bicarbonate buffer with 0.15 M NaCl added (final pH 8.2). Last, cultures were rinsed and the chamber slides were mounted in Glycergel. In some experiments, macrophages were labeled with the fluorescent cholesterol-binding agent filipin, as described previously (20).

Elimination of cholesterol labeling with cholesterol oxidase

Monocyte-macrophage cultures were incubated for 2 days in RPMI 1640 medium with AcLDL (50 μg/ml) plus ACAT inhibitor (4 μg/ml) to induce plasma membrane cholesterol microdomains. Cultures were then rinsed and fixed before being incubated for 3 h at 37°C in 0.1 M potassium phosphate buffer (pH 7.4) without or with cholesterol oxidase (1.5 U/ml). After this treatment, cultures were labeled with anti-cholesterol MAb 58B1.

Microscopy

Cell fluorescence was evaluated by conventional and confocal fluorescence microscopy. The approximate frequency of positive and negative cells was estimated for all conditions after examining the entire culture with a ×10 objective. This estimate was then confirmed by examination with a ×25 objective of at least 10 microscopic fields for each culture condition. Each microscopic field contained about 40 to 60 cells. All experiments were carried out at least twice, and the findings were reproducible.

Confocal images were collected on a microscope (Axioplan; Carl Zeiss, Thornwood, NY) equipped with a laser scanning confocal attachment (model MRC-1024; Bio-Rad, Hercules, CA) using a ×63 1.4 NA oil objective lens. Fluorescein was excited at a wavelength of 488 nm generated with an argon-krypton mixed gas laser, and its emission was detected through a 522DF32 filter. Generally, between 20 and 40 Z-series digital images (the number of images depended on macrophage thickness) with a Z-step of 0.5 μm were obtained at a resolution of 512 × 512. In some cases (i.e., Figs. 2 and 5), photomicrographs were produced from the collapsed Z-series digital images, using Confocal Assistant software (Bio-Rad).

RESULTS

Induction of cholesterol microdomains by an ACAT inhibitor

Because cells were not permeabilized before labeling with the cholesterol MAb, only the external plasma membrane surface of cells, not intracellular structures, were labeled. MAb 58B1 did not label macrophage plasma membranes under basal conditions when macrophages were incubated without (**Fig. 2a**) or with 10% human serum. Only when macrophages were incubated with AcLDL (the acetylated derivative of LDL taken up by macrophage scavenger receptors) or AcLDL plus an ACAT inhibitor (S58-035) did macrophage plasma membranes label with MAb 58B1. The ACAT inhibitor blocked esterification of excess unesterified cholesterol and by that increased the cellular content of unesterified cholesterol (**Table 1**). Incubation of macrophages with ACAT inhibitor alone did not induce MAb 58B1 labeling. MAb 58B1 labeled macrophages only rarely after incubation with AcLDL (Fig. 2b), but after incubation with AcLDL plus ACAT inhibitor many macrophages (often >50%) were labeled (Fig. 2c). When these cholesterol-enriched macrophages were further incubated without AcLDL and ACAT inhibitor, the plasma membrane cholesterol microdomains disappeared. This reflects removal of the block in cholesterol esterification and mobilization of cholesterol from the microdomains.

Once macrophages esterify cholesterol, the esterified cholesterol undergoes a continuous cycle of hydrolysis and re-esterification (21). Thus, after AcLDL-derived cholesterol is initially esterified, addition of ACAT inhibitor should also cause the buildup of unesterified cholesterol in macrophages. We confirmed this with chemical experiments (data not shown). Although this buildup of unesterified cholesterol occurred after AcLDL loading of macrophages, it nevertheless caused plasma membrane cholesterol microdomains to appear. Thus, many macrophages showed

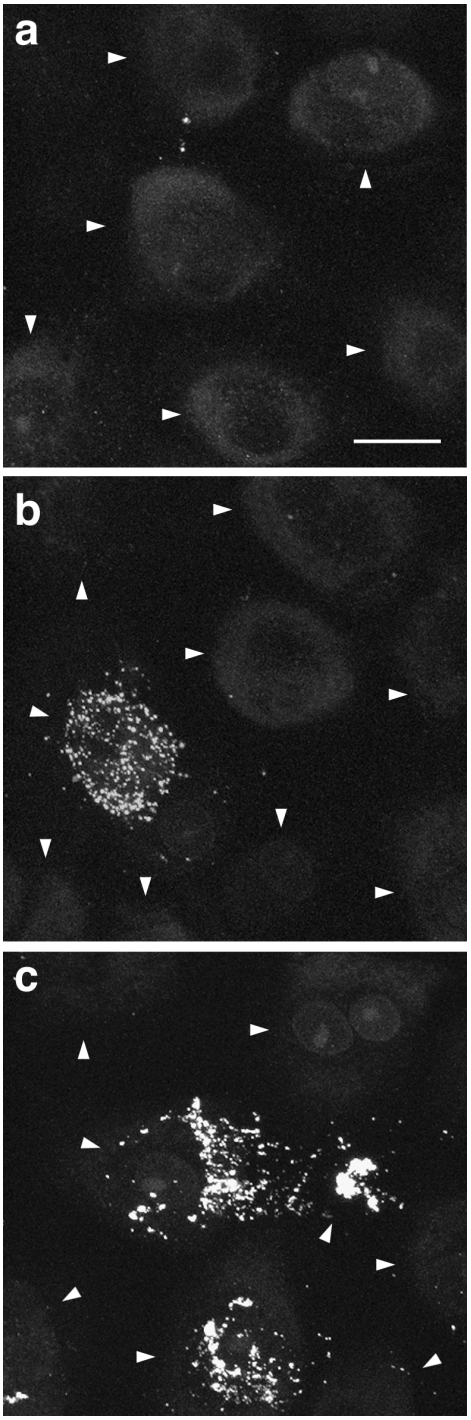

Fig. 2. Plasma membrane cholesterol microdomains induced by macrophage cholesterol enrichment. Two-week-old macrophage cultures were incubated for 2 days in RPMI 1640 medium with no addition (a), AcLDL at 50 μg/ml (b), or AcLDL at 50 μg/ml plus ACAT inhibitor at 4 μg/ml (c). Cultures were then rinsed, fixed, and labeled with anti-cholesterol MAb 58B1. Arrowheads indicate each macrophage in the microscopic field. The fluorescence intensity of macrophages in (a) was no different from the fluorescence intensity of macrophages labeled with an irrelevant monoclonal antibody substituted for MAb 58B1, under all incubation conditions. Thus, the macrophages in (a) show the background levels of fluorescence. Arrowheads indicating macrophages with no detectable fluorescence in the photomicrograph were located by computer enhancement of the digitally collected images. This allowed low levels of macrophage background fluorescence to be visualized. Bar: 10 μm.

TABLE 1. Effect of ACAT inhibitor, ketoconazole, and progesterone on macrophage cholesterol accumulation

Condition	Cholesterol		
	Unesterified	Esterified	Total
	nmol/mg cell protein		
0 day	80 ± 4	5 ± 1	85 ± 4
AcLDL 2 days	111 ± 1	109 ± 5	220 ± 5
AcLDL + ACAT inhibitor (4 μg/ml) 2 days	190 ± 6	2 ± 2	192 ± 5
AcLDL + ketoconazole (10 μg/ml) 2 days	172 ± 4	22 ± 8	194 ± 13
AcLDL + progesterone (10 μg/ml) 2 days	186 ± 5	17 ± 5	202 ± 9

Two-week-old human macrophage cultures were incubated with AcLDL (50 μg/ml) for 2 days with the indicated additions. Macrophages were then rinsed, harvested, and analyzed for their cholesterol and protein contents as described previously (55). Values represent means ± SE of triplicate cultures.

MAb 58B1 labeling when macrophages were incubated for 2 days with AcLDL, followed by incubation for an additional 2 days with basal medium containing ACAT inhibitor. Only rare macrophages showed MAb 58B1 labeling when AcLDL loading was followed by incubation in basal medium without ACAT inhibitor.

The pattern of cholesterol labeling was punctate, suggesting the presence of cholesterol-rich microdomains (see Fig. 2b). Often, in more flattened macrophages, plasma membrane labeling was more focal and possibly represented clustered microdomains of cholesterol (Fig. 2c and 4a). In contrast, macrophage plasma membranes labeled uniformly under all conditions with the fluorescent polyene cholesterol-binding agent filipin (data not shown). The intensity and pattern of MAb 58B1 labeling were similar for macrophages labeled at 4°C or at room temperature.

Besides cholesterol microdomains associated with the plasma membrane, MAb 58B1 also labeled small extracellular particles. These particles extended beyond the plasma membrane like a comet's tail (**Fig. 3**). Only a rare macrophage (<1%) from 2-week-old cultures showed this type of labeling. However, the comets were more often observed (≅10–20% of macrophages) with macrophages from 4- and 7-day-old cultures. As with induced plasma membrane labeling, comets were induced by incubating macrophages in the presence of AcLDL, and the number of comet-like macrophages increased when cholesterol enrichment was carried out in the presence of an ACAT inhibitor. Plasma membrane cholesterol microdomains and extracellular labeled particles in comets were induced not only by incubating macrophages with AcLDL, but also by incubating macrophages with microcrystalline cholesterol (50 μg/ml). Again, the intensity and number of macrophages labeled were increased when macrophages were incubated with microcrystalline cholesterol in the presence of the ACAT inhibitor. Macrophages were observed that showed labeling only of extracellular particles, only plasma membrane, or labeling of both extracellular particles and plasma membrane.

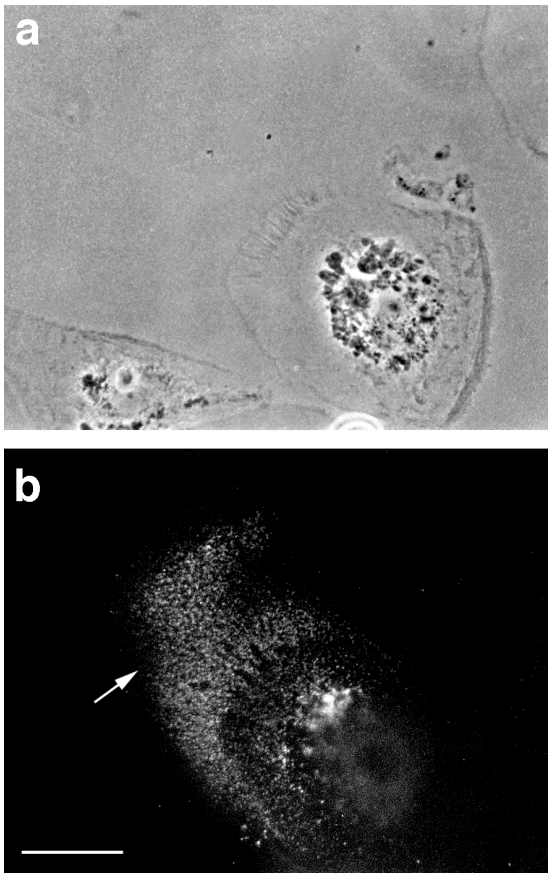

Fig. 3. Extracellular cholesterol particles induced by macrophage cholesterol enrichment. Shown is a 1-week-old macrophage culture that was incubated for 2 days in RPMI 1640 medium with microcrystalline cholesterol (50 μg/ml) plus ACAT inhibitor (4 μg/ml). After incubation, the culture was rinsed, fixed, stained with anti-cholesterol MAb 58B1, and viewed by conventional fluorescence microscopy. Phase (a) and fluorescence (b) images of the same microscopic field. Arrow in (b) indicates MAb 58B1-labeled extracellular particles extending beyond the macrophage plasma membrane that is visualized in (a). These extracellular particles were attached to the well surface. Bar: 28 μm.

Several findings showed the specificity of MAb 58B1 for cholesterol. MAb 58B1 labeling of macrophages was eliminated if macrophages were treated with 70% ethanol or absolute methanol solvents (three 30-min incubations at room temperature) to extract cholesterol just before labeling. Cholesterol oxidase treatment of aldehyde-fixed macrophages effectively oxidizes plasma membrane cholesterol (1, 22). Oxidation of plasma membrane cholesterol by treating macrophages with cholesterol oxidase also eliminated subsequent MAb 58B1 labeling (**Fig. 4**). On the other hand, treatment of macrophages with trypsin (20 μg/ml at 37°C for 30 min after macrophage incubations with AcLDL plus ACAT inhibitor) did not eliminate MAb 58B1 labeling. Last, macrophages did not show labeling when MAb 58B1 was substituted with an irrelevant isotype-matched monoclonal antibody.

To determine whether the cholesterol microdomains were raftlike (i.e., resisted extraction with Triton X-100), macrophages with induced cholesterol microdomains were

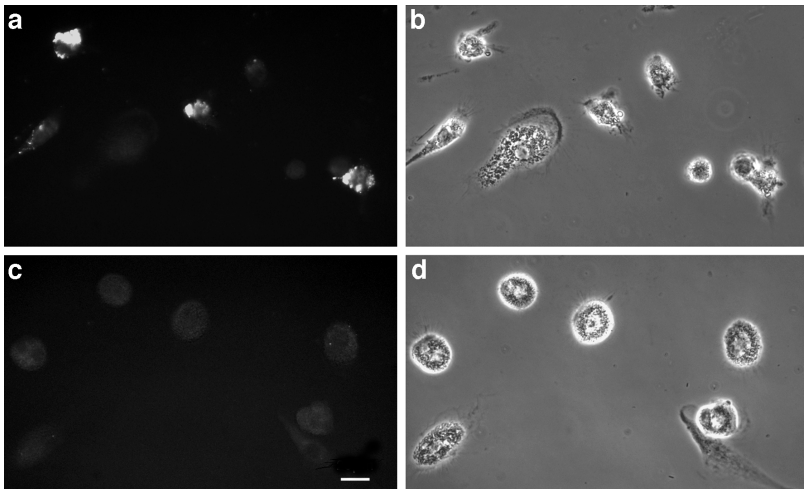

Fig. 4. Elimination of cholesterol labeling after treatment of macrophages with cholesterol oxidase. Two-week-old macrophage cultures were incubated for 2 days in RPMI 1640 medium with AcLDL (50 μg/ml) plus ACAT inhibitor (4 μg/ml). Cultures were rinsed and treated without (a and b) or with cholesterol oxidase (c and d) for 3 h before fluorescence labeling of cholesterol with MAb 58B1. Fluorescence and phase photomicrographs of the same microscopic fields are shown in the left- and right-hand columns, respectively. Bar: 20 μm.

exposed to 1% ice-cold Triton X-100 for 30 min after standard fixation in paraformaldehyde but before MAb 58B1 labeling. The Triton X-100 treatment eliminated MAb 58B1 labeling of macrophages.

Effects of agents that modulate cholesterol trafficking on ACAT inhibitor-induced plasma membrane cholesterol microdomains

Next, we evaluated whether agents that interfere with cholesterol trafficking affected the development of plasma membrane cholesterol microdomains. Chloroquine is a drug that inhibits lysosomal function and as a result blocks lysosomal acid cholesterol esterase from hydrolyzing lipoprotein-derived cholesteryl esters (21) (Fig. 1). Thus, this agent should block extralysosomal (e.g., plasma membrane) accumulation of unesterified cholesterol (23). This was the case. No cholesterol microdomains developed when macrophages were incubated with AcLDL plus ACAT inhibitor in the presence of chloroquine (100 μM).

Ketoconazole inhibits cholesterol transport from lysosomes to the cell surface (24) (Fig. 1). Here, like the ACAT inhibitor, it caused predominantly unesterified cholesterol

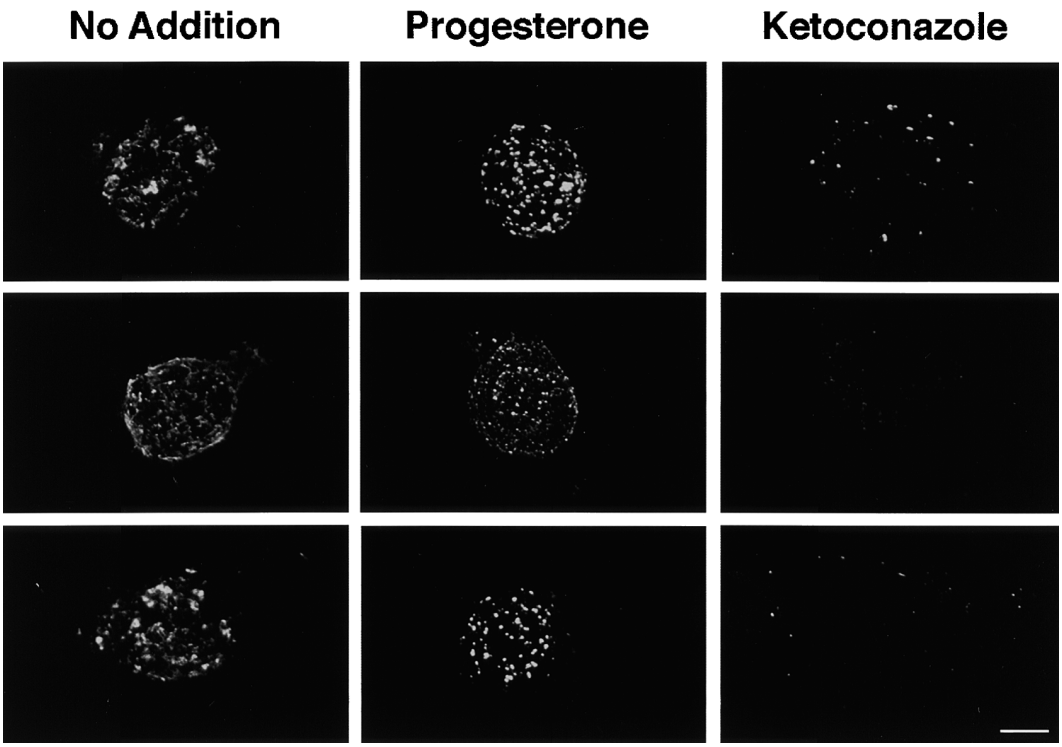

Fig. 5. Effect of ketoconazole and progesterone on plasma membrane cholesterol microdomain formation. Two-week-old macrophage cultures were incubated for 2 days in RPMI 1640 medium with AcLDL (50 μg/ml) plus ACAT inhibitor (4 μg/ml) without or with ketoconazole (10 μg/ml) or progesterone (10 μg/ml). Three representative macrophages are shown for each condition. After incubations, cultures were rinsed, fixed, and labeled with anti-cholesterol MAb 58B1. Bar: 10 μm.

to accumulate within human monocyte-macrophages during incubation with AcLDL (Table 1). Ketoconazole (10 μg/ml) inhibited (but did not completely block) the induction of plasma membrane cholesterol microdomains in macrophages incubated with AcLDL plus ACAT inhibitor (**Fig. 5**). The results show that agents that interfere with cholesterol trafficking to the plasma membrane, also inhibited the development of plasma membrane cholesterol microdomains detected by MAb 58B1.

Progesterone is another agent that causes accumulation of unesterified cholesterol in macrophages incubated with AcLDL (Table 1). Progesterone (10 μg/ml) did not block the appearance of cholesterol microdomains induced in macrophage plasma membranes during incubation with AcLDL plus ACAT inhibitor (Fig. 5), nor did incubation of macrophages with progesterone and AcLDL together induce plasma membrane cholesterol microdomains. Thus, the effect of progesterone on development of membrane cholesterol microdomains was different from that of ketoconazole or the ACAT inhibitor.

Plasma membrane cholesterol microdomain development in normal and NPC mutant fibroblasts

MAb 58B1 labeled plasma membranes of normal human fibroblasts enriched with unesterified cholesterol by incubation with LDL and ACAT inhibitor (**Fig. 6a** and **b**) (25). Greater than 40% of fibroblasts labeled. Fibroblasts incubated with LDL but without ACAT did not show labeling, and no extracellular labeling was observed under either condition. Trafficking of LDL-derived cholesterol to the plasma membrane has been reported to be defective in NPC mutant fibroblasts (26–30). Consistent with this was the complete lack of MAb 58B1 labeling of NPC mutant fibroblasts after their incubation with LDL and ACAT inhibitor (Fig. 6c and d).

Effect of cholesterol acceptors on plasma membrane cholesterol microdomain development

We examined whether the cholesterol microdomains induced by ACAT inhibition were responsive to agents that stimulate cholesterol efflux from macrophages. We tested three types of cholesterol acceptors: the amphipathic apoE and apoA-I, the plasma lipoprotein HDL, and a chemical agent that binds cholesterol, 2-hydroxypropyl-β-cyclodextrin, which has been shown to induce cholesterol efflux from macrophages (12). All these cholesterol acceptors, apoE (20 μg/ml), apoA-I (20 μg/ml), human HDL (100 μg/ml), and cyclodextrin (1 mM), when added to incubations inhibited the formation of AcLDL plus ACAT inhibitor-induced plasma membrane cholesterol microdomains, thus linking these cholesterol microdomains with the cholesterol efflux pathway. ApoE was tested and found to inhibit the development of the comet-like extracellular particles that were common in less differentiated macrophage cultures.

While cyclodextrin eliminated AcLDL plus ACAT inhibitor-induced plasma membrane cholesterol microdomains from all macrophages, induced cholesterol microdomains in rare macrophages were unaffected by addition of apoE, apoA-I, or HDL. The lack of removal of the cholesterol microdomains in these macrophages may reflect an absence of cellular amphipathic apolipoprotein receptors or other cellular factors required for cholesterol efflux. Consistent with this conclusion, cyclodextrin caused loss of induced cholesterol microdomains even if macrophages were first fixed with 4% paraformaldehyde and then incubated for 15 h with cyclodextrin. On the other hand, apoE and HDL could not cause loss of induced cholesterol microdomains from paraformaldehyde-fixed macrophages, showing that the process of removal of cholesterol microdomains by these latter agents required living cells.

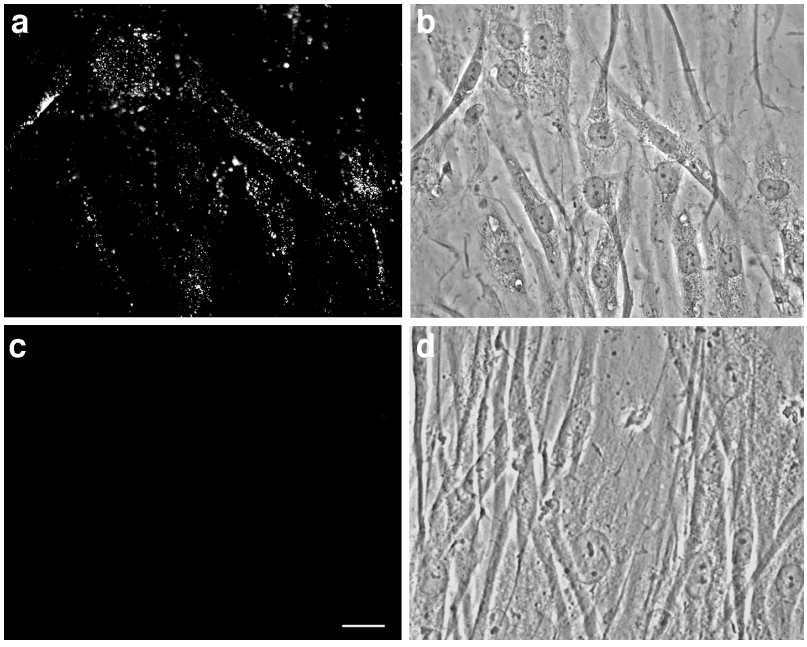

Fig. 6. Plasma membrane cholesterol microdomains in cholesterol-enriched normal and NPC mutant fibroblasts. Normal (a and b) and NPC mutant (c and d) fibroblasts were incubated in DMEM plus 10% human lipoprotein-deficient serum, and then incubated for 3 days in DMEM with LDL (50 μg/ml) and ACAT inhibitor (4 μg/ml). After this procedure, which enriches the fibroblasts with unesterified cholesterol, cultures were rinsed, fixed, and labeled with anti-cholesterol MAb 58B1. Fluorescence and phase photomicrographs of the same microscopic fields are shown in the left- and right-hand columns, respectively. Bar: 20 μm.

DISCUSSION

We have directly visualized plasma membrane cholesterol microdomains that respond to agents that modulate cholesterol trafficking in cells. The cholesterol microdomains were induced in macrophages when esterification of excess LDL-derived cholesterol was blocked with an ACAT inhibitor. The appearance of the cholesterol microdomains could be inhibited by agents such as chloroquine and ketoconazole, which interfere with trafficking of cholesterol from lysosomes to the plasma membrane, and by agents such as HDL and HDL-associated apolipoproteins, known to function in removing cholesterol from the plasma membrane. While plasma membrane cholesterol microdomains developed in normal fibroblasts during enrichment with cholesterol, the microdomains failed to develop in NPC mutant fibroblasts, where trafficking of cholesterol to the plasma membrane has been reported to be impaired (26–30).

Progesterone, an agent that interferes with cholesterol esterification, did not block development of plasma membrane cholesterol microdomains when macrophages were incubated with AcLDL in the presence of the ACAT inhibitor. Investigators disagree about the mechanism of progesterone inhibition of cholesterol esterification. Some investigators suggest that progesterone blocks transport of cholesterol from lysosomes to the plasma membrane (24, 31), whereas others suggest that progesterone interferes with cholesterol transport at some point after it enters the plasma membrane (10, 32–35). Our results support the latter hypothesis but further investigation of this finding is required.

The physical nature of the plasma membrane cholesterol microdomains detected by MAb 58B1 remains to be determined. However, the characteristics of MAb 58B1 binding to cholesterol suggest that the recognized epitope is different from a single molecule of cholesterol, either in the solubilized form or dispersed in the cell membrane. MAb 58B1 specifically recognizes cholesterol when it is organized in the crystalline form (13). The antibody also recognizes cholesterol when deposited at air-water interfaces as a homogeneous monolayer, but does not recognize monomeric cholesterol (14). MAb 58B1 thus interacts with several cholesterol molecular moieties, as they are exposed and organized at these interfaces. It is possible that cholesterol in the plasma membrane must show an orientation and organization similar to that exposed on the surface of cholesterol crystals before MAb 58B1 can cooperatively bind to it. The cholesterol microdomains detected with MAb 58B1 may be related to lateral immiscible cholesterol microdomains shown by X-ray diffraction to coexist within the liquid crystalline lipid bilayer of arterial smooth muscle plasma membranes isolated from aortas of cholesterol-fed rabbits (36). Such cholesterol microdomains form in model membranes when the membranes are enriched with cholesterol (37–40).

Induction of plasma membrane cholesterol microdomains on inhibition of ACAT suggests one way that ACAT inhibition promotes cholesterol efflux from cells. The plasma membrane is where amphipathic apolipoproteins such as apoE and apoA-I, used here, are believed to complex with phospholipid, forming nascent HDL (5). Amphipathic apolipoproteins stimulate cholesterol efflux from a specific limited pool of cholesterol (41, 42). Because apoE and apoA-I eliminated the cholesterol microdomains shown here, it is possible that these microdomains represent sites where nascent HDL can access and remove cholesterol from the plasma membrane. HDL can solubilize crystalline cholesterol (43–45), the putative physical state of cholesterol in the plasma membrane cholesterol microdomains detected by MAb 58B1. Formation of cholesterol microdomains could be one mechanism by which cholesterol enrichment of macrophages and other cells enhances the rate of apolipoprotein-stimulated cellular cholesterol efflux (7, 46).

Detergent-insoluble lipid microdomains enriched in sphingomyelin and cholesterol associated with rafts and caveolae have been implicated in cholesterol efflux (47, 48). However, the cholesterol microdomains we detected in the plasma membrane with MAb 58B1 were sensitive to extraction with 1% ice-cold Triton X-100, whereas lipid rafts and caveolae are not (49, 50). Thus, by this definition, the cholesterol-rich domains labeled by MAb 58B1 do not seem equivalent to rafts or caveolae. ApoA-I was shown to mediate cholesterol efflux from Triton X-100 soluble (i.e., nonraft and noncaveolae) plasma membrane lipid domains (51). Both apoA-I and apoE bind plasma membrane protuberances of cholesterol-enriched human fibroblasts and THP-1 macrophages (52). It will be of interest in future studies to determine the topological relationship of apoA-I and apoE binding to these plasma membrane protuberances with the cholesterol microdomains detected by MAb 58B1.

Besides detecting plasma membrane cholesterol microdomains, the anti-cholesterol antibody also labeled extracellular particles that were bound to the extracellular matrix next to regions of the macrophage plasma membrane. The focal comet-like appearance of these cholesterol-containing extracellular particles is reminiscent of the appearance of filipin-stained cholesterol-phospholipid liposomes that occur in the extracellular spaces of atherosclerotic lesions (53, 54). Macrophages excrete cholesterol not only in the small discoidal phospholipid particles (nascent HDL) containing amphipathic apolipoproteins such as macrophage-produced apoE, but also in large liposomal phospholipid particles that contain an unidentified 22-kDa protein (7, 55–59). Thus, the extracellular particles that labeled with MAb 58B1 could be one of these two particle types. It is more likely that the extracellular cholesterol particles would be related to the liposomal particles because incubation of macrophages with exogenous apoE, which stimulates formation of discoidal particles (5), prevented development of the extracellular cholesterol particles. This could have occurred either by apoE stimulating preferential cholesterol excretion in discoidal phospholipid particles or through solubilization of extracellular liposomes by the discoidal phospholipid particles. It has been shown that such discoidal phospholipid parti-

cles solubilize cholesterol-phospholipid liposomes isolated from human atherosclerotic plaques (60). The extracellular cholesterol particles were more frequent in less differentiated macrophages (4 days old) compared with more differentiated macrophages (14 days old). This may reflect that as macrophages differentiate, increased production of apoE-phospholipid discoidal particles (nascent HDL) may be sufficient to decrease formation of the extracellular cholesterol particles.

Our results show that MAbs that are useful for detecting and studying cholesterol microdomains in the plasma membrane can be isolated and selected through their interactions with cholesterol crystals. Because of the epitope specificity of antibodies, the potential exists for the development of other monoclonal antibodies that will detect other pools of cholesterol within the plasma membrane. In this regard, we are currently studying another monoclonal antibody that detects a more extensive pool of cholesterol, possibly structural, associated with all cell plasma membranes even when the cells are not enriched with cholesterol. In contrast, MAb 58B1 detects a specific plasma membrane pool of cholesterol that responds to agents that modulate trafficking of excess cholesterol within macrophages. MAb 58B1 also detected similar cholesterol microdomains when cultured human skin fibroblasts were enriched with cholesterol. Because these cholesterol microdomains did not form in NPC mutant fibroblasts, MAb 58B1 could be useful in screening for drugs that normalize defective cholesterol trafficking in these cells. Also, anti-cholesterol MAb 58B1 should be generally useful for studying gene products that regulate cholesterol trafficking and efflux in cells.

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