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ORIGINAL ARTICLE

Lipids-induced Apoptosis Is Aggravated by Acyl-coenzyme A: Cholesterol Acyltransferase Inhibitor[△]

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Objective To investigate the role of acyl-coenzyme A: cholesterol acyltransferase inhibitor (ACATI) in apoptosis induced by lipids and whether lipids-induced apoptosis is accompanied by increase of free cholesterol in endoplasmic reticulum (ER), in order to further understand the mechanism of lipids-induced apoptosis in advanced atherosclerosis.

Methods Human vascular smooth muscle cells (VSMCs) and phorbol 12-myristate 13-acetate (PMA) differentiated THP-1 macrophages were used. Tritiated thymidine incorporation was applied to detect cell proliferation. Cytotoxicity was assessed by lactate dehydrogenase (LDH) release. 4',6-diamidino-2-phenylindole (DAPI) staining, caspase-3, -7 assay, and Annexin-V/propidium iodide (PI) staining were used to detect apoptosis. High performance liquid chromatography was used in intracellular free cholesterol and cholesterol ester assay. ER free cholesterol was quantified.

Results Different lipids had different effects on proliferation and cytotoxicity of VSMCs. 25-hydroxycholesterol (25OHC) had biphasic effects on the proliferation of VSMCs. At low concentration, it stimulated cell proliferation, but turned to proliferation inhibition as concentration reached 15 µg/mL. 25OHC and acetylated low density lipoprotein (AcLDL) could respectively induce apoptosis in human VSMCs and PMA differentiated THP-1 macrophages, which was aggravated by ACATI, accompanied by increase of intracellular free cholesterol content. There was also an increase of cholesterol content in ER with AcLDL-induced apoptosis in THP-1 macrophages.

Conclusions Lipids could induce apoptosis, accompanied by increase of intracellular free cholesterol content, which could be augmented by ACATI, suggesting that insults resulting in ER free cholesterol rise might be the initiator of apoptosis.

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THE concept of "glomerular atherosclerosis" was proposed in 1982,¹ suggesting that atherosclerosis and glomerulosclerosis share common pathogenic mechanisms.² Cardiovascular complications caused by accelerated atherosclerosis constitute the largest single cause of mortality in chronic renal failure patients.³ Atherosclerotic plaques develop as a consequence of the accumulation of circulating lipids and the subsequent migration of inflammatory cells (macrophages and T cells) and vascular smooth muscle cells (VSMCs).

Apoptosis has been found in atherosclerosis and is increasingly observed in plaque development.^{4,5} Both macrophages and VSMCs, the two major cell types involved, bear markers of apoptosis.⁶ Apoptosis of VSMCs may contribute to the accumulation of plaque.^{7,8} Apoptotic macrophages were detected in the lipid-rich core of atherosclerotic lesions.⁹ Macrophage apoptosis is significantly higher at sites of plaque rupture and thrombosis in patients with sudden coronary death.¹⁰

The uptake of modified lipoproteins mainly by macrophages leads to the accumulation of cholesterol esters (CE) and the formation of macrophage-derived foam cell, the hallmark of fatty streak.¹¹ As the lesion progressed, free cholesterol (FC) accumulated and resulted in apoptosis.^{12,13}

The enzyme responsible for cholesterol esterification is acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1) located in endoplasmic reticulum (ER) in cells.¹⁴ Chang et al¹⁵ pointed out that the main role of ACAT in intracellular cholesterol homeostasis is to guard against excessive buildup of cholesterol in ER. It would be advantageous to maintain the cholesterol concentration in ER low enough so that subtle changes in this parameter could produce sensitive signals for regulating sterol-regulated enzymes or proteins located in ER.¹⁶

When an ACAT inhibitor (ACATI) 58-035 was added to reduce the amount of CE, significant toxicity and cell death were observed.¹⁷ It was shown that cholesterol loading in macrophages with ACATI added could induce accumulation of FC in ER and lead to apoptosis mediated by ER stress pathway.^{18,19}

In this study, we observed the apoptosis of the cells involved in atherogenesis induced by oxysterol or modified lipoprotein, and the effect of ACATI on apoptosis in the context of intracellular cholesterol homeostasis change.

MATERIALS AND METHODS

Low density lipoprotein (LDL) isolation and acetylated LDL (AcLDL) preparation

Human LDL was isolated from fresh human plasma by sequential ultracentrifugation.²⁰ The degree of lipid peroxidation of the LDL was estimated as the concentration of thiobarbituric acid reactive substances (TBARS)²¹ and the result was expressed as nmol malondialdehyde (MDA)/mg LDL. The levels of TBARS in native LDL used in this study were lower than 0.1 nmol MDA/mg LDL. AcLDL was prepared by reaction of LDL with acetic anhydride.²²

THP-1 cells culture

THP-1 monocytes (American Type Culture Collection, Manassas, VA, USA) were maintained at 37°C in humidified 5% CO₂ atmosphere, in suspension at a cell density of 2.0×10⁵-8.0×10⁵/mL in RPMI 1640 containing 10% fetal bovine serum (FBS) (v/v), 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.05 mmol/L 2-mercaptoethanol (Sigma, Poole, UK). The cells were then cultured in phorbol 12-myristate 13-acetate (PMA) (Sigma) at a final concentration of 160 nmol/L for five days until complete differentiation into macrophage-like cells. All reagents for cell culture were obtained from GIBCO BRL (Paisley, UK) unless stated otherwise.

Human vascular smooth muscle cells (VSMCs) culture

Human aortic smooth muscle cells (TCS Cellworks, Liverpool, UK) were grown in TCS smooth muscle growth medium supplemented with 5% FBS. The cells were incubated at 37°C in humidified 5% CO₂ atmosphere. Medium was changed every 2-3 days, and passages 3-6 were used for experiments.

Protein determination and cell proliferation and cytotoxicity assay

Protein concentration was determined by modified Lowry assay.^{23,24} Human VSMCs were plated in 96-well plates (Falcon, Oxford, UK) at a density of 8 000 cells/well in standard culture medium (TCS Cellworks) until confluent. The cells were synchronized to the quiescent state by incubation in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 0.2% bovine serum albumin (BSA) (essentially fatty acid free) (Sigma) for 24 hours, then each well was washed with phosphate buffered saline (PBS) intensively. The cells were incubated for 24 hours in five different types of medium: DMEM containing 5% lipoprotein-deficient serum (LPDS) (GIBCO) as control, 1-15 µg/mL of 25-hydroxycholesterol (25OHC), 10-75 µg/mL of cholesterol, 25-200 µg/mL of LDL, and 25-100 µg/mL of AcLDL. For proliferation assay, the cells were then labelled for 18 hours with 1 µCi [³H] thymidine (Amersham, Buc-

kinghamshire, UK), washed with PBS, trypsinized for 5-10 minutes, and harvested. Radioactivity associated with replicative DNA synthesis of proliferating cells was measured with a scintillation counter. In the [^3H] incorporation experiment, the proliferation was represented by counts per minute (CPM). In toxicity assay, we detected lactate dehydrogenase (LDH) release in the supernatant and LDH in lysed cells with an enzyme assay following the instructions from In Vitro Toxicity Assay Kit Lactate Dehydrogenase (Sigma, Saint Louis, MS, USA). The cytotoxicity was represented by the ratio of LDH detected in the supernatant (assumed to be released from the dead cells) to the total amount of LDH detected. The sum was assumed as the total cell number, which was the final normalization index.

4',6-diamidino-2-phenylindole (DAPI) staining

Human VSMCs were plated into eight-well plates (Nunc, Naperville, IL, USA) at 1×10^4 cells/well. After reaching 60%-70% confluence, the cells were washed with DMEM and the culture medium were replaced by DMEM containing 0.2% BSA (as control), 200 nmol/L of ACATI FR 179254 (Calbiochem, Nottingham, UK), 15 $\mu\text{g/mL}$ of 25OHC, or 200 nmol/L of ACATI FR179254 plus 15 $\mu\text{g/mL}$ of 25OHC for 24-hour incubation. THP-1 cells were plated into eight-well plates at 8×10^4 cells/well until fully differentiated by PMA for five days. The differentiated THP-1 cells were washed with serum-free RPMI and then cultured for 24 hours in four types of medium: serum-free RPMI medium (as control), 50 $\mu\text{g/mL}$ of AcLDL, 100 $\mu\text{g/mL}$ of AcLDL, and 50 $\mu\text{g/mL}$ of AcLDL plus 800 nmol/L of ACATI FR 179254.

The treated cells were washed once with 1 $\mu\text{g/mL}$ of DAPI (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in methanol, then incubated with the stain for 15 minutes at 37°C in dark, and washed again with methanol. The plates were analyzed by fluorescence microscopy with 340/380 nm excitation filter and LP (long-pass) 430 nm barrier filter. Digital images were obtained with a C-4000 ZOOM Olympus digital camera (Olympus America, Inc.). The percentage of apoptotic cells was calculated based on the results of cell count in five different visual fields, the total number of cells counted in each field being more than 500.

Annexin-V and propidium iodide (PI) staining

Human VSMCs were seeded into 12-well plates (Nunc Inc., Naperville, IL, USA) at a density of 7×10^4 cells/well. The following treatment prior to harvest was the same as in DAPI staining. The cell pellets were washed with PBS twice, then stained with Annexin-V-FLUOS (Roche Applied

Science, Nonnenwald, Penzberg, Germany) labeling solution following the manufacturer's instructions. Stained cells were immediately analyzed by fluorescence-activated cell sorter (FACS) with a flow cytometer EPICS XL-MCL (Coulter, CA, USA).

Caspase-3, -7 activities and cell viability assay

THP-1 cells were seeded into opaque-walled 96-well plates (Nunc, Naperville, IL, USA) at a density of 2×10^4 cells/well in 100 μL of RPMI 1640 medium. After fully differentiated, the cells were washed with RPMI 1640 medium twice, and cultured for 24 hours in three different media: 100 μL of RPMI 1640 medium (as control), 100 $\mu\text{g/mL}$ of AcLDL, and 100 $\mu\text{g/mL}$ of AcLDL plus 10 $\mu\text{g/mL}$ of ACATI 58-035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl] propanamide, kindly donated by Novartis, Basel, Switzerland). Caspase-3, -7 activities and viability of the cells were studied according to the protocols provided by the manufacturer of CellTiter-Glo[®] Reagent and Caspase-Glo[®] 3/7 Reagent (Promega, Madison, WI, USA). Briefly, 100 μL of each of these two reagents were added into two sets of identically treated wells. The wells containing only experimental medium without cells were set as background. The mixtures were incubated at room temperature for 10-30 minutes. Measure the luminescence of each set of wells with a plate-reading luminometer, following the instructions provided by the manufacturer. The final reading was defined by subtracting the average reading detected from the background wells. The apoptosis was represented by the relative caspase activities after normalization by cell viability.

Intracellular FC and CE assay

THP-1 cells were seeded into 12-well plates at a density of 4×10^5 in 2 mL of culture medium until fully differentiated. The cells were cultured for 24 hours in serum-free RPMI 1640 (as control), 100 $\mu\text{g/mL}$ of AcLDL, or 100 $\mu\text{g/mL}$ of AcLDL plus 10 $\mu\text{g/mL}$ of ACATI 58-035. Modified method for determination of intracellular FC and total cholesterol (TC) was applied.^{25,26} Briefly, re-suspend the cell pellet in 1 mL of chloroform/methanol 2 : 1 (v/v); leave the pellet dry after sonification and dissolve it in 10 N of NaOH for protein assay. Remove the liquid phase into clean glass tubes. After chloroform was evaporated in vacuum, heat the tubes for 20 minutes at 105°C in an oven. Re-suspend the samples in 95% ethanol. Cholesterol and cholesteryl oleate were prepared in chloroform in stock at a concentration of 10 $\mu\text{g/mL}$ and the standard solutions containing 0-300 ng cholesterol or 0-600 ng cholesteryl oleate were successively diluted in 95% ethanol. For the compositions of

solutions used for determination of FC and TC, we resorted to references 25 and 26. Take aliquots of samples for FC and TC assays, and add prepared assay solutions. After incubation for 30 minutes at 37°C, fluorescence was measured with a spectrophotometer (excitation, 325 nm; emission, 415 nm). The actual amounts of FC and TC were obtained from the standard curves, and normalized by protein mass. The CE amount was determined by subtracting FC from TC. All the chemicals and organic solvents used in this assay were produced by Sigma.

Statistical analysis

All results in this study were expressed as the relative ratio over control (as 100%) unless otherwise indicated. Within an experiment, duplicate, triplicate, or quadruplicate wells were used for each condition or treatment. Data were collected from at least three separate experiments and presented as means $\pm$ SD. SPSS11.0 software was used to analyze difference between samples with one-way analysis of variance (ANOVA), and S-N-K post-test for difference between selected pairs of samples. $P<0.05$ was considered statistically significant.

RESULTS

Cytotoxicity and effects of lipids on the proliferation of VSMCs

In our assay, 25OHC had biphasic effects on VSMC proliferation: at low concentration (below 5 μ g/mL), it stimulated cell proliferation, but turned to inhibition as concentration reached 15 μ g/mL. Meanwhile, in LDH release assay, the cytotoxicity of 25OHC appeared at the concentration of 5 μ g/mL and intensified as concentration increased up to 15 μ g/mL. In the concentration range studied (10–75 μ g/mL), cholesterol *in vitro* inhibited VSMC proliferation only at concentrations above 50 μ g/mL, and exerted cytotoxicity only at the concentration of 75 μ g/mL. LDL at the concentration of 25–200 μ g/mL demonstrated dose-dependent stimulation on VSMC proliferation as the [^{3} H] incorporation increased significantly compared with control, but no toxic effects were observed. AcLDL exhibited dose-dependent inhibition on cell proliferation in the studied range of 25–100 μ g/mL, and showed cytotoxicity at the concentration ranging from 25 to 100 μ g/mL (Fig. 1).

25OHC-induced apoptosis in VSMCs and the additive effect of ACATI on apoptosis

Since 15 μ g/mL of 25OHC inhibited VSMC proliferation significantly, we investigated whether there existed apoptosis, and what the effect of ACATI was on this phe-

nomenon. Three methods were used for detecting apoptosis, including Annexin-V and PI staining, DAPI staining, and caspase-3, -7 assay.

Compared with control, 200 nmol/L of ACATI FR 179254 alone could not induce apoptosis after incubation in DMEM medium containing 2% LPDS for 24 hours, as the percentage of Annexin-V positive cells detected by FACS did not increase significantly. 25OHC at 15 μ g/mL significantly induced VSMC apoptosis as the ratio of apoptotic cell percentage between treated cells over control increased to 248.79% $\pm$ 12.51%, which was further enhanced by 200 nmol/L of FR 179254 to 293.97% $\pm$ 44.93% over control. The results from DAPI staining conformed with those from Annexin-V staining. After treatment with 25OHC, the apoptotic cells with condensed nuclei became remarkably visible. In cells treated with both ACATI FR179254 and 25OHC, more apoptotic cells were observed than in those treated with 25OHC alone (Fig. 2).

AcLDL-induced apoptosis in PMA differentiated THP-1 cells and the additive effect of ACATI on apoptosis

Macrophage is the main type of cell involved in the pathogenesis of atherosclerosis as it uptakes modified lipids including AcLDL and oxidized LDL (oxLDL) *via* scavenge receptors, the expression of which is not regulated by intracellular cholesterol contents. Therefore, we studied if AcLDL could induce apoptosis in PMA differentiated THP-1 cells and further observed if ACATI could augment the apoptosis. In terms of the percentage of apoptotic cells, more DAPI stained cells were observed in 50 μ g/mL AcLDL treated group than in control (5.60% $\pm$ 1.68% vs. 1.90% $\pm$ 0.14%, $P<0.05$). With the concentration of AcLDL doubled, the apoptotic cell percentage increased to 24.57% $\pm$ 3.75%, significantly higher than that with 50 μ g/mL AcLDL ($P<0.001$). Adding 800 nmol/L of FR 179254 significantly increased the apoptotic cell percentage compared with 50 μ g/mL AcLDL treatment (11.93% $\pm$ 3.75% vs. 5.60% $\pm$ 1.68%, $P<0.05$) (Fig. 3).

The above data led us to expect an increase in caspase-3, -7 activity when macrophages were lipid-loaded and a further increase when another ACATI, 58-035, was tested. As revealed in observation, 100 μ g/mL of AcLDL significantly increased the caspase-3, -7 activity to 246.67% $\pm$ 39.65% over control ($P<0.01$ compared with control), and 10 μ g/mL of ACATI 58-035 increased the caspase-3, -7 activity to 330.91% $\pm$ 68.29% ($P<0.05$ compared with AcLDL treated cells), which showed that ACATI augmented the effect of AcLDL.

Augmentative effect of ACATI on apoptosis possibly caused by FC accumulation

By measuring the content of intracellular FC and CE, we tried to find out if intracellular cholesterol homeostasis was changed in THP-1 cells in apoptosis. THP-1 cells cultured in 100 $\mu\text{g}/\text{mL}$ of AcLDL for 24 hours demonstrated significant

increase in both FC and CE content, $153.72\% \pm 21.38\%$ and $240.12\% \pm 16.94\%$ respectively compared with control. In the presence of 10 $\mu\text{g}/\text{mL}$ of ACATI 58-035, FC content was significantly increased to $198.94\% \pm 35.65\%$ over control, while no significant change was observed in CE content (Fig. 4).

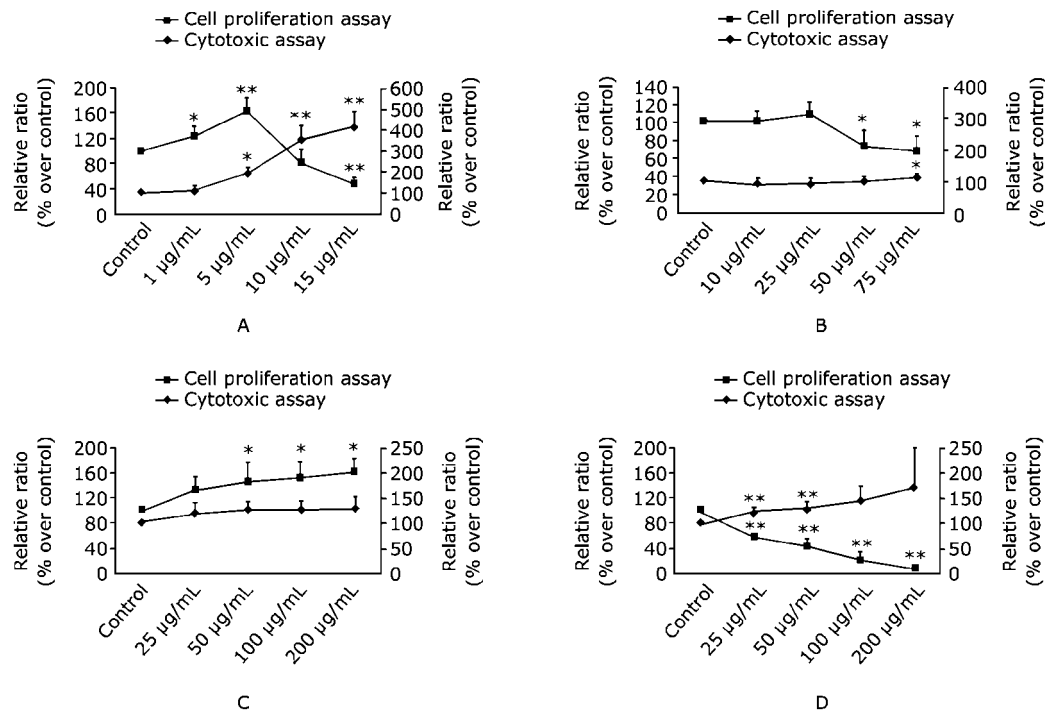


Figure 1. Cytotoxicity of 25OHC, cholesterol, LDL, and AcLDL and their effects on proliferation of human VSMCs.

The proliferation results are expressed as ratios of CPM of treated wells over those of controls. The cytotoxicity results are expressed as ratios of readings in $\text{OD}_{490\text{nm}-690\text{nm}}$ of the supernatant over the sum (supernatant plus lysed cells). The left axis represents the relative ratio over control in $[\text{}^3\text{H}]$ incorporation as proliferation index; the right axis is the relative ratio over control in the toxicology assay based on LDH detection. The values are represented as means $\pm$ SD of quadruplicate wells from four experiments.

A. 25OHC; B. cholesterol; C. LDL; D. AcLDL.

25OHC: 25-hydroxycholesterol; LDL: low density lipoprotein; AcLDL: acetylated low density lipoprotein; VSMCs: vascular smooth muscle cells; CPM: counts per minute; OD: optical density; LDH: lactate dehydrogenase.

* $P < 0.05$, ** $P < 0.001$ compared with control.

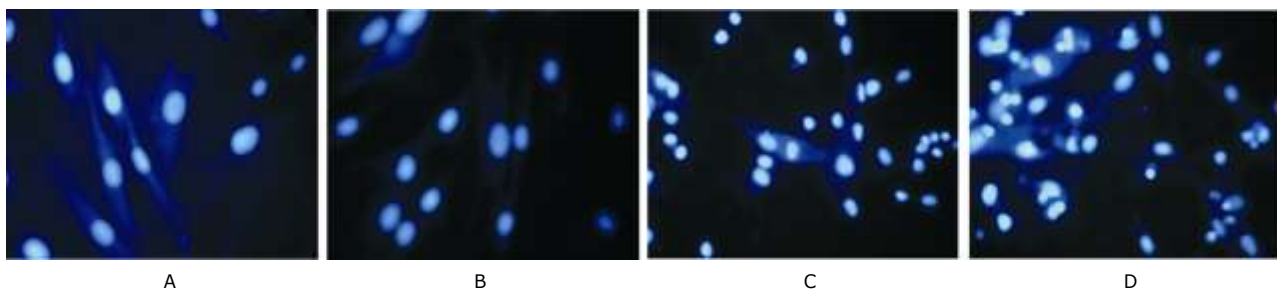


Figure 2. Morphological changes of human VSMCs after treatment with either or both of 25OHC and ACATI FR 179254. DAPI staining $\times 400$ Human VSMCs were incubated for 24 hours in four different types of media: DMEM containing 0.2% bovine serum albumin (control) (A), 200 nmol/L of ACATI FR 179254 (B), 15 $\mu\text{g}/\text{mL}$ of 25OHC (C), and 15 $\mu\text{g}/\text{mL}$ of 25OHC plus 200 nmol/L of ACATI FR 179254 (D).

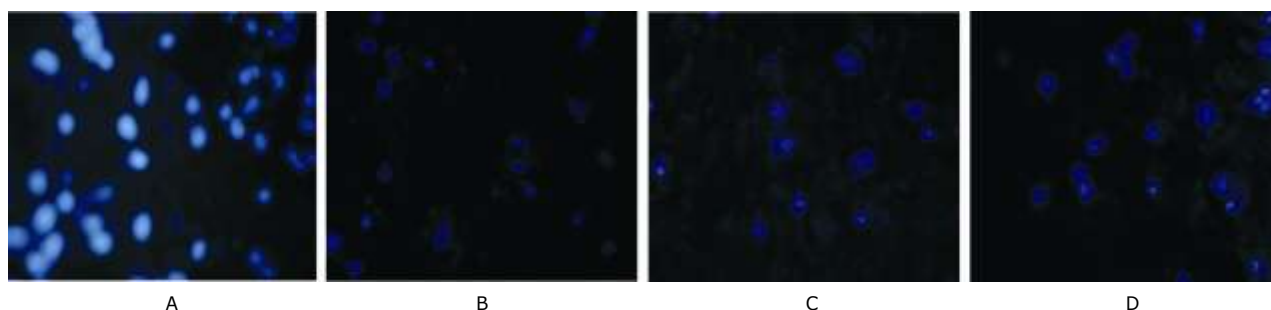


Figure 3. Morphological changes of THP-1 macrophages after treatment with either or both of AcLDL and ACATI FR 179254. DAPI staining $\times 400$

THP-1 cells were differentiated by culturing in phorbol 12-myristate 13-acetate (PMA) at a concentration of 160 nmol/L for five days in 10% fetal bovine serum/RPMI 1640 medium. For the next 24-hour culture, the culture medium was replaced by RPMI 1640 (control) (A), or RPMI with 50 $\mu\text{g/mL}$ of AcLDL (B), with 100 $\mu\text{g/mL}$ of AcLDL (C), or with 50 $\mu\text{g/mL}$ of AcLDL plus 800 nmol/L of ACATI FR 179254 (D).

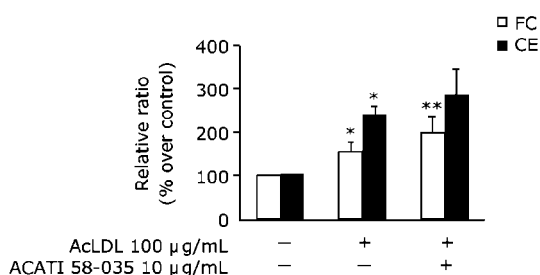


Figure 4. Effect of ACATI on the intracellular free cholesterol (FC) and cholesterol ester (CE) in AcLDL-loaded THP-1 macrophages.

THP-1 cells were differentiated by culturing in PMA at a concentration of 160 nmol/L in 10% FBS/RPMI 1640 medium for five days, then cultured for another 24 hours in RPMI 1640 medium (control), or in RPMI 1640 with 100 $\mu\text{g/mL}$ of AcLDL, or with 100 $\mu\text{g/mL}$ of AcLDL plus 10 $\mu\text{g/mL}$ of ACATI 58-035. The intracellular FC and CE were assayed. The results are expressed as means $\pm$ SD of the ratios over control ($n=3$).

* $P<0.01$ compared with control, ** $P<0.05$ compared with AcLDL treated cells.

DISCUSSION

Cholesterol and its oxides are believed to be involved in initiation and progression of atherosclerosis.²⁷ Many types of cholesterol oxides have been found in atheromatous plaques in hypercholesterolaemic patients.²⁸ Kandutsch et al²⁹ found that purified cholesterol only had weak inhibitory effect on proliferation of smooth muscle cells (SMCs) *in vitro*. 25OHC is a much-studied oxysterol that is both formed endogenously and found in the diet. It is also

present in atheromatous plaque.³⁰ 25OHC has been found to inhibit VSMC proliferation in a dose-dependent manner, and to be a potent inducer of apoptosis in VSMCs which were cultured for 24 hours at the concentration of 25 $\mu\text{g/mL}$ in 15% FBS.²⁷

Setting 15% FBS/DMEM as control, Yin et al²⁷ also demonstrated that cholesterol up to 50 $\mu\text{g/mL}$ did not either inhibit rabbit VSMC proliferation or induce apoptosis, 25OHC less than 10 $\mu\text{g/mL}$ did initially stimulate VSMC proliferation, and turned to dose-dependent inhibitory effect thereafter until reaching 50 $\mu\text{g/mL}$, the maximum concentration tested.

To exclude the interference from the unmeasured lipids in FBS, we used culture medium containing 5% LPDS as control. Our findings coincided with the results of previous studies: cholesterol itself had no inhibitory effect on proliferation of human VSMCs until the concentration exceeded 50 $\mu\text{g/mL}$. Interestingly, in the concentration range from 1 to 5 $\mu\text{g/mL}$, 25OHC did stimulate VSMC proliferation, but exerted dose-dependent inhibitory effect at higher concentrations. This will challenge further research on specific concentration-dependent effects of 25OHC on key enzymes in cholesterol turnover at transcriptional and posttranscriptional levels^{31,32} and on consequent distinct cell biology.

The inhibition of proliferation may be explained by several mechanisms. For example, 25OHC may induce the cells to remain in G0, or cause necrosis or apoptosis. Vascular cells possess two major apoptotic pathways commonly referred to as the death receptor pathway and the mitochondrial pathway, both of which could be mobilized in oxysterol-induced apoptosis.^{33,34} What we found based on that understanding was that *in vitro* 25OHC-induced apoptosis in human VSMCs was enhanced by

ACATI FR 179254.

Foam cells found *in vivo* have been demonstrated to be of either macrophage or SMC origin.¹⁹ Acetylation of LDL removes positive charges from the ϵ -amino groups of lysine and therefore converts a weakly anionic lipoprotein into a strongly anionic one. AcLDL is one of the candidates binding to the scavenger receptors expressed in macrophages, and the non-regulated uptake of modified lipoproteins leads to the formation of foam cells. PMA treated THP-1 cells have been employed as a representative macrophage cell line and used as a model in the study of atherosclerosis and foam cell formation *in vitro*.³⁵

Apart from the extensive investigation on oxLDL-induced apoptosis in macrophages, some studies have also been conducted on AcLDL-induced apoptosis and produced affirmative results. Nhan et al³⁶ found that while about 100% of wild-type peritoneal macrophages harvested from C57BL/6/J male mice were TUNEL-positive by 4-day's incubation with 30 $\mu\text{g/mL}$ of oxLDL, AcLDL at the same concentration could also induce a modest increase in TUNEL frequency to approximately 15%. Using double labeling with green fluorescent fluorescein (FITC) and annexin V, Rosenson-Schloss et al³⁷ detected the apoptosis in IC21 macrophages cultured in 10% FBS medium containing 25 $\mu\text{g/mL}$ of AcLDL for a maximum of seven hours. In spite of methodological differences, our study also produced evidence of AcLDL-induced apoptosis in PMA fully differentiated THP-1 macrophages by using DAPI staining and caspase assay. Moreover, AcLDL-induced apoptosis in THP-1 macrophages was aggravated by ACAT inhibition.

The mechanisms by which ACATI aggravates lipid-induced apoptosis deserve further exploration, suggesting that FC increase might be an important signal in apoptosis initiation. Some previous work established a FC loading model.^{19,38} After intraperitoneal injection of 40 μg concanavalin A in 0.5 mL PBS in adult C57BL/6 female mice for three days, the peritoneal macrophages were harvested and incubated with 100 $\mu\text{g/mL}$ of AcLDL in the presence of 10 $\mu\text{g/mL}$ ACATI 58-035 for five hours. Since cholesterol homeostasis differs between human macrophages and those of murine origin,^{39,40} it was reasonable to investigate whether ACATI 58-035 could also lead to FC loading in AcLDL treated THP-1 macrophages.⁴¹ The present study provided strong support to the correlation between apoptosis in lipid-laden macrophages and FC loading because apoptosis was aggravated by ACAT inhibition and further FC accumulation.

FC accumulation has been detected in apoptotic lipid-laden macrophages, and the functioning target of ACATI was found in ER.^{42,43} Therefore, by directly measuring the

sterol content in ER, we found that in AcLDL-induced apoptosis in THP-1 macrophages, there was a significant rise of cholesterol in ER, conforming with the finding of a previous study.⁴⁴ Further research is needed on the mechanism by which ER sterol stress regulating key enzymes and proteins located in ER are involved in cholesterol homeostasis associated with macrophage foam cell formation and apoptosis.

Patients with end-stage renal disease (ESRD) suffer from secondary complex dyslipidemia consisting of both quantitative and qualitative abnormalities in serum lipoproteins resulting from alterations in lipoprotein metabolism and composition.⁴⁵ Atherosclerosis itself is a chronic inflammatory disease; together with disrupted lipid metabolism in chronic kidney disease and inflammatory state, a vicious cycle is formed in ESRD. Therefore, atherosclerosis constitutes the major concern in patients suffering from chronic kidney disease. The present study demonstrated the multiple pathways of atherogenesis, such as LDL-stimulated VSMC proliferation, 25OHC-induced VSMC apoptosis, and AcLDL-induced THP-1 macrophage apoptosis, both of the latter two augmented by ACAT inhibition. The study also confirmed that FC accumulation was a potent inducer of apoptosis, and that sterol stress in ER was the key to initiation of the apoptotic cascade.

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