



Anti-atherogenic effects of the acyl-CoA:cholesterol acyltransferase inhibitor, avasimibe (CI-1011), in cultured primary human macrophages

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Abstract

Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitors have been shown to reduce atherosclerotic lesions in animals; however, the mechanism(s) for this effect remains unclear. Therefore, we used cultured primary human monocyte-derived macrophages (HMMs) to examine the effect of the ACAT inhibitor, avasimibe (CI-1011), during foam cell formation and during cholesterol efflux from established foam cells. To examine the effect of CI-1011 on foam cell development, HMMs were incubated with aggregated acetylated LDL (ag-acLDL) \pm CI-1011 for 48 h. Total cholesterol (TC) was 29% lower in HMMs incubated with ag-acLDL and CI-1011 compared with ag-acLDL ($P < 0.05$). To determine if TC reduction was due to reduced ag-acLDL uptake by CI-1011, ¹²⁵I-acLDL binding at 4 °C for 4 h to HMMs preincubated with acLDL or ag-acLDL, CI-1011, acLDL + CI-1011, or ag-acLDL + CI-1011 for 48 h was measured. Specific binding was 40% lower in cells preincubated with acLDL + CI-1011, 52% lower in cells preincubated with ag-acLDL + CI-1011 and 49% lower in cells preincubated with CI-1011 compared with cells preincubated with acLDL ($P < 0.0003$). Because CI-1011 appeared to directly affect acLDL binding, ¹²⁵I-acLDL (3–80 μ g protein/ml) binding was done in HMMs preincubated with CI-1011 (0–10 μ g/ml) for 48 h. The calculated B_{\max} decreased in HMMs exposed to increasing concentrations of CI-1011, suggesting that CI-1011 altered scavenger receptor function and/or number. To examine the effects of CI-1011 on cholesterol efflux from established foam cells, we first examined whether CI-1011 was cytotoxic. HMMs were preincubated with ag-acLDL for 24 h, and then radiolabeled with [¹⁴C]adenine for 2 h (time zero). The radiolabeled cells were exposed to control RPMI medium or the same medium + HDL, CI-1011, or HDL + CI-1011 for 24 h. The release of [¹⁴C]adenine into the medium was not significantly different between cells exposed to RPMI, HDL, CI-1011, or HDL + CI-1011, suggesting that CI-1011 was not cytotoxic. Foam cells exposed to RPMI and CI-1011 (1–10 μ g/ml) for 48 h showed time dependent reduction in cellular TC mass, with a corresponding increase in radiolabeled unesterified cholesterol into the medium. We then asked whether CI-1011 enhanced apoE mediated cholesterol efflux. Although cellular apoE increased between 2- and 7-fold in foam cells compared to control macrophages, apoE secreted into the medium was not significantly different between cells exposed to RPMI or CI-1011. Thus, CI-1011 exerted anti-atherogenic effects by reducing TC accumulation, inhibiting acLDL binding, and by limiting lipid storage in HMMs. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

The presence of cholesteryl ester-enriched macrophages (foam cells) is a characteristic finding within atherosclerotic lesions. Foam cells develop via

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receptor and non-receptor mediated uptake of modified lipoproteins, particularly low density lipoproteins. Internalized modified LDL is degraded in lysosomes and the hydrolyzed unesterified or free cholesterol (FC) can then be transported to the plasma membrane. Excess FC is re-esterified by the enzyme, acyl-CoA:cholesterol acyltransferase (ACAT), to form esterified cholesterol (EC) [1]. It is the excess accumulation of EC droplets

that imparts a foamy appearance to the cells under light microscopy. The pivotal role of ACAT in foam cell formation has been determined through the use of ACAT inhibitors. We recently reported novel effects of the ACAT inhibitor, 58-035, during foam cell development in HMMs [2]. In the presence of acLDL, 58-035 significantly lowered cholesterol accumulation two ways: by decreasing high affinity binding of ^{125}I -acLDL and enhancing cholesterol efflux [2]. Additionally, 58-035 was not cytotoxic to the cells [2]. However, in this study we did not directly test the effect of 58-035 alone on acLDL binding, nor did we test its effect on cholesterol efflux after cells were enriched. The pattern of lipid accumulation in HMMs exposed to acLDL plus 58-035 was consistent with those reported by Meiner et al. [3]; they showed that total cholesterol (TC) and EC accumulation in peritoneal macrophages isolated from ACAT knockout mice and exposed to acLDL were lower (88 and 7.9 μg protein/ml, respectively) compared with control macrophages (166 and 130 μg protein/ml, respectively). Interestingly, FC accumulation was not significantly different between the control and ACAT knockout mouse macrophages [3].

Many ACAT inhibitors have been developed to reduce the formation and progression of atherosclerotic lesions in various in vivo animal models [4–6]. A newer compound, avasimibe (CI-1011), is an oxysulfonylcarbamate that inhibits ACAT function, and is bioavailable [7]. Delsing et al. [8] studied the effect of this inhibitor on atherosclerosis development and found that lesions were significantly lower in apolipoprotein E (apoE)-Leiden transgenic animals fed a cholesterol diet plus CI-1011 compared with control animals. In a study of regression, Bocan et al. [9] reported that New Zealand white rabbits fed a high cholesterol, high fat diet and then treated with CI-1011 showed reduced atherosclerotic lesions compared with control animals. Cullen et al. [10] reported greater FC efflux from HMM foam cells incubated with CI-1011 and/or atorvastatin compared to control macrophages.

The mechanism(s) for the potential benefit of ACAT inhibitors in reducing atherosclerotic lesions, however, is still unclear. In this present work, we have used cultured HMMs to further explore the mechanism(s) for ACAT inhibition during the process of foam cell development and during cholesterol efflux from established foam cells. Specifically, we examined the effects of CI-1011 on cholesterol accumulation, ^{125}I -acLDL binding, cytotoxicity, cholesterol efflux, and apoE secretion.

2. Materials and methods

2.1. Materials

Tissue culture plates (Falcon Primaria) were purchased from Becton Dickinson. Heat inactivated, pooled human

serum was purchased from PelFreeze. RPMI-1640 medium was purchased from Life Technologies. Ficoll-Paque was purchased from Pharmacia Biotech, Inc. [^{14}C]adenine (287 mCi/mmol) and [^{125}I] (15 Ci/mg iodide) were purchased from Amersham Life Sciences, Inc. All other chemicals were reagent grade or higher.

2.2. Cell culture

HMMs were isolated from leukocyte preparations obtained from the Johns Hopkins Hemapheresis Center. Monocytes were isolated and differentiated into macrophages after being plated on Falcon Primaria dishes for up to 2 weeks as previously described [2].

2.3. Lipoprotein isolation

LDL (1.019–1.063 g/ml) and HDL (1.063–1.210 g/ml) were isolated from healthy male and female donors using sequential density ultracentrifugation [11]. LDL was acetylated (acLDL) as described by Frankel-Conrat [12], and the completeness of acetylation and purity of the acLDL was assessed by agarose electrophoresis. Aggregated acLDL (ag-acLDL) was obtained by subjecting an aliquot of acLDL to vortexing (9000 rpm) at room temperature for 10 min before use in experiments [13].

2.4. Lipid enrichment of cells

In earlier work we incubated HMMs with larger quantities of acLDL (250–500 μg protein/ml) to induce foam cell formation [2]. We subsequently found that cells exposed to ag-acLDL showed greater levels of cholesterol accumulation in a shorter time (manuscript submitted). For foam cell formation, the growth medium (RPMI medium containing 10% human serum) was aspirated and the cells were rinsed four times with RPMI medium, and then HMMs were exposed to RPMI medium containing bovine serum albumin (BSA, 0.2%) and dimethylsulfoxide (DMSO, 0.2%, vehicle for CI-1011) (control medium) with and without ag-acLDL (100 μg protein/ml) and CI-1011 (1 μg /ml) for 48 h. For cholesterol efflux experiments, HMMs were preincubated with ag-acLDL (100 μg protein/ml) for 24 h, and then exposed to control RPMI medium with and without HDL (100 μg protein/ml), CI-1011 (2 μg /ml) or HDL plus CI-1011 (2 μg /ml) for 24–48 h. Additionally, the appearance of [^{14}C]FC in the medium was monitored by first preincubating HMMs with RPMI medium containing ag-acLDL (100 μg protein/ml) radiolabeled with [^{14}C]FC (0.5 μCi /ml) in an ethanolic spritz (final concentration, 0.1%) for 24 h. The medium was removed, cells rinsed three times with RPMI medium, and then cells were exposed to control RPMI medium with and without CI-1011 (1–10 μg /ml) for

4–48 h. At each time point, the medium was aspirated and centrifuged to pellet nonadherent cells. The appearance of [^{14}C]FC in the medium was measured by liquid scintillation spectroscopy. Cellular lipids were extracted using hexane:isopropanol (3:2, v/v) for 1 h [1]. The distribution of cellular radiolabeled cholesterol was measured by subjecting an aliquot of the cell extract and FC and EC standards to thin layer chromatography using petroleum ether:hexane:glacial acetic acid solvent system (85:15:2, v/v). The percent FC efflux was calculated as: medium [^{14}C]FC dpm/ cell [^{14}C] dpm \times 100. FC and TC mass were quantified by gas liquid chromatography using stigmaterol (1 mg/ml) as an internal standard. EC mass was calculated as the difference between TC and FC [14], and all values were normalized to cell protein [15].

2.5. Binding experiments

2.5.1. ^{125}I -acLDL labeling

^{125}I -LDL was prepared by the method of McFarlane [16] as previously described [17]. Unbound ^{125}I was removed by dialysis against four changes (4 l each) of 0.15 M NaCl containing 0.05% (w/v) Na_2EDTA , pH 7.5. The labeled lipoprotein was then acetylated as described above and dialyzed against four changes of 0.15 M NaCl- Na_2EDTA buffer, pH 7.5. The labeled acLDL was 98–99% precipitable with 10% (w/v) trichloroacetic acid (TCA). More than 95% of the radioactivity migrated with acLDL upon electrophoresis in 3% agarose gels. The specific activity of ^{125}I -acLDL from two separate preparations varied between 38.5 and 315 cpm/ng cell protein.

2.5.2. ^{125}I -acLDL binding

HMMs were preincubated with ag-acLDL (100 μg protein/ml) or acLDL (500 μg protein/ml) \pm CI-1011 (2 μg /ml) for 48 h, the medium was aspirated and the cells were rinsed four times with RPMI medium, and then incubated in RPMI medium containing lipoprotein deficient serum (LPDS, 5 mg/ml) for 30 min at 37 $^\circ\text{C}$. The medium was then aspirated and the cells were incubated with fresh LPDS medium containing ^{125}I -ag-acLDL or ^{125}I -acLDL (5 μg /ml) alone or with unlabeled ag-acLDL or acLDL (500 μg protein/ml) for an additional 4 h at 4 $^\circ\text{C}$. At the end of the binding period, the medium was discarded and the cells were washed five times with cold phosphate buffered saline (PBS) containing BSA (0.5%), and then three times with cold PBS alone. The cells were lysed in 1.0 ml of 1.0 N NaOH, dried overnight, and reconstituted with 1.0 ml distilled H_2O . Aliquots were taken for the measurement of bound ^{125}I -acLDL and cell protein.

To measure the effects of CI-1011 on acLDL binding, HMMs were preincubated with control RPMI medium with and without CI-1011 alone (1, 2, and 10

$\mu\text{g}/\text{ml}$) for 48 h. The medium was aspirated, the cells rinsed four times with RPMI medium, and then incubated with LPDS medium for 30 min at 37 $^\circ\text{C}$. Cells were then incubated with LPDS medium containing ^{125}I -acLDL (3–80 μg protein/ml) for 4 h at 4 $^\circ\text{C}$. Nonlinear regression and Scatchard analysis were done using Sigmaplot (v4.0).

2.6. Cytotoxicity assays

2.6.1. [^{14}C]adenine release

The cellular release of radiolabeled adenine was measured and calculated as described by Warner et al. [18]. HMMs were preincubated with RPMI medium containing ag-acLDL (100 μg protein/ml) for 24 h. The medium was removed, the cells rinsed four times with RPMI medium, and then incubated with RPMI medium containing [^{14}C]adenine (0.5 $\mu\text{Ci}/\text{ml}$) for 2 h (time zero). This medium was aspirated and the cells rinsed three times with serum free medium. Radiolabeled cells were incubated with control RPMI medium with and without CI-1011 (2 $\mu\text{g}/\text{ml}$), HDL (100 μg protein/ml) or HDL plus CI-1011 for 24 h. Experiments were ended by removing the medium and subjecting it to centrifugation at 9000 rpm for 10 min at room temperature to pellet nonadherent cells. Aliquots of the medium were measured for released [^{14}C]adenine by liquid scintillation spectrometry and the values were normalized to cell protein. The percent adenine release was calculated as: ([^{14}C] dpm in the medium/cell [^{14}C] dpm at time zero) \times 100. Cellular cholesterol mass was quantified by GLC.

2.7. Apolipoprotein E assays

HMMs were incubated with control RPMI medium or ag-acLDL (100 μg protein/ml) for 24 h. Triplicate wells from foam cells and control macrophages were lysed with 5% SDS, 50 mM Tris-HCl (pH 6.8) and pooled for measurement of cellular apoE levels. Foam cells were then exposed to control RPMI medium with and without CI-1011 (2 $\mu\text{g}/\text{ml}$) for 24 h (efflux). The medium was collected and subjected to centrifugation at 9000 rpm for 10 min at room temperature to pellet nonadherent cells. Various proteinase inhibitors were added to the medium (aprotinin 10 $\mu\text{g}/\text{ml}$, leupeptin 20 $\mu\text{g}/\text{ml}$, pepstatin 10 $\mu\text{g}/\text{ml}$, and PMSF 1 mg/ml) and then the samples were frozen at -80 $^\circ\text{C}$. NUNC 96-well microtiter plates, coated with the human apoE specific monoclonal antibody E10 7F4 and blocked for 1 h with ELISA diluent (borate-saline buffer containing 0.1% casein, 0.1% Tween 20, 0.01% Na_2EDTA , and 0.01% NaN_3), were used to trap apoE bearing lipoproteins in appropriately diluted samples. After an overnight incubation at room temperature with shaking, the plates were washed with PBS buffer containing

0.1% Tween 20 and incubated for 2 h with the reporting antibody, rabbit anti-human apoE IgG. The plates were again washed and incubated for 2 h with a goat anti-rabbit IgG antibody conjugated to alkaline phosphatase. After a final washing, substrate was added and the plates read using a Dynex microtiter plate reader set at a 405 nm wavelength. Samples and controls were done in duplicate and the standards (Wako) in triplicate.

2.8. Statistical analysis

Student's *t*-test was used to compare group means. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. The effect of CI-1011 on foam cell formation

To determine if CI-1011 lowered cholesterol accumulation in cells exposed to ag-acLDL, we examined foam cell formation in HMMs incubated with RPMI medium containing ag-acLDL (100 µg protein/ml) with and without CI-1011 (1 µg/ml) for 48 h. TC was 29% lower ($P < 0.05$), EC was 67% lower ($P < 0.003$), and FC was 38% higher ($P < 0.002$) in cells incubated with ag-acLDL plus CI-1011 compared with ag-acLDL alone (Fig. 1). While FC was higher, the reductions in TC and EC mass were consistent with results in cells co-incubated with acLDL and 58-035 [2]. CI-1011 significantly lowered TC in HMMs exposed to ag-acLDL. We have shown that the uptake, cell association, and degradation of ag-acLDL occurs via receptor and non-receptor mediated mechanisms, and is associated with

Table 1

High affinity binding characteristics of ^{125}I -ag-acLDL or ^{125}I -acLDL in human macrophages preincubated with modified LDL with and without CI-1011 for 48 h

	High affinity binding (ng/cell protein)
<i>Exp. 1</i>	
ag-acLDL (100 µg protein/ml)	24.2 ± 3.9
ag-acLDL + CI-1011 (2 µg/ml)	11.5 ± 1.1*
CI-1011	12.1 ± 1.8**
<i>Exp. 2</i>	
acLDL (500 µg protein/ml)	56.5 ± 4.6
acLDL + CI-1011 (2 µg/ml)	34.0 ± 2.0*
CI-1011	29.7 ± 3.0*

In the first experiment, HMMs were preincubated with ag-acLDL (100 µg protein/ml), CI-1011 (2 µg/ml) or ag-acLDL plus CI-1011 for 48 h. Cells were rinsed with serum-free medium and then incubated with ^{125}I -ag-acLDL (5 µg protein/ml) ± unlabeled ag-acLDL (500 µg protein/ml) for 4 h at 4 °C. In the second experiment, HMMs were similarly treated but were preincubated with acLDL (500 µg protein/ml) for 48 h. High affinity binding was calculated as the difference in binding between cells incubated with ^{125}I -ag-acLDL (or ^{125}I -acLDL) and those incubated with ^{125}I -ag-acLDL (or ^{125}I -acLDL) ± unlabeled ag-acLDL (or acLDL). The results shown represent the mean ± SE of triplicate dishes.

* $P < 0.0006$ compared with ag-acLDL or acLDL alone.

** $P < 0.01$ compared with ag-acLDL.

lysosomal and cytoplasmic lipid accumulation (manuscript submitted). Thus, structurally different ACAT inhibitors exert a similar effect in reducing TC and EC accumulation in HMMs, a necessary step in foam cell formation.

One mechanism for the lower TC by CI-1011 could be its effect in limiting ag-acLDL uptake. Therefore, we next examined the effect of CI-1011 on ^{125}I -ag-acLDL binding by first preincubating HMMs for 48 h with ag-acLDL (100 µg protein/ml), CI-1011 (2 µg/ml), or ag-acLDL plus CI-1011, and then incubating cells with ^{125}I -ag-acLDL (5 µg/ml) in the presence or absence of excess unlabeled ag-acLDL (500 µg protein/ml) for 4 h at 4 °C. As shown in Table 1, experiment 1, high affinity binding of ^{125}I -ag-acLDL was significantly lower in cells preincubated with CI-1011 or ag-acLDL plus CI-1011 compared with cells preincubated with ag-acLDL ($P < 0.01$). To compare the effect of CI-1011 to 58-035, we performed a high affinity binding experiment using unaggregated ^{125}I -acLDL. HMMs were preincubated for 48 h with acLDL (500 µg protein/ml), CI-1011 (2 µg/ml), or acLDL plus CI-1011, and then cells were incubated with ^{125}I -acLDL (5 µg/ml) in the presence or absence of excess unlabeled acLDL (500 µg protein/ml) for 4 h at 4 °C. As shown in Table 1, experiment 2, high affinity binding of ^{125}I -acLDL was

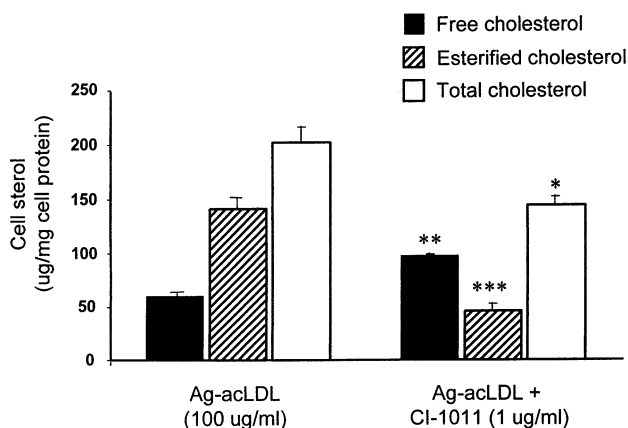


Fig. 1. Cellular sterol mass in HMMs incubated with ag-acLDL (100 mg protein/ml) with and without CI-1011 (1 µg/ml) for 48 h. The results represent mean ± SE of triplicate wells. * $P < 0.05$, ** $P < 0.002$, *** $P < 0.003$ compared with ag-acLDL.

significantly reduced in cells preincubated with CI-1011 or acLDL + CI-1011 compared with acLDL ($P < 0.0006$). These results were consistent with our earlier studies using 58-035, in that CI-1011 could directly reduce binding of acLDL or ag-acLDL. Additionally, CI-1011 reduced acLDL binding under conditions in which cells were not cholesteryl ester-enriched, suggesting the inhibitor directly altered scavenger receptor (SR) function or number.

To measure the direct effect of CI-1011 on SR function, HMMs were preincubated with control RPMI medium with and without CI-1011 (1–10 $\mu\text{g}/\text{ml}$) for 48 h, but without exposure to acLDL. After the preincubation period, the cells were then incubated with ^{125}I -acLDL (3–80 μg protein/ml) for 4 h at 4 $^{\circ}\text{C}$. The calculated B_{max} decreased in HMMs preincubated with increasing concentrations of CI-1011 (443, 342, 268, 258 ng/mg cell protein for control macrophages and cells treated with CI-1011 (1, 2, and 10 $\mu\text{g}/\text{ml}$, respectively) (Fig. 2). Since the calculated K_d values did not

decrease with increasing concentrations of inhibitor (mean was 11 $\mu\text{g}/\text{ml}$), this suggested that CI-1011 directly decreased SR function and/or number.

3.2. Effect of CI-1011 on cholesterol efflux from established HMM foam cells

Warner et al. [18] had previously shown that the ACAT inhibitor, CP-113,818, could induce cytotoxicity in cultured mouse peritoneal macrophage foam cells secondary to the intracellular accumulation of excess FC. To determine whether CI-1011 was cytotoxic, HMMs were first exposed to ag-acLDL (100 μg protein/ml) for 24 h, and then incubated with RPMI medium containing [^{14}C]adenine (0.5 $\mu\text{Ci}/\text{ml}$) for 2 h (time zero control). The medium was removed, cells were rinsed four times with RPMI medium, and then incubated with control RPMI medium with and without HDL (100 μg protein/ml), HDL + CI-1011 (2 $\mu\text{g}/$

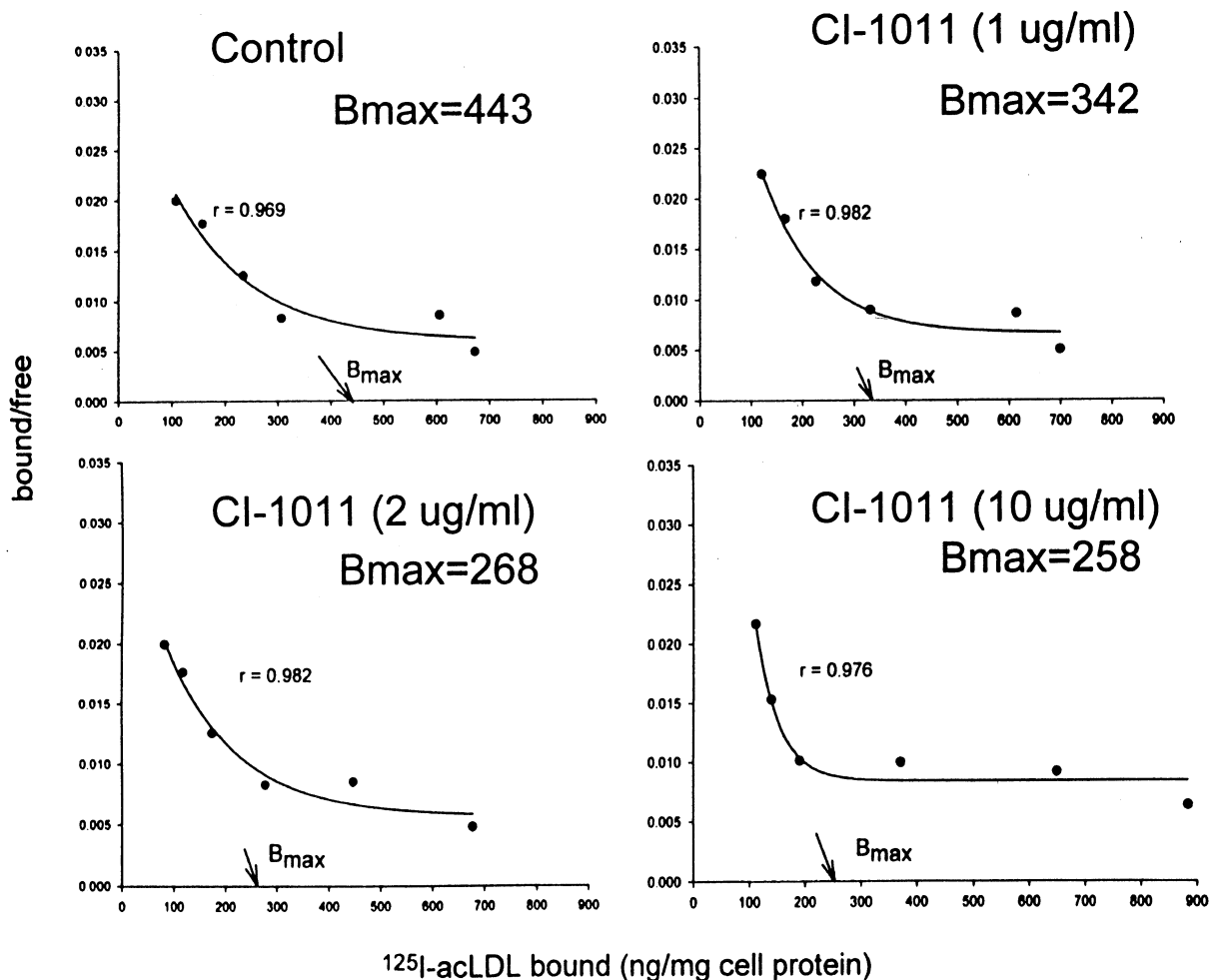


Fig. 2. Nonlinear regression analysis of ^{125}I -acLDL binding in HMMs preincubated with RPMI medium or CI-1011 (1–10 $\mu\text{g}/\text{ml}$) for 48 h. Results represent the mean \pm SD of duplicate wells.

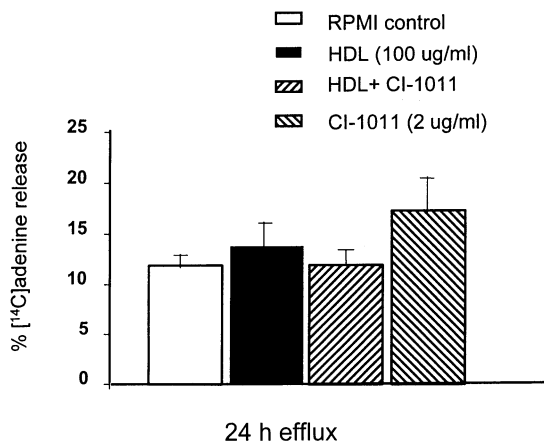


Fig. 3. Effect of CI-1011 on cytotoxicity as measured by the percent of cellular [^{14}C]adenine released to the medium. HMM foam cells were radiolabeled with [^{14}C]adenine (0.5 $\mu\text{Ci}/\text{ml}$) for 2 h (time zero) and then incubated with control RPMI medium with and without HDL (100 μg protein/ml), HDL + CI-1011, or CI-1011 (2 $\mu\text{g}/\text{ml}$) for 24 h. The percent of [^{14}C]adenine released was calculated as: (^{14}C dpm in the medium/cell [^{14}C] dpm at time zero) \times 100. The results represent two independent experiments, each in triplicate.

ml), or CI-1011 for an additional 24 h. Cytotoxicity was measured as the percent of [^{14}C]adenine released to the medium relative to that present at time zero. The percent of [^{14}C]adenine released into the medium was not significantly different between the groups (Fig. 3). Thus, CI-1011 was not cytotoxic to cells.

A probable explanation for the lack of cytotoxicity by CI-1011 was its effect on FC efflux. The data in Fig. 4 represent the sterol mass from the four different conditions shown in Fig. 3. The data are expressed as the percent of control foam cells (after 24 h cholesteryl ester-enrichment period) from two independent experiments (in the first experiment TC mass in the control foam cells was 89.7 ± 4.7 $\mu\text{g}/\text{mg}$ cell protein, while in the second experiment TC mass was 228.4 ± 6.2 $\mu\text{g}/\text{mg}$ cell protein). TC mass was 15% lower in cells exposed to RPMI ($P < 0.002$), 20% lower in cells exposed to HDL ($P < 0.002$), 28% lower in cells incubated with HDL plus CI-1011 ($P < 0.002$), and 44% lower in cells exposed to CI-1011 ($P < 0.0001$) compared to control foam cells. FC mass was 24% lower in cells exposed to RPMI ($P < 0.003$), 20% lower in cells exposed to HDL ($P < 0.0007$), 12% higher in cells incubated with HDL plus CI-1011 ($P < 0.08$, not significant), and 10% lower in cells exposed to CI-1011 alone ($P < 0.08$, not significant) compared to control foam cells. EC mass was 9% lower in cells exposed to RPMI ($P < 0.02$), 20% lower in cells exposed to HDL ($P < 0.001$), 56% lower in cells incubated with HDL plus CI-1011 ($P < 0.0001$), and 68% lower in cells exposed to CI-1011 ($P < 0.0001$) compared to control foam cells. Thus, TC mass was significantly reduced, but without a significant increase in FC mass, in foam cells incubated with CI-1011.

We next examined the dose effect of CI-1011 alone (1–10 $\mu\text{g}/\text{ml}$) on cholesterol efflux. HMMs were preincubated with [^{14}C]FC–ag–acLDL (100 μg protein/ml) for 24 h, and then control RPMI medium with and without CI-1011 (1–10 $\mu\text{g}/\text{ml}$) for 4–48 h. As shown in Fig. 5, TC mass decreased in a time-dependent manner ($r^2 = 0.998$, 0.920, and 0.955, for RPMI, CI-1011 (1 $\mu\text{g}/\text{ml}$), and CI-1011 (2 $\mu\text{g}/\text{ml}$), respectively). TC mass did not decrease in a linear fashion in cells exposed to the highest concentration of CI-1011. It appeared that this concentration was cytotoxic as the protein concentration was lower compared with the other treatment groups (data not shown). FC mass decreased in a time dependent manner only in cells exposed to RPMI ($r^2 = 0.94$). At 24 h, FC mass increased 28% in cells exposed to CI-1011 (2 and 10 $\mu\text{g}/\text{ml}$) compared with foam cells ($P < 0.002$). The variability in FC accumulation in foam cells exposed to CI-1011 in this figure compared with Fig. 4 could have been attributed to the variability in cholesterol enrichment and localization of FC during foam cell formation. Nonetheless, by 48 h FC mass was not significantly different in cells exposed to CI-1011 (2 $\mu\text{g}/\text{ml}$) compared with foam cells. With the exception of

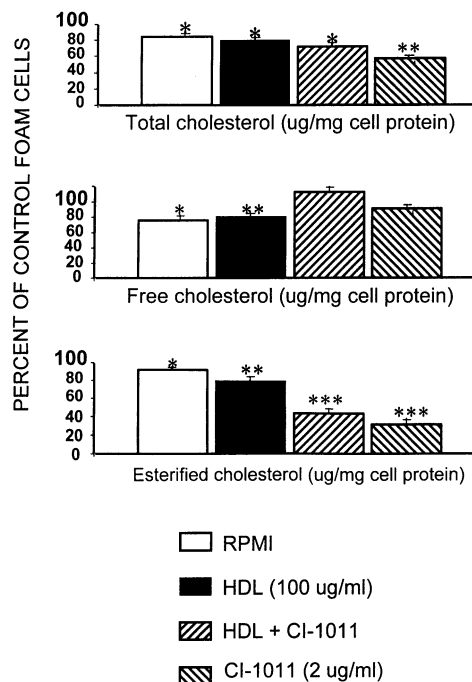


Fig. 4. Effect of CI-1011 on cellular sterol mass in HMM foam cells that were exposed to RPMI, HDL (100 μg protein/ml), HDL + CI-1011 (2 $\mu\text{g}/\text{ml}$), or CI-1011 for 24 h. The results from two independent experiments are expressed as the percent of control (n = 9, one experiment with triplicate wells, control foam cell was TC 89.7 ± 4.7 μg protein/ml, and one experiment with six wells, control foam cell was TC mass 228.4 ± 6.2 μg protein/ml). * $P < 0.002$, ** $P < 0.0001$, *** $P < 0.00001$ compared to control foam cells.

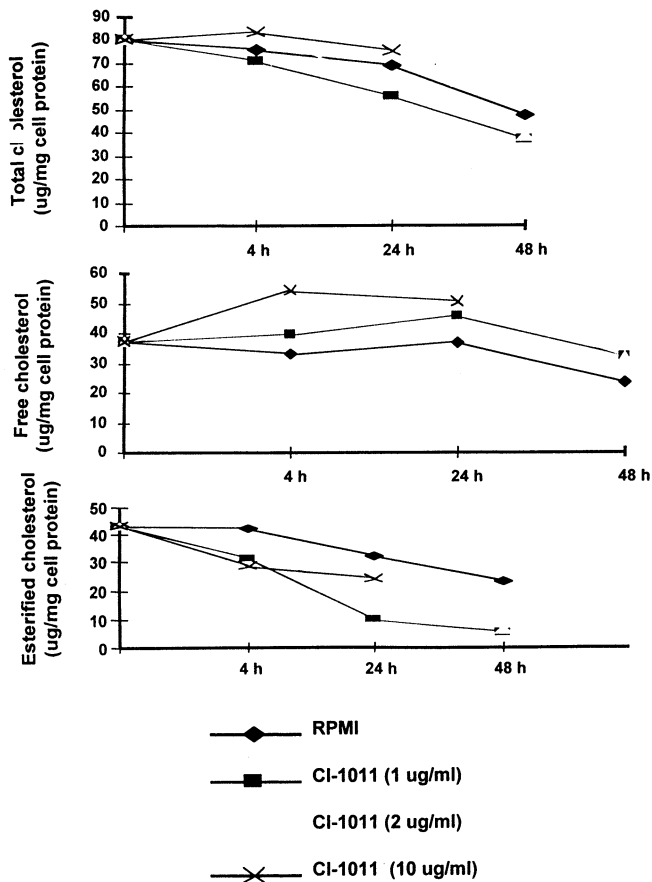


Fig. 5. Cellular sterol mass from HMM foam cells preincubated with [14 C]FC–ag-acLDL (100 μ g protein/ml) for 24 h, and then exposed to control RPMI medium with and without CI-1011 (1–10 μ g/ml) for 4–48 h. The results are the mean \pm SE from two independent experiments, each in triplicate.

cells exposed to CI-1011 (10 μ g/ml), EC mass also decreased in a time dependent manner ($r^2 = 0.996$, 0.821, and 0.884 for RPMI, CI-1011 (1 μ g/ml), and CI-1011 (2 μ g/ml), respectively). In Fig. 6, [14 C]FC in the medium increased in a time-dependent manner regardless of whether cells were exposed to RPMI alone or to increasing concentrations of CI-1011. These results showed that HMM foam cells released FC to the medium under basal conditions (RPMI alone) and when ACAT was inhibited.

The reduction of TC mass in HMM foam cells exposed to CI-1011 suggested that FC efflux was occurring in medium without added cholesterol acceptors. We therefore studied the effect of CI-1011 on apoE expression, a secreted macrophage protein thought to mediate cholesterol efflux to an acceptor-free medium [19]. HMMs were incubated with RPMI medium or the same medium plus ag-acLDL (100 μ g protein/ml) for 24 h. At the end of the 24 h enrichment period, the medium was removed, and cells from triplicate dishes were lysed and samples pooled for measurement of cellular apoE (foam cells and control). The remainder

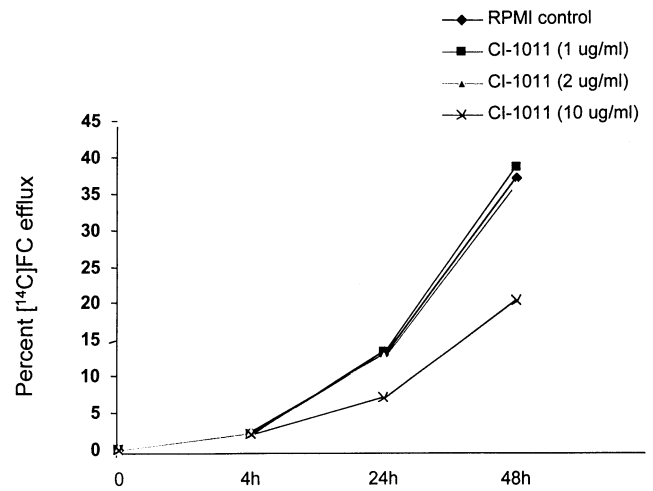


Fig. 6. Percent [14 C]FC efflux from HMM foam cells preincubated with [14 C]FC–ag-acLDL (100 μ g protein/ml) for 24 h, and then exposed to control RPMI medium with and without CI-1011 (1–10 μ g/ml) for 4–48 h. The results are from the experiments described in Fig. 5.

of the foam cells were incubated with control RPMI medium with and without CI-1011 (2 μ g/ml) for an additional 24 h (efflux phase). As shown in Fig. 7, cellular apoE mass increased approximately 2-fold in cells incubated with ag-acLDL compared with control macrophages. The results represent two independent experiments (the second experiment showed a 7-fold increase in apoE levels in foam cells compared to control macrophages (data not shown)). The reduction in TC between the two experiments varied 57–79% in

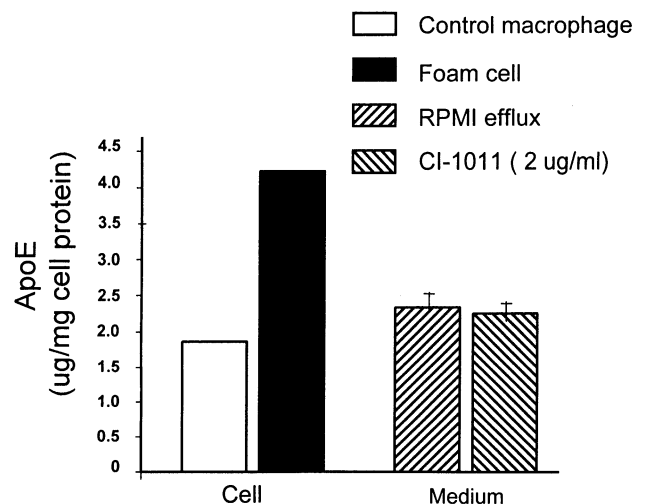


Fig. 7. Effect of CI-1011 on apoE secretion from HMM foam cells. HMMs were incubated with control RPMI medium or ag-acLDL (100 μ g protein/ml) for 24 h. Triplicate wells from foam cells and control macrophages were lysed with 5% SDS, 50 mM Tris–HCl and pooled for measurement of cellular apoE levels. Foam cells were then exposed to control RPMI medium with and without CI-1011 (2 μ g/ml) for 24 h. The results are the mean \pm SE of triplicate wells for medium apoE levels and represent two independent experiments.

cells exposed to CI-1011 compared with foam cells (data not shown). ApoE levels in the medium were not significantly different from foam cells exposed to RPMI compared with cells exposed to CI-1011.

4. Discussion

In the present study we found that CI-1011 significantly lowered TC accumulation during foam cell formation and enhanced cholesterol efflux from established HMM foam cells, without evidence for increased cytotoxicity. TC and EC mass were significantly lower in HMM foam cells after exposure to CI-1011 alone, without an excess accumulation of FC mass. Cholesterol efflux in the presence of CI-1011 occurred without the addition of known cholesterol acceptors to the medium. In addition, apoE levels in the medium were not affected by the presence of CI-1011.

We had previously shown that the ACAT inhibitor, 58-035, decreased ^{125}I -acLDL binding and cholesterol accumulation in HMMs when present during foam cell formation [2]. Our present studies show that CI-1011 reduced cholesterol accumulation in cells exposed to ag-acLDL, and directly reduced ^{125}I -acLDL binding in cells that were not cholesteryl ester enriched. Saturation binding experiments in HMMs preincubated with and without varying concentrations of CI-1011 were done to examine the direct effect of the inhibitor on ^{125}I -acLDL binding. Our results showed that B_{max} for ^{125}I -acLDL binding decreased in HMMs exposed to increasing concentrations of CI-1011.

These results suggested that ACAT inhibition altered the function of scavenger receptors. Our results are consistent with those reported by Meiner et al. in the ACAT knockout mouse model. These investigators reported that cultured peritoneal macrophages from the ACAT^{-/-} mouse showed significantly less cholesterol accumulation after exposure to acLDL than control macrophages [3]. It is highly probable that reduced foam cell formation in these macrophages will be secondary to decreased uptake of acLDL and enhanced FC efflux. Studies are currently underway to examine the effect of ACAT inhibition and deficiency on scavenger receptor mRNA expression. We have hypothesized that ACAT inhibition or deficiency affects transcriptional regulation of scavenger receptor(s).

Our findings contrasted with studies using cultured rodent macrophages, in which the addition of other ACAT inhibitors caused a redistribution of FC and EC mass but did not change net total cholesterol (TC) mass [20]. Differences in cell types and method of cholesterol enrichment may offer possible explanations for the discordant results.

CI-1011 did not induce cytotoxicity in HMM foam cells and this was likely due, in part, to a lack of excess FC accumulation. Using electron microscopy, we have shown a lack of cellular blebbing and crystal formation in HMM foam cells exposed to CI-1011 (unpublished results, data not shown). Our findings were different from those reported by Kellner-Weiber et al. [20], in which these investigators found that mouse macrophage foam cells exposed to 58-035 showed increased FC mass accumulation and increased lactate dehydrogenase (LDH) release, another measure of cytotoxicity. That differences in the localization of FC pools could influence the metabolic state of the cell was suggested by their study. J774 macrophage foam cells incubated with 58-035 and U18666A, a compound that affects intracellular cholesterol movement, did not have increased LDH release compared with cells exposed to 58-035 alone, despite an equivalent accumulation of excess FC mass [20]. Thus, even in the presence of excess FC mass, the results suggested that different pools of cellular FC could influence cell viability. We are also conducting studies to examine the apparent differences in mouse and human macrophage responses to ACAT inhibition.

The overall lack of FC accumulation in the presence of CI-1011 was likely secondary to FC efflux to the medium (as shown in Fig. 6). Kruth et al. [21] had previously reported that HMM foam cells could release intracellular cholesterol to acceptor free medium. These investigators subsequently reported that apoE was the rate-limiting step in cholesterol efflux to RPMI medium [19]. The addition of 58-035 to the RPMI medium did not increase cholesterol efflux or decrease TC mass but caused a redistribution of FC and EC mass [19]. Our results shown in Figs. 5 and 6 were consistent with those reported by Zhang et al. [19], in that net cholesterol efflux was seen in cells exposed to RPMI alone. This represents another apparent difference in the behavior of mouse and human macrophage foam cells to release cholesterol to acceptor free medium, another line of investigation that is currently being pursued. One difference between our data and that of Zhang et al. was that we were able to detect significant changes in cholesterol efflux within 24 h compared with their observations at 48 h. One possible explanation could have been the method of cholesteryl ester enrichment. We used ag-acLDL to induce foam cell formation compared with the acLDL used by Kruth et al. We have shown a relatively flat dose-response curve for TC accumulation in HMMs exposed to increasing concentrations of acLDL, while the dose response for HMMs exposed to increasing concentrations of ag-acLDL is linear (manuscript submitted). Zhang et al. [22] had also reported that aggregated LDL uptake was associated with surface connected compartments and was independent of LDL receptor activity. It could be

possible that cholesterol efflux to serum and acceptor free medium might be dependent on the mechanism of cholesterol enrichment and localization of FC.

The second possibility for the observed differences may have been due to the different ACAT inhibitors used in the experiments. However, this seemed less likely because 58-035 and CI-976 were effective in enhancing cholesterol efflux from HMM foam cells (unpublished observations). In general, FC efflux to medium without added acceptors was observed from HMM foam cells in the presence and absence of CI-1011. The level of the candidate cholesterol acceptor protein, apoE, in the medium however, was not significantly different for cells exposed to RPMI and those exposed to CI-1011, a finding also reported by Zhang et al. [19]. These investigators also noted that medium levels of apoE were not significantly different between control macrophages and foam cells incubated in the presence or absence of the ACAT inhibitor, 58-035 [19]. Interestingly, they also reported that cellular levels of apoE were not different between control macrophages and foam cells, an observation that contrasted with ours and Kayden et al.'s [23]. The lack of an expected increase in cellular apoE in the studies by Zhang et al. might have been because HMMs were enriched with cholesterol in ethanol dispersions and not by incubation with modified LDL [19].

That apoE was thought to be the rate-limiting step in FC efflux to an acceptor free medium was based on inhibition of FC efflux when apoE antibodies were added to the medium [19]. Our studies did not examine the effect of adding apoE antibodies to the medium during the efflux phase because we consistently found that, in our model, the presence of CI-1011 significantly reduced TC and EC without a significant rise in FC mass. Clearly, a cholesterol acceptor was present in the medium for net FC efflux to occur. We have performed 2D gel analysis of secreted proteins from HMM foam cells, but as of yet are unable to specifically identify one or more of the candidate cholesterol acceptor proteins (unpublished results). Further studies will continue to explore the effect of ACAT inhibitors on FC efflux, and on the secretion of cholesterol acceptor proteins from HMM foam cells.

In summary, CI-1011 exhibited anti-atherogenic properties by limiting foam cell formation and enhancing cholesterol efflux from HMM foam cells. These effects were not associated with enhanced cytotoxicity as evidenced by a lack of increased adenine release or cholesterol crystal formation. One novel finding was the reduced B_{\max} for ^{125}I -acLDL binding in macrophages exposed to increasing concentrations of the inhibitor, suggesting that ACAT inhibitors might alter scavenger receptor function. This possibility is currently being investigated. These anti-atherogenic effects of CI-1011 may explain the reduced atherosclerotic lesions ob-

served in animals fed various ACAT inhibitors, and suggested in those animals that are genetically deficient in ACAT [24]. ACAT inhibitors are currently under study in patients with dyslipidemia and coronary artery disease (Parke–Davis files). These agents may prove useful in the treatment of atherosclerosis.

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References

- [1] Brown MS, Ho YK, Goldstein JL. The cholesteryl ester cycle in macrophage foam cells. *J Biol Chem* 1980;255:9344–52.
- [2] Rodriguez A, Bachorik PS, Wee S-B. Novel effects of the acyl-Coenzyme A:cholesterol acyltransferase inhibitor 58-035 on foam cell development in primary human monocyte-derived macrophages. *Arterioscler Thromb Vasc Biol* 1999;19:2199–206.
- [3] Meiner VL, Cases S, Myers HM, et al. Disruption of the acyl-CoA:cholesterol acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals. *Proc Natl Acad Sci USA* 1996;93:14041–6.
- [4] Lee HT, Slikskovic DR, Picard JA, et al. Inhibitors of acyl-CoA:cholesterol *O*-acyl transferase (ACAT) as hypocholesterolemic agents. *J Med Chem* 1996;39:5031–4.
- [5] Ishii I, Yokoyama N, Yanagimachi M, et al. Stimulation of cholesterol release from rabbit foam cells by the action of a new inhibitor for acyl CoA:cholesterol acyltransferase (ACAT), HL-004. *J Pharm Exp Ther* 1998;287:115–21.
- [6] Tanaka H, Ohtsuka I, Kogushi M, et al. Effect of the acyl-CoA:cholesterol acyltransferase inhibitor, E5324, on experimental atherosclerosis in rabbits. *Atherosclerosis* 1994;107:187–201.
- [7] Sorbera LA, Rabasseda X, Castaner J. Avastimibe, treatment of lipoprotein disorders. *Drugs Future* 1999;24:9–15.
- [8] Delsing DJM, Offerman EH, van der Room H et al. The acyl-CoA:cholesterol acyltransferase (ACAT)-inhibitor avastimibe reduces atherosclerosis independently of its lipid-lowering effect in cholesterol-fed apoE3-Leiden transgenic mice. *Circulation (Suppl.)* 1999. Abstract 3232.
- [9] Bocan TMA, Krause BR, Rosebury WS, et al. The ACAT inhibitor avastimibe reduces macrophages and matrix metalloproteinase expression in atherosclerotic lesions of hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol* 2000;20:70–9.
- [10] Cullen P, Cignarella A, Assmann G. The effects of atorvastatin, the ACAT inhibitor CI-1011, and progesterone on storage and secretion of cholesterol in human monocyte-derived macrophages. *Circulation* 1998. Abstract 2828.
- [11] Havel RJ, Eder HA, Bragdon JH. Distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 1955;34:1345–53.
- [12] Frankel-Conrat H. Methods for investigating the essential groups for enzyme activity. *Methods Enzymol* 1957;4:247–69.
- [13] Khoo JC, Miller E, McLoughlin P, et al. Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis* 1988;8:348–58.
- [14] Ishikawa TT, MacGee J, Morrison JA, et al. Quantitative analysis of cholesterol in 5 to 20 microliters of plasma. *J Lipid Res* 1974;15:286–91.

- [15] Markwell MK, Haas SM, Bieber LL, et al. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978;87:206–10.
- [16] McFarlane AS, Koj A. Short-term measurement of catabolic rates using iodine-labeled plasma proteins. *J Clin Invest* 1970;49:1903–11.
- [17] Bachorik PS, Franklin FA, Virgil DG, et al. High affinity uptake and degradation of apolipoprotein E-free high density lipoprotein in cultured porcine hepatocytes. *Biochemistry* 1982;21:4675–84.
- [18] Warner GJ, Stoudt G, Bamberger M, et al. Cell toxicity induced by inhibition of acyl Coenzyme A:cholesterol acyltransferase and accumulation of unesterified cholesterol. *J Biol Chem* 1995;270:5772–8.
- [19] Zhang W-Y, Gaynor PM, Kruth HS. Apolipoprotein E produced by human monocyte-derived macrophages mediates cholesterol efflux that occurs in the absence of added cholesterol acceptors. *J Biol Chem* 1996;271:28641–6.
- [20] Kellner-Weibel G, Jerome WG, Small DM, et al. Effects of intracellular free cholesterol accumulation on macrophage viability, a model for foam cell death. *Arterioscler Thromb Vasc Biol* 1998;18:423–31.
- [21] Kruth HS, Skarlatos SI, Gaynor PM, et al. Production of cholesterol-enriched nascent high density lipoproteins by human monocyte-derived macrophages is a mechanism that contributes to macrophage cholesterol efflux. *J Biol Chem* 1994;269:24511–8.
- [22] Zhang W-Y, Gaynor PM, Kruth HS. Aggregated low density lipoprotein induces and enters surface-connected compartments of human monocyte macrophages. *J Biol Chem* 1997;272:31700–6.
- [23] Kayden HJ, Maschio F, Traber MG. The secretion of apolipoprotein E by human monocyte-derived macrophages. *Arch Biochem Biophys* 1985;239:388–95.
- [24] Accad M, Smith SJ, Newland DL, et al. Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl-CoA:cholesterol acyltransferase I. *J Clin Invest* 2000;105:711–9.