

Anti-bis(monoacylglycero)phosphate antibody accumulates acetylated LDL-derived cholesterol in cultured macrophages

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Abstract Bis(monoacylglycero)phosphate (BMP), also called lysobisphosphatidic acid, is a phospholipid highly enriched in the internal membranes of multivesicular late endosomes, in which it forms specialized lipid domains. It has been suggested that BMP-rich membranes regulate cholesterol transport. Here, we examine the effects of an anti-BMP antibody on cholesterol metabolism and transport in two macrophage cell lines, RAW 264.7 and THP-1, during loading with acetylated low density lipoprotein (AcLDL). Anti-BMP antibody was internalized and accumulated in both macrophage cell types. Cholesterol staining with filipin and mass measurements indicate that AcLDL-stimulated accumulation of free cholesterol (FC) was enhanced in macrophages that had accumulated the antibody. Unlike the hydrophobic amine U18666A (3- β -[2-(diethylamino)ethoxy]androst-5-en-17-one), esterification of AcLDL-derived cholesterol by ACAT was not modified after anti-BMP treatment. AcLDL loading led to an increase of FC in the plasma membrane. This increase was further enhanced in anti-BMP-treated macrophages. However, cholesterol efflux to HDL was reduced in antibody-treated cells. **These results suggest that the accumulation of anti-BMP antibody alters cholesterol homeostasis in AcLDL-loaded macrophages.**—Delton-Vandenbroucke, I., J. Bouvier, A. Makino, N. Besson, J-F. Pageaux, M. Lagarde, and T. Kobayashi. **Anti-bis(monoacylglycero)phosphate antibody accumulates acetylated LDL-derived cholesterol in cultured macrophages.** *J. Lipid Res.* 2007. 48: 543–552.

Supplementary key words endocytosis • late endosome • lipid domain • cholesterol efflux • low density lipoprotein

Cholesterol homeostasis in cells is regulated by a complex set of mechanisms that include cholesterol synthesis, uptake of LDL, cholesterol esterification, and cholesterol efflux (1–3). Macrophages acquire the bulk of cholesterol by receptor-mediated endocytosis of LDL, a process that is

normally under regulatory control. During atherogenesis, macrophages take up modified LDL in an unregulated manner, via scavenger receptors, ultimately resulting in the deposition of the large stores of cholesteryl esters (CEs) and free cholesterol (FC) that characterize foam cell phenotypes. The formation of cholesterol-laden macrophages is a prominent feature of atherosclerotic lesions (4, 5).

Endocytosed LDL and modified lipoprotein particles are first transported to early endosomes and then late endosomes/lysosomes. Late endosomes function not only as an obligatory station for LDL and other endocytosed ligands destined to be degraded but also as a major protein- and lipid-sorting compartment (6, 7). When endosomal function is altered, as observed in Niemann-Pick type C cells or after treatment with drugs that mimic Niemann-Pick type C, cholesterol accumulates in late endosomes and membrane trafficking is impaired (8, 9). Previously, a monoclonal antibody against endosomal membranes was isolated that recognizes a unique lipid, lysobisphosphatidic acid (LBPA) [also called as bis(monoacylglycero)phosphate (BMP)] (10). BMP is highly enriched in the internal membranes of multivesicular late endosomes, in which it forms specialized lipid domains (11–13). Antibody against BMP, when internalized from the medium, alters both the organization of internal membranes and membrane traffic from late endosomes (10, 14). These results suggest that the BMP-rich membrane domains contribute to membrane sorting and/or trafficking from late endosomes. Treatment of the cells with anti-BMP antibody also resulted in a massive accumulation of cholesterol in late

Abbreviations: AcLDL, acetylated low density lipoprotein; BMP, bis(monoacylglycero)phosphate; CE, cholesteryl ester; FC, free cholesterol; [³H-CO]LDL, low density lipoprotein labeled with [³H]cholesteryl oleate; LBPA, lysobisphosphatidic acid; M β CD, methyl- β -cyclodextrin; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PMA, phorbol myristate acetate.

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endosomes, suggesting that the characteristic network of BMP-rich membranes contained within multivesicular late endosomes regulates cholesterol transport. Recent results indicate that *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol alters cellular cholesterol homeostasis by modulating the BMP domains (15). The role of BMP in vascular thrombosis and atherosclerosis has been suggested by the finding that BMP is a specific antigen of the antibodies found in patients with antiphospholipid syndrome (10, 16–18). BMP domains also seem to play an important role in glycolipid degradation in the lumen of late endosomes (19–22). Recent results suggest that BMP itself has the ability to create multivesicular membrane organization (23).

As in other cell types, BMP is enriched in late endosomes in cultured macrophages (24). In this study, we examined the effect of anti-BMP antibody on cholesterol metabolism and transport in two macrophage cell lines, RAW 264.7 and THP-1. Acetylated low density lipoprotein (AcLDL)-derived cholesterol accumulated in macrophages that had accumulated anti-BMP antibody. Our results indicate that in addition to the increase of intracellular cholesterol, the anti-BMP antibody increased cell surface cholesterol. Anti-BMP antibody accumulation alters HDL-mediated cholesterol efflux. These results suggest that BMP is important for the transport of LDL-derived cholesterol from the endosomal compartment to the plasma membrane domains, where cholesterol is preferentially removed by HDL.

MATERIALS AND METHODS

Materials

Tissue culture media were purchased from Eurobio (Les Ulis, France). [9,10-³H]oleic acid (23 Ci/mmol) and [1,2,6,7-³H]cholesteryl oleate (60 Ci/mmol) were from Perkin-Elmer Life Science (Paris, France). Alexa 488- and Alexa 546-conjugated anti-mouse IgGs and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-AcLDL were from Molecular Probes (Eugene, OR). Monoclonal antibody against BMP (anti-LBPA antibody, 6C4) was obtained as described (10). U18666A (3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one) was purchased from Biomol, amphotericin B from Calbiochem, and filipin, methyl-β-cyclodextrin (MβCD), stigmasterol, cholesteryl heptadecanoate, phorbol myristate acetate (PMA), mevinolin, and mevalonolactone from Sigma. The cell proliferation kit [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)] was from Roche Diagnostics. All solvents were analytical grade from SDS. Silica gel 60 plates were supplied by Merck.

Lipoprotein preparation

Human LDLs and HDLs were isolated from plasma by sequential ultracentrifugation. LDLs were acetylated with acetic anhydride by the method of Basu et al. (25). In some experiments, LDLs were first labeled with [³H]cholesteryl oleate ([³H-CO]LDL) (40 μCi/mg LDL protein; incubation overnight at 40°C) as described previously (26, 27). [³H-CO]LDL was then reisolated by ultracentrifugation and acetylated. Average radioactivity was 37.1 ± 5.3 μCi/mg LDL protein (mean ± SD of eight preparations).

Cell culture and treatment

Human monocytic leukemia cell line THP-1 and murine macrophage-like RAW 264.7 cells were obtained from the RIKEN Bioresource Center (Tsukuba, Japan). THP-1 monocytes were grown in suspension at 37°C in an atmosphere of 5% CO₂ in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (basal culture conditions). Cells were subcloned periodically to maintain a cellular density of ~2 × 10⁵ cells/ml. RAW macrophages were routinely grown in 100 mm dishes in MEM supplemented with nonessential amino acids and containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (basal culture conditions). They were subcultured by trypsinization at a 1:5 ratio. For THP-1 cells, experiments were started on day 0 by differentiation into macrophage phenotype (3 × 10⁶ cells per 60 mm diameter dish, unless otherwise indicated) with 100 nM PMA. PMA was maintained throughout the experiment to keep fully differentiated macrophages. PMA treatment did not modify lipid metabolism, whereas it improved the cell viability of differentiated macrophages (28). RAW macrophages were seeded on day 0 into 60 mm diameter dishes (1 × 10⁶ cells per dish, unless otherwise indicated). From day 1 (RAW) or day 2 (THP-1), macrophages were cultured in the absence (control) or in the presence of anti-BMP antibody (50 μg/ml) or U18666A (3 μg/ml) in basal culture conditions. When applied, incubation with AcLDL (50–100 μg/ml) was performed on day 2 (RAW) or day 3 (THP-1) for up to 24 h. The concentration of serum in the culture medium was then decreased from 10% to 1% to minimize the amount of lipoproteins supplied by the serum. Macrophages not incubated with AcLDL (unloaded cells) were also prepared in the same conditions. At the end of the experiments, cells were rinsed two times with PBS, scraped, and pelleted by centrifugation. Cell pellets were kept frozen at –20°C until analysis.

Fluorescence microscopy

THP-1 cells were seeded in glass-bottom 35 mm dishes (0.5 × 10⁶ cells per dish), differentiated with PMA, and exposed to anti-BMP antibody for 48 h. All subsequent manipulations were performed at room temperature. Cells were first washed with PBS and then fixed with 3% paraformaldehyde in PBS for 20 min, quenched with 50 mM NH₄Cl, and blocked with 0.1% BSA in PBS. Cells were then permeabilized by treatment with 50 μg/ml saponin for 5 min followed by incubation with Alexa 546-conjugated anti-mouse antibody for 1 h. After washing with PBS, cells were further incubated with 50 μg/ml filipin. The specimens were mounted with Mowiol and examined with a Zeiss LSM 510 confocal microscope equipped with a C-Apochromat 63XW Korr (1.2 numerical aperture) objective. To compare the distribution of internalized anti-BMP antibody and AcLDL, RAW cells grown on glass-bottom 35 mm dishes were treated with 50 μg/ml anti-BMP antibody for 24 h in 10% FBS-containing medium. Cells were then incubated with 10 μg/ml DiI-AcLDL in 1% FBS-containing medium in the presence of the anti-BMP antibody for 1 h. Cells were washed with PBS and kept in normal medium for another 2 h. Cells were then fixed and stained with Alexa 488-conjugated anti-mouse antibody (to visualize the internalized anti-BMP antibody) as described above.

Determination of cholesterol and CE mass

Total lipids were extracted from cell lysates (0.1% Triton in water) by the method of Bligh and Dyer (29). Stigmasterol (10 μg) and cholesteryl heptadecanoate (5 μg) were added to serve as internal standards. Lipids were separated by TLC (silica gel 60 plates) using the solvent system hexane-diethylether-acetic

acid (80:20:1, v/v). FC and CE were revealed under ultraviolet light after spraying 0.05% 2',7'-dichlorofluorescein in methanol and were identified by comparison with authentic standards spotted on the same plate. The silica gel containing FC was scraped, and FC was extracted with 2 ml of chloroform-methanol (2:1, v/v). The samples were dried under nitrogen and resuspended in a known volume of acetone. FC was analyzed by GC using an Econo-Cap EC-5 capillary column (30 m × 0.32 μm, 0.25 μm) with helium as the carrier gas and quantified using stigmaterol as the internal standard. The silica gel containing CE was scraped, and CE was transmethylated by heating at 100°C for 90 min in 1 ml of methanol containing 5% H₂SO₄. The resulting fatty acid methyl esters were analyzed by GC, and the percentage and mass of each fatty acid were calculated using the internal standard (pentadecanoic acid methyl ester) as described previously (30).

Incorporation of [³H]oleate into CE

Macrophages were exposed to 0.5 μCi/ml (22 nM) [³H]oleate during 24 h of AcLDL loading (100 μg/ml). Unloaded macrophages were also labeled. [³H]oleate was added into the medium from an ethanolic stock solution with final ethanol concentration of <0.1%. Total lipids were extracted from cell lysates, and radioactivity in an aliquot was determined by liquid scintillation counting. Lipids were separated by TLC as described above. The labeled compounds were detected with a Berthold radioactivity analyzer, and the radioactivity of the spot, scrapped off the plate, was quantified by liquid scintillation counting. Cholesterol esterification was expressed as the percentage of cholesteryl [³H]oleate.

Cellular uptake of [³H-CO]AcLDL

Macrophages were incubated overnight with 50 μg/ml [³H-CO]AcLDL in 1% serum-containing medium for 24 h. Cells were lysed in 0.1% Triton, and radioactivity in an aliquot was determined by liquid scintillation counting. Total uptake was calculated as nCi/mg cell protein and converted to μg AcLDL protein/mg cell protein based on the specific radioactivity of [³H-CO]AcLDL. Total lipids were extracted from cell lysates and separated by TLC as described above. The labeled compounds (FC and CE) were detected with a Berthold radioactivity analyzer, and the radioactivity of the spot, scrapped off the plate, was quantified by liquid scintillation counting.

Amphotericin B-mediated cell killing

RAW macrophages were seeded on 96-well plates (10,000 cells/well). AcLDL-loaded and unloaded macrophages were treated with amphotericin B (25 or 50 μg/ml, 3 h) in 1% serum-containing medium. After treatments, cells were washed with PBS and cell viability was assessed using a colorimetric MTT assay according to the manufacturer's instructions. MTT cleavage was determined by reading the absorbance at 560 nm. Cell viability in control and anti-BMP-treated macrophages was expressed as a percentage of the maximum cell viability of untreated cells.

Cholesterol oxidase treatment

RAW macrophages were seeded on six-well plates (500,000 cells/well). Control and anti-BMP-treated macrophages were loaded with 50 μg/ml [³H-CO]AcLDL. Cells were then treated with cholesterol oxidase (31, 32). After AcLDL incubation, cells were washed three times with PBS, fixed with 1% (v/v) glutaraldehyde in PBS (10 min at room temperature), and then washed with PBS. The cells were incubated for 30 min with MEM containing 2 U/ml cholesterol oxidase. After washing with PBS, cells were scraped in 1 ml of PBS and lipids were extracted

with 2 ml of hexane-isopropyl alcohol (3:2, v/v). [³H]cholesterol and [³H]cholestenone were separated by TLC using hexane-diethylether-acetic acid (130:30:2, v/v) and visualized with a Berthold radioactivity analyzer. Radioactivity was measured by liquid scintillation counting. [³H]cholestenone formation was expressed as a percentage of total cellular [³H]cholesterol and [³H]cholestenone.

MβCD- and HDL-mediated [³H]cholesterol efflux

Macrophages were incubated with 50 μg/ml [³H-CO]AcLDL in 1% serum-containing medium for 24 h (THP-1) or 8 h (RAW). Cells were then washed three times with PBS and incubated with 5 or 10 mM MβCD or 100 μg/ml HDL in 1% serum-containing medium for various periods of time. The medium was then collected and centrifuged at 1,000 rpm for 10 min to remove cell debris, and radioactivity in an aliquot was determined by liquid scintillation counting. Cells were lysed in 0.1% Triton, and radioactivity in an aliquot was determined. Cholesterol efflux was expressed as the percentage of radioactivity released from the cells into the medium relative to the total radioactivity in cells and medium. Analysis of radioactivity in medium showed that >95% was recovered as FC.

Statistical analysis

The results are presented as means ± SD. Significant differences between groups were assessed by Student's *t*-test for paired samples when the values were means of at least three independent experiments, each represented by the average of three wells per condition. Student's *t*-test for unpaired samples was applied when the values corresponded to means of at least four wells in one representative experiment, repeated at least two times.

RESULTS

Anti-BMP antibody alters the intracellular distribution of cholesterol but not cholesterol content in cultured macrophages grown in normal medium

Previously, it was shown that the anti-LBPA (anti-BMP) antibody added to the medium is accumulated in late endosomes in cultured fibroblasts. Accumulation of the antibody is accompanied by the accumulation of FC in late endosomes (8). Similar to fibroblasts, the antibody was internalized and accumulated when cultured macrophages were incubated with anti-BMP antibody. In THP-1 macrophages, the degree of accumulation of the antibody was heterogeneous: some cells highly accumulated the antibody, whereas others did not. The accumulation of anti-BMP antibody correlated and colocalized with accumulated FC, as revealed by the higher intensity of filipin staining (Fig. 1), compared with cells that did not significantly accumulate the antibody. However, cholesterol mass measurements showed that there was no significant change in the total amounts of cellular FC (25.9 ± 3.9 vs. 24.6 ± 2.5 μg/mg protein in control cells, mean ± SD of three experiments in duplicate wells). We also examined the effects of the hydrophobic amine U18666A, a potent inhibitor of cholesterol metabolism and transport (33, 34). After 24 h of incubation with U18666A (3 μg/ml), FC mass increased by 30% (35 ± 3.9 μg/mg protein, mean ± SD of three experiments in duplicate wells). In contrast to THP-1, most of the RAW macrophages significantly accumulated

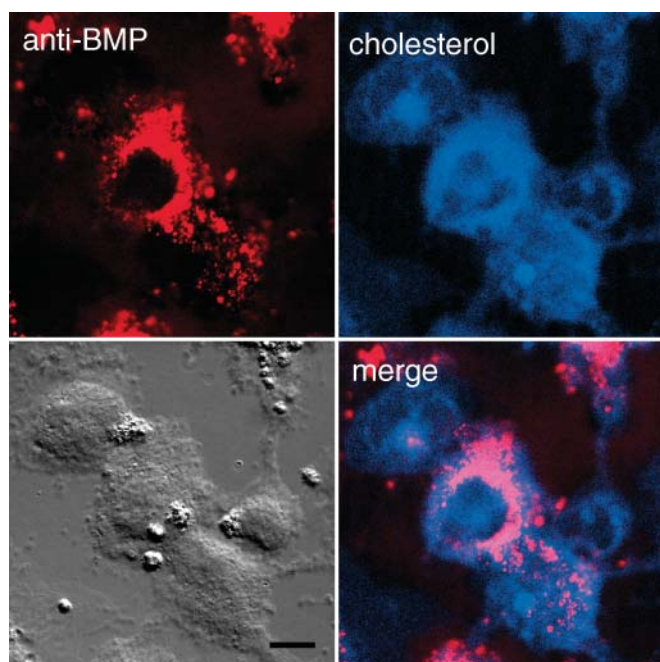


Fig. 1. Effect of anti-bis(monoacylglycero)phosphate (BMP) antibody on cholesterol accumulation in THP-1 macrophages. THP-1 macrophages were incubated with 50 $\mu\text{g/ml}$ anti-bis(monoacylglycero)phosphate (BMP) antibody for 48 h as described in Materials and Methods. Cells were fixed and double stained with Alexa 546-conjugated anti-mouse antibody (to visualize the internalized anti-BMP antibody) and filipin. Bar = 10 μm .

anti-BMP antibody. However, we saw no difference of filipin staining after the antibody treatment, because of the strong filipin staining in the control cells (data not shown). Anti-BMP antibody did not significantly affect the total cholesterol content in RAW cells. Because RAW cells display homogeneous incorporation of the antibody, we performed most of the subsequent experiments using RAW cells.

Anti-BMP antibody enhances AcLDL-stimulated cholesterol accumulation

In the experiments described above, macrophages were cultured in basal conditions (i.e., in medium containing 10% serum), which provides a relatively low amount of LDL (8.6 $\mu\text{g/ml}$). To promote cholesterol loading, macrophages were incubated with AcLDL. These modified LDLs have been shown to be good inducers of the accumulation of FC and especially of CE in macrophages (28). Before starting the experiments, we first investigated whether internalized AcLDL was recovered in endosomes that contain anti-BMP antibody. RAW macrophages were treated with 50 $\mu\text{g/ml}$ anti-BMP antibody overnight followed by incubation with DiI-labeled AcLDL (10 $\mu\text{g/ml}$, 1 h pulse and 2 h chase). Most of the DiI fluorescence was detected in structures that had accumulated the antibody, indicating that AcLDL and anti-BMP antibody share the same endocytic pathway (Fig. 2). In addition, there are compartments that accumulated anti-BMP but did not contain DiI-AcLDL. This may be because of the heterogeneity of late endocytic compartments (33). Alternatively AcLDL was

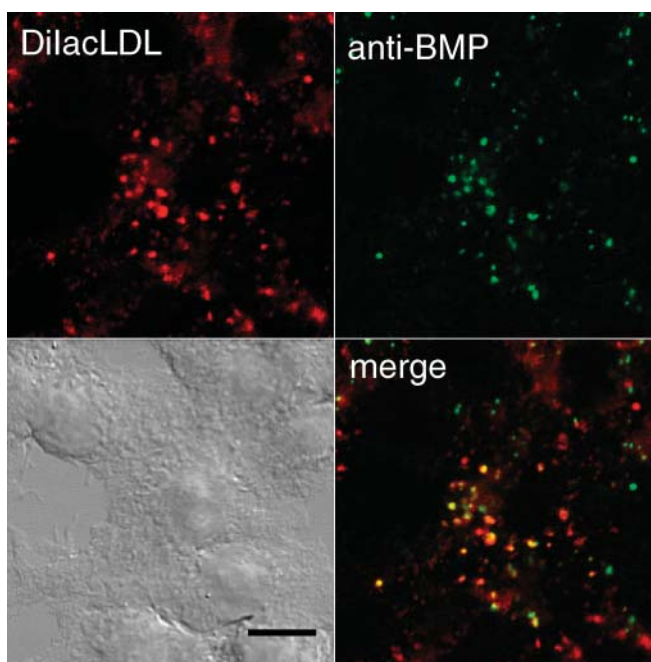


Fig. 2. Colocalization of internalized anti-BMP antibody and DiI-acetylated low density lipoprotein (AcLDL) in RAW macrophages. RAW macrophages were incubated with 50 $\mu\text{g/ml}$ anti-BMP antibody for 24 h as described in Materials and Methods. Cells were then incubated with 10 $\mu\text{g/ml}$ DiI-AcLDL for 1 h. Cells were washed with PBS and kept in normal medium for another 2 h. Cells were fixed and stained with Alexa 488-conjugated anti-mouse antibody (to visualize the internalized anti-BMP antibody). Bar = 10 μm .

degraded in these compartments. When RAW cells were incubated with AcLDL, the FC mass was increased by $\sim 40\%$ compared with unloaded cells (Fig. 3A). FC accumulation induced by AcLDL loading was further enhanced, by $\sim 30\%$, in cells that had accumulated the antibody. In contrast, incubation with a control mouse antibody had no effect. After the addition of U18666A, AcLDL-stimulated FC accumulation was increased by 90%, in agreement with the known action of the drug (34). The time course of AcLDL-stimulated FC accumulation was then examined (Fig. 3B). Accumulation of FC was observed during incubation with AcLDL. The addition of anti-BMP antibody further increased the FC content. Similar results were obtained in THP-1 macrophages (data not shown).

Anti-BMP antibody does not affect cholesterol esterification

Incubation with 100 $\mu\text{g/ml}$ AcLDL for 8 h caused a 4-fold increase of CE content in RAW macrophages (Fig. 4). This increase was slightly augmented (+18%) in anti-BMP-treated cells, whereas U18666A almost completely inhibited AcLDL-stimulated CE accumulation. Table 1 shows the fatty acid composition of cellular CE in RAW macrophages. Fatty acid composition was modified after AcLDL loading. The proportions of oleate (C18:1) and arachidonate (C20:4) esterified to cholesterol were significantly augmented compared with their levels in the CE of unloaded cells. These changes are consistent with the selec-

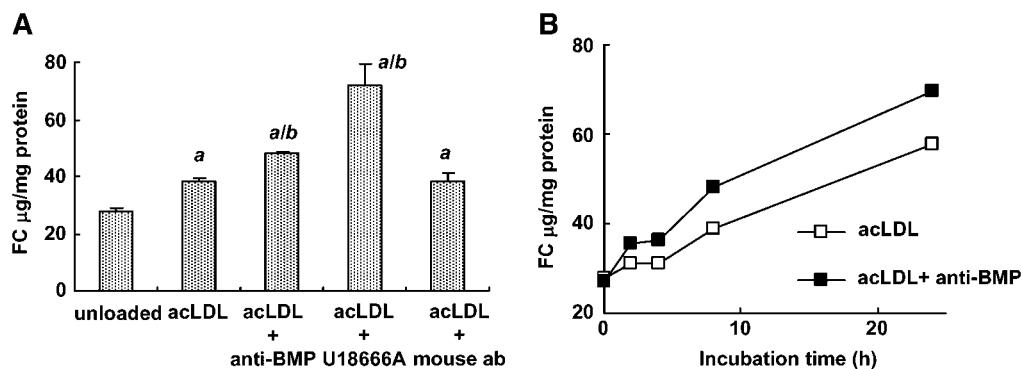


Fig. 3. AcLDL-stimulated free cholesterol (FC) accumulation. Cells were cultured in the absence or presence of 50 µg/ml anti-BMP antibody, 1 µg/ml U18666A (3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one), or 50 µg/ml mouse IgG for 24 h in 10% FBS-containing medium. The medium was then changed to 1% FBS-containing medium with 100 µg/ml AcLDL in the absence or presence of antibodies or U18666A. Cells were incubated for 8 h (A) or for various periods of time (B). FC was measured by GC as described in Materials and Methods. The amount of cholesterol is expressed as µg/mg protein and represents the mean ± SD of three independent experiments in triplicate wells (A). For the time-course study (B), data represent the average of triplicate wells from one experiment. ^a $P \leq 0.05$ compared with unloaded cells; ^b $P \leq 0.05$ compared with AcLDL-treated cells using a paired *t*-test.

tivity toward fatty acids of the ACAT in the endoplasmic reticulum (35). This is important to consider in view of the recent finding that under certain conditions, FC could be reesterified in late endosomes, independently of the canonical ACAT (36). The fatty acid composition of CE isolated from AcLDL was quite different from that of cellular CE, with linoleate accounting for ~60% of the total fatty acids. No enrichment in linoleate was observed in cellular CE. This suggests that CE that accumulated in cells after AcLDL loading resulted mainly from the reesterification of LDL-derived cholesterol. It is noteworthy that the fatty acid composition of the cellular CE recovered after AcLDL loading was the same in control and

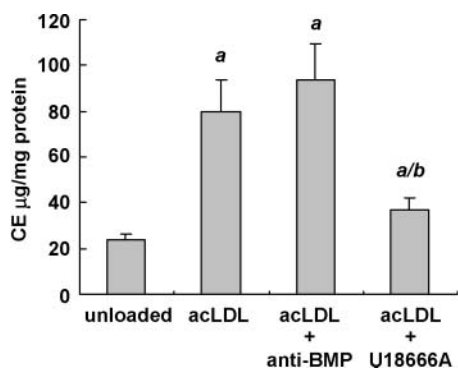


Fig. 4. AcLDL-stimulated cholesteryl ester (CE) accumulation. Cells were cultured in the absence or presence of 50 µg/ml anti-BMP antibody or 1 µg/ml U18666A for 24 h in 10% FBS-containing medium, followed by loading with 100 µg/ml AcLDL for 8 h in 1% FBS-containing medium in the presence of anti-BMP antibody or U18666A. CE was quantified by GC analysis of fatty acid content as described in Materials and Methods. The amount of CE is expressed as µg/mg protein and represents the mean ± SD of three independent experiments in triplicate wells. ^a $P \leq 0.05$ compared with unloaded cells; ^b $P \leq 0.05$ compared with AcLDL-treated cells using a paired *t*-test.

anti-BMP-treated macrophages. Similar observations were made in THP-1 macrophages (data not shown). These results suggest that anti-BMP antibody accumulation affects neither the hydrolysis of AcLDL-associated CE nor the esterification of LDL-derived cholesterol.

To further examine the ACAT pathway, the effect of the anti-BMP antibody on the ability of AcLDL to stimulate cholesterol esterification was studied in RAW macrophages (Fig. 5). Cells were incubated with 0.5 µCi/ml [³H]oleate during 8 h of AcLDL loading (100 µg/ml) in the absence or presence of anti-BMP antibody. As the total uptake of [³H]oleate (total radioactivity/mg protein) was the same for all groups (data not shown), [³H]oleate incorporation into [³H]CE was expressed as a percentage of total cell radioactivity. In unloaded cells, only a trace proportion of total radioactivity was recovered in the CE pool, indicating a very low rate of esterification. After AcLDL loading, [³H]oleate incorporation into CE was increased significantly in both control and anti-BMP-treated cells, with no significant difference between the two groups. In contrast, AcLDL-stimulated cholesterol esterification was completely abolished in U18666A-treated cells, as described (32). Together, these findings demonstrate that anti-BMP antibody accumulation does not affect the esterification of AcLDL-derived cholesterol, indicating that the transport of FC to the endoplasmic reticulum was not impaired.

Anti-BMP antibody does not affect the uptake of [³H-CO]AcLDL

One possible mechanism that could contribute to FC accumulation in anti-BMP-treated macrophages is an enhanced uptake of AcLDL. To clarify this point, control and anti-BMP-treated RAW macrophages were incubated with 50 µg/ml [³H-CO]AcLDL, and total radioactivity in cell homogenates was counted. As reported in Table 2, the uptake of AcLDL was not significantly different between control and anti-BMP-treated cells. Analyses were done to

TABLE 1. Fatty acid composition of cellular CE and AcLDL-associated CE

Fatty Acid	Cellular CE			AcLDL-Associated CE
	Unloaded	AcLDL	AcLDL + anti-BMP	
			<i>mol%</i>	
14:0	6.70 ± 1.55	5.39 ± 1.27	5.49 ± 1.48	0.43 ± 0.1
16:0	41.71 ± 4.31	25.30 ± 2.44 ^a	25.25 ± 1.72 ^a	11.8 ± 0.5
18:0	8.42 ± 2.53	7.97 ± 2.22	8.25 ± 1.29	0.6 ± 0.1
18:1	19.90 ± 2.86	37.52 ± 3.64 ^a	36.68 ± 3.65 ^a	20.9 ± 0.2
18:2	25.91 ± 5.56	19.88 ± 4.29	20.99 ± 5.02	55.2 ± 0.9
20:4	1.20 ± 0.50	3.94 ± 1.39 ^a	3.33 ± 1.43 ^a	7.4 ± 0.1

AcLDL, acetylated low density lipoprotein; BMP, bis(monoacylglycero)phosphate; CE, cholesteryl ester. RAW macrophages were cultured in the absence or presence of the anti-BMP antibody (50 µg/ml) for 24 h in 10% FBS-containing medium. The culture medium was then changed to 1% FBS-containing medium with 100 µg/ml AcLDL in the absence or presence of anti-BMP antibody. Cellular CE and AcLDL-associated CE were isolated from total lipids by TLC, and their fatty acid composition was determined as described in Materials and Methods. The values are expressed as mol% of total fatty acids and are means ± SD of three independent experiments in triplicate wells.

^a $P \leq 0.05$ compared with unloaded cells using a paired *t*-test.

determine the distribution of the radioactivity between FC and CE. There was no difference between the control and anti-BMP-treated cells. The proportion of radioactive CE recovered in the cells depends on both the hydrolysis of AcLDL-associated CE and the esterification of AcLDL-derived cholesterol. Because the cholesterol esterification pathway was not modified in the anti-BMP-treated cells, these data also suggest that there was no difference in the rate of AcLDL-CE hydrolysis after anti-BMP antibody treatment. Similar results were obtained in THP-1 macrophages.

Anti-BMP antibody increases plasma membrane cholesterol

It is assumed that most LDL-derived cholesterol transits through the plasma membrane before reaching the endo-

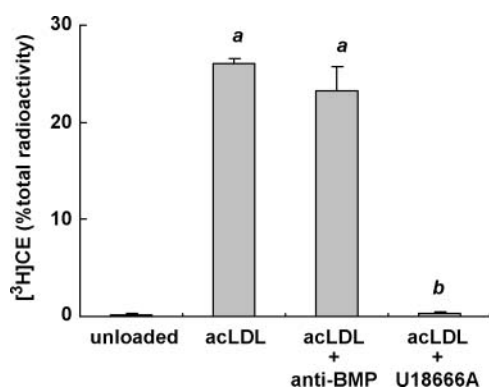


Fig. 5. AcLDL-stimulated cholesterol esterification. RAW macrophages were cultured in the absence or presence of 50 µg/ml anti-BMP antibody or 1 µg/ml U18666A for 24 h in 10% FBS-containing medium, followed by loading with 100 µg/ml AcLDL for 8 h in the presence of 0.5 µCi/ml [³H]oleate in 1% FBS-containing medium in the absence or presence of anti-BMP antibody or U18666A. The cellular content of cholesteryl [³H]oleate was measured as described in Materials and Methods. The values are expressed as percentages of total cellular radioactivity and represent means ± SD of four wells in two independent experiments. ^a $P \leq 0.05$ compared with unloaded cells; ^b $P \leq 0.05$ compared with AcLDL-treated cells.

plasmic reticulum (37), although direct transport to the endoplasmic reticulum has also been reported in fibroblasts (38). Our data show that AcLDL-derived cholesterol esterification was not affected by anti-BMP antibody, suggesting that cholesterol can be transported to the plasma membrane. To evaluate the level of plasma membrane-associated cholesterol, we used three procedures: the sensitivity of cells to cholesterol binding toxin, the sensitivity of cellular cholesterol to cholesterol oxidase, and the extraction of cholesterol by cyclodextrin. Amphotericin B is a polyene antibiotic that forms pores in cholesterol-rich membranes, causing cell death. Cytolytic susceptibility of cells to amphotericin B has been suggested to represent a semiquantitative measure of plasma membrane-associated cholesterol (38–40). Cell viability in control and anti-BMP-treated macrophages was assessed by colorimetric MTT assay, and amphotericin B-mediated cell killing was expressed as a percentage of maximum cell viability determined in untreated cells. Anti-BMP antibody alone did not significantly change cell viability in unloaded or AcLDL-loaded cells in the absence of amphotericin B (data not shown). In unloaded RAW macrophages, cell viability decreased by 25% and 40%, respectively, with

TABLE 2. Uptake of [³H-CO]AcLDL

Variable	Control	anti-BMP
Uptake (µg AcLDL/mg cell protein)	9.2 ± 0.9	9.9 ± 1.7
Radioactivity distribution (% total)		
FC	34.8 ± 2.8	33.6 ± 6.0
CE	65.2 ± 2.8	66.4 ± 6.0

FC, free cholesterol; [³H-CO]AcLDL, AcLDL labeled with [³H]cholesteryl oleate. RAW macrophages were grown in the absence or presence of anti-BMP antibody (50 µg/ml) for 24 h in 10% FBS-containing medium before 8 h of loading with 50 µg/ml [³H-CO]AcLDL in 1% FBS-containing medium in the absence or presence of anti-BMP antibody. Cellular uptake of [³H-CO]AcLDL was measured as described in Materials and Methods; values are means ± SD of three independent experiments in triplicate wells. The radioactivity associated with FC and CE was quantified by liquid scintillation counting after separation of cellular lipids by TLC; values are means ± SD of three independent experiments in triplicate wells.

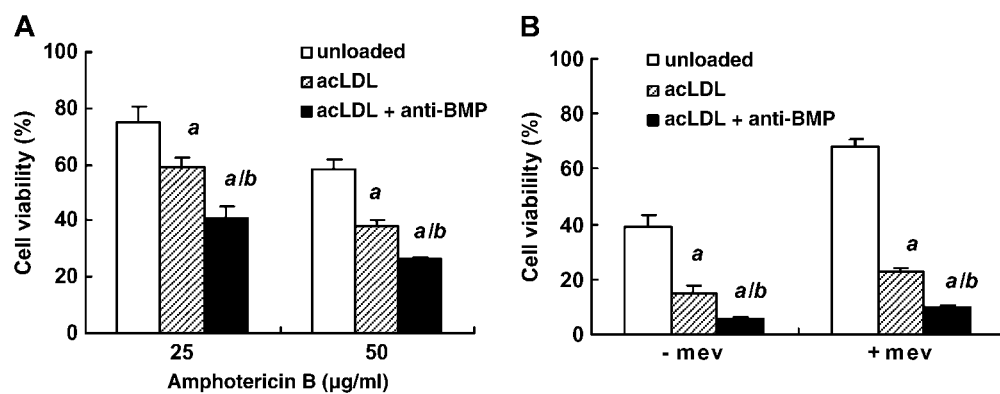


Fig. 6. Amphotericin B-mediated killing of macrophages. Cells were cultured in the absence or presence of 50 µg/ml anti-BMP antibody for 24 h in 10% FBS-containing medium before loading with 100 µg/ml AcLDL in 1% FBS-containing medium in the absence or presence of anti-BMP antibody. After overnight incubation, cells were treated with amphotericin B as described in Materials and Methods. A: Killing of RAW macrophages with the indicated concentrations of amphotericin B. B: Killing of RAW macrophages with 50 µg/ml amphotericin B in the presence or absence of mevinolin/mevalonate (mev). Cell viability was assessed using a colorimetric 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Results are expressed as percentages of maximum cell viability in untreated cells. The values are means \pm SD of four to five wells and are representative of three independent experiments. ^a $P \leq 0.05$ compared with unloaded cells; ^b $P \leq 0.05$ compared with AcLDL-loaded cells.

25 and 50 µg/ml amphotericin B (**Fig. 6A**). After AcLDL loading, amphotericin B-mediated cell killing was enhanced. This is consistent with an increase of plasma membrane-associated cholesterol rendering the cells more sensitive to amphotericin B treatment. Interestingly, cell susceptibility to amphotericin B was further increased in anti-BMP-treated cells, suggesting an enrichment of plasma membrane-associated cholesterol. One possible explanation for this enrichment is an increased cholesterol synthesis. To address this question, experiments were performed on macrophages treated with 20 µM mevinolin, to inhibit endogenous cholesterol synthesis, and 0.5 mM mevalonate, which enters the cholesterol biosynthetic pathway after the point of mevinolin inhibition and provides essential nonsteroidal isoprenoids (40). Macrophages treated with mevinolin/mevalonate were approximately twice as resistant to amphotericin B-mediated cell killing, consistent with a lower level of plasma membrane-associated cholesterol after inhibition of cholesterol synthesis (**Fig. 6B**). Even under these conditions, a decrease in cell survival in the anti-BMP-treated cells was observed. These results suggest that the FC that accumulates in the plasma membrane of anti-BMP antibody-treated cells originates from AcLDL-derived cholesterol.

Plasma membrane cholesterol was also assayed using cholesterol oxidase treatment according to the method of Slotte et al. (31). The cholesterol oxidase-sensitive pool of cellular cholesterol is largely at the plasma membrane (41). In control RAW macrophages, $56 \pm 1\%$ of the AcLDL-derived [³H]cholesterol was converted to [³H]cholestenone. This proportion was increased to $64 \pm 1.5\%$ in anti-BMP-treated cells (mean \pm SD of five wells, representative of three independent experiments; $P \leq 0.01$). Cyclodextrins serve as high-affinity acceptors for cholesterol. Short-time incubation with M β CD was shown to remove

cholesterol from the plasma membrane (37, 42). We have examined the effect of anti-BMP antibody on the M β CD-mediated efflux of cholesterol. RAW macrophages were first loaded with 50 µg/ml [³H-CO]AcLDL, and [³H]cholesterol removal by M β CD was then measured. **Figure 7** shows that M β CD induced a time- and concentration-dependent efflux of [³H]cholesterol in both control and anti-BMP-treated macrophages. It is noteworthy that [³H]cholesterol efflux was significantly enhanced in the

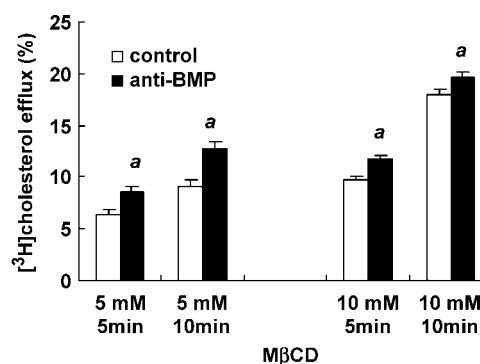


Fig. 7. Methyl- β -cyclodextrin (M β CD)-stimulated cholesterol efflux. RAW macrophages were cultured in the absence or presence of 50 µg/ml anti-BMP antibody for 24 h in 10% FBS-containing medium before loading with 50 µg/ml AcLDL labeled with [³H]cholesteryl oleate ([³H-CO]AcLDL) for 8 h in 1% FBS-containing medium in the presence or absence of anti-BMP antibody. Cholesterol efflux was then stimulated by incubation with 5 or 10 mM M β CD for the indicated intervals. Cholesterol efflux was expressed as a percentage of [³H]cholesterol in medium over total ³H radioactivity (sum of cellular and medium [³H]cholesterol plus cellular [³H]CE). The values are means \pm SD of four wells and are representative of three independent experiments. ^a $P \leq 0.05$ compared with control.

anti-BMP-treated cells compared with control cells. Collectively, these experiments using cholesterol binding toxins, cholesterol oxidase treatment, and M β CD-mediated cholesterol efflux support the conclusion that AcLDL-derived cholesterol was transported out of late endosomes to the plasma membrane and suggest the enrichment of cell surface cholesterol in the antibody-treated cells.

Anti-BMP antibody reduces HDL-mediated cholesterol efflux

Next, we examined whether HDL-induced cholesterol efflux would be affected after treatment with anti-BMP antibody. Kinetic data show that HDL (100 μ g/ml) caused an obvious time-dependent efflux of [3 H]cholesterol from RAW macrophages (Fig. 8). At any time point, there was a slight but significant decrease of [3 H]cholesterol efflux in the anti-BMP-treated macrophages. After 24 h of incubation with HDL, cholesterol efflux was reduced by 15% compared with the control. Similar results were obtained in THP-1 macrophages (data not shown).

DISCUSSION

Previous studies reported that treatment of cells with anti-BMP antibody (anti-LBPA antibody) induces the intracellular accumulation of cholesterol. Yet, the consequences of anti-BMP antibody accumulation on the different pathways of LDL-derived cholesterol metabolism and transport have not been fully examined. In this study, we examined the effects of anti-BMP antibody on the regulation of cellular cholesterol homeostasis in the cultured macrophage cell lines RAW 264.7 and THP-1. It has been shown that anti-BMP antibody accumulates in late endosomes upon binding to its antigen, causing the emergence of a population of abnormal, electron-dense late endosomes with

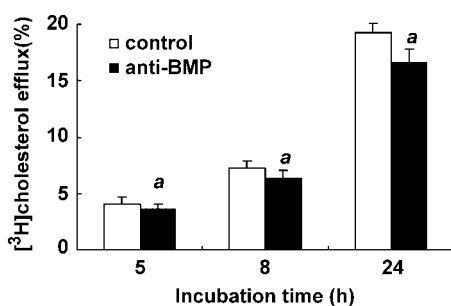


Fig. 8. HDL-stimulated cholesterol efflux. Macrophages were cultured in the absence or presence of 50 μ g/ml anti-BMP antibody for 24 h in 10% FBS-containing medium before loading with 50 μ g/ml [3 H-CO]AcLDL for 8 h in 1% FBS-containing medium in the presence or absence of anti-BMP antibody. Cholesterol efflux was then stimulated by incubation with 100 μ g/ml HDL for the indicated times. Cholesterol efflux was expressed as a percentage of [3 H]cholesterol in medium over total 3 H radioactivity (sum of cellular and medium [3 H]cholesterol plus cellular [3 H]CE). The values represent means \pm SD of three independent experiments in duplicate wells. ^a $P \leq 0.05$ compared with control using a paired t -test.

altered inner membrane organization (10). The importance of BMP in the formation of multivesicular liposomes that resemble the multivesicular endosomes, in which BMP is found in vivo, has been demonstrated (23). Anti-BMP antibody, therefore, may alter the functional organization of endosomal membranes. Accumulating evidence suggests the involvement of BMP-rich membrane domains in cholesterol transport and protein sorting from late endosomes (8, 10, 15, 43, 44). This study shows that the anti-BMP antibody leads to FC accumulation in RAW and THP-1 macrophages.

Similar to the hydrophobic amine U18666A, anti-BMP antibody induces the accumulation of cholesterol in intracellular structures, including late endosomes. Extensive studies have indicated that U18666A blocks the transport of LDL-derived cholesterol from late endosomes/lysosomes to the plasma membrane and from the plasma membrane to the endoplasmic reticulum. In addition, the drug blocks the transport of cholesterol from endosomes/lysosomes to the endoplasmic reticulum that is distinct from the pathway from endosomes/lysosomes to the plasma membrane. These accumulated effects lead to a complete inhibition of LDL-stimulated cholesterol esterification (32, 34, 38). In this study, we found that U18666A enhanced AcLDL-stimulated accumulation of FC in both THP-1 and RAW macrophages, whereas AcLDL-stimulated cholesterol esterification was almost completely inhibited, supporting the mechanism described above. Unlike U18666A, the reesterification of CE derived from AcLDL was not inhibited in anti-BMP-treated macrophages, suggesting that cholesterol was not totally trapped in late endosomes. Recently, we showed that *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol accumulates in late endosomes and inhibits acid lipase activity in fibroblasts by changing the ultrastructure of BMP-rich membrane domains (15). The results presented here indicate that unlike *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, anti-BMP antibody does not inhibit acid lipase under our experimental conditions. Sugii et al. (42) showed that in several cell types, including THP-1 macrophages, acid lipase is located mainly in the early endosomal compartment that is distinct from the late endosomal/lysosomal compartment, where Niemann-Pick type C1 resides. Therefore, it is possible that in macrophages, the exit of AcLDL-derived cholesterol does not totally depend on the function of the late endosomes. Although anti-BMP may affect only the late endosomal compartment, U18666A may affect the function of both the acid lipase compartment and the late endosomal compartment in delivering cholesterol to the endoplasmic reticulum.

In addition to the intracellular accumulation of cholesterol, anti-BMP antibody was found to cause the accumulation of cholesterol in the plasma membrane after loading with AcLDL. The cholesterol enrichment of the plasma membrane is not attributable to increased cholesterol synthesis. A potential source of cholesterol for the plasma membrane is the pool of CE in the endoplasmic reticulum. Thus, it may be considered that the increased plasma membrane-associated cholesterol reflects a higher level of CE hydrolysis. However, this is most unlikely, as we

demonstrated that both the CE content and the level of cholesterol esterification were the same in the control and anti-BMP-treated macrophages.

Another consequence of anti-BMP accumulation in macrophages is a decrease of HDL-mediated cholesterol efflux. Studies from Tabas and coworkers (45) have shown that FC loading in mouse peritoneal macrophages moderately inhibits the cholesterol efflux mediated by HDL. In their report, a massive FC accumulation (6-fold increase compared to unloaded cells) was obtained by loading macrophages with AcLDL in the presence of an ACAT inhibitor to prevent CE synthesis (46). FC overloading in the presence of an ACAT inhibitor has also been reported to trigger cell death, whereas the lower FC loading induced by AcLDL alone exerts no cytotoxic effect (5, 47, 48). In our experimental conditions, FC accumulation did not exceed twice the level of the unloaded cells and did not induce significant cell death. Therefore, we conclude that inhibition of cholesterol efflux caused by the anti-BMP antibody does not result from primary massive FC loading in macrophages. There is increasing evidence for the existence of distinct cholesterol domains in the plasma membrane that are differently modulated depending on the specific pathway involved in cholesterol efflux (49–51). In macrophages, efflux by HDL was shown to depend on the ABC transporter ABCG1 (52, 53). Our results may suggest that the anti-BMP antibody affects the ABCG1-dependent cholesterol pool.

In conclusion, these results support the concept that BMP plays a role in the regulation of cholesterol transport and the cellular distribution of cholesterol in macrophages. BMP thus contributes to the efflux of LDL-derived cholesterol and helps prevent macrophages from excessive FC loading. This might be of particular importance for pathophysiological study, as the intracellular accumulation of cholesterol is considered to be a determinant step in the development of atherosclerotic lesions. ■

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