Regulation of sterol carrier protein 2 (SCP2) gene expression in rat peritoneal macrophages during foam cell formation. A key role for free cholesterol content.

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Research Article

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Regulation of Sterol Carrier Protein 2 (SCP_2) Gene Expression in Rat Peritoneal Macrophages during Foam Cell Formation

A Key Role for Free Cholesterol Content

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Abstract

Sterol carrier protein 2 (SCP_2) has been shown to be involved in intracellular transport and metabolism of cholesterol. However, there have been no reports concerning SCP_2 in macrophages, the major source of atheromatous foam cells. We investigated whether SCP_2 is present in rat peritoneal macrophages and determined the changes of SCP_2 and its mRNA levels in macrophages during foam cell formation induced by acetylated LDL (AcLDL).

Immunoblot analysis and Northern blot analysis demonstrated that both SCP_2 and its mRNA are expressed in rat peritoneal macrophages. Incubations with AcLDL caused a dose- and time-dependent increase of cellular esterified cholesterol, SCP_2 and its mRNA in rat peritoneal macrophages. The inhibitor of acyl-CoA:cholesterol acyltransferase further enhanced AcLDL-induced increase of SCP_2 protein and its mRNA. Incubations with 25-hydroxy cholesterol also caused a dose-dependent stimulation of SCP_2 gene expression in macrophages, while incubation with maleylated BSA had no effect. These results suggest that the increment of cellular-free cholesterol is responsible for enhanced SCP_2 gene expression in macrophages. The enhancement of SCP_2 gene expression by AcLDL suggests that SCP_2 may play an important role during foam cell formation induced by AcLDL which may be most important step for the atherosclerosis. (*J. Clin. Invest.* 1994. 94:2215–2223.) Key words: macrophage • sterol carrier protein 2 • acetylated LDL • oxysterol • foam cell

Introduction

One of the major histological characteristics of atherosclerosis is the accumulation of large amounts of lipids, especially cholesteryl esters, in the arterial wall (1). In the atherosclerotic plaques, macrophages are thought be the major source of atheromatous foam cells (2). Since they are reported to uptake the modified LDL such as acetylated LDL (AcLDL)[1] via the scavenger receptors (3). In the macrophages, modified LDL is hydrolyzed in lysosomes, and much of the liberated cholesterol is transported to the endoplasmic reticulum for the formation of cholesteryl esters by acyl-CoA:cholesterol acyltransferase (ACAT) (4). ACAT-catalyzed esterification of cholesterol is markedly accelerated by the liberated cholesterol in the macrophages uptaking AcLDL, resulting in the accumulation of large amount of cholesteryl esters within the cells (5).

Since cholesterol is relatively water insoluble, transfer proteins which can facilitate cytoplasmic cholesterol transport and affect distribution of cholesterol in cell organelles could be of importance in the regulation of intracellular cholesterol metabolism (6). In contrast to the vast amount of information available on the identification of lipoproteins and receptors that may be involved in foam cell formation, very little is known about the regulation of pathways that govern intracellular trafficking of cholesterol in macrophage foam cells. Recent studies have suggested that this sterol translocation may be mediated by specific sterol transfer proteins. Sterol carrier protein 2 (SCP_2), also called nonspecific lipid transfer protein (7), is a low molecular weight protein (13.2 kD) and thought to be one of highly touted candidates for an intracellular cholesterol carrier (6–8). SCP_2 has been reported to facilitate enzymatic reactions involved in cholesterol biosynthesis in the liver (6), and in ACAT-catalyzed cholesterol esterification in various tissues such as the liver, intestine, adrenal cortex, and aorta (9–11). On the other hand, no relationship between SCP_2 level and ACAT-dependent cholesterol esterification in vivo has been shown in the experiments using SCP_2-deficient cells (12, 13). The protein has also been reported to participate in mitochondrial production of steroids from cholesterol in the adrenal cortex (14, 15). Recently, SCP_2 is reported to exhibit a tissue-specific distribution and expression, which is closely correlated with the rate of cholesterol metabolism in each tissues (16).

These evidences suggest that SCP_2 might play a certain role for the esterification of cholesterol during the formation of foam cell from the macrophages by AcLDL, which is one of the most important steps in the atherosclerosis. However, there have been no reports concerning not only the existence of SCP_2, but also the changes of SCP_2 during foam cell formation in macrophages. The present investigation was performed in order to explore a new insight into the regulation of intracellular cholesterol metabolism by lipid transfer protein during foam cell formation.

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1. *Abbreviations used in this paper:* 25-OH cholesterol, 25-hydroxy cholesterol; ACAT, acyl-CoA:cholesterol acyltransferase; AcLDL, acetylated LDL; Apo E, apoprotein E; EIA, enzyme immunoassay; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; Mal-BSA, maleylated BSA; p-APMSF, *p*-amidinophenyl methanesulfonyl fluoride hydrochloride; SCP_2, sterol carrier protein 2.

For this purpose, we studied the following questions: (*a*) Is SCP_2 present in rat peritoneal macrophages from the view of both protein and mRNA? (*b*) Are SCP_2 and its mRNA regulated by uptake of AcLDL in macrophages?

Methods

Preparation of SCP_2 from rat liver and anti-SCP_2 IgG. SCP_2 was purified ~5,000-fold to homogeneity from 105,000 *g* supernatant of rat liver using a two-step fast flow ion-exchange and gel permeation column chromatography as reported previously (17). SCP_2 activity was determined by the stimulation of microsomal conversion of 7-dehydrocholesterol to cholesterol according to the method of Noland et al. (6). The purified SCP_2 was used as antigen for the immunization of a rabbit according to a previously reported method (11). IgG fraction was prepared from the antiserum using Protein G–Sepharose 4 FF column. Specific antibody against SCP_2 was prepared from the IgG fraction by affinity chromatography using SCP_2-conjugated CNBr-activated Sepharose 4B (1.5 mg SCP_2/1g CNBr-activated Sepharose 4B) as previously reported (11).

Study design. Rat peritoneal macrophages which were collected by the method of Saitoh et al. (18) were suspended in RPMI 1640 medium containing 20% FCS, 40 μg/ml gentamicin, at 2×10^6 cells/ml. 10-ml aliquots of cells were dispensed into 100-mm plastic dishes and incubated in a humidified CO_2 (5%) incubator at 37°C. After 2 h, medium was changed to remove non-adherent cells. The adherent cells were incubated for 22 h at 37°C in 10 ml of RPMI 1640 medium containing 20% FCS. After 22 h of incubation (day 1), each macrophage monolayer was washed with RPMI medium. The dishes then received 10 ml of RPMI 1640 medium with or without the indicated concentrations of AcLDL. AcLDL was prepared by the reaction with acetic anhydride as described by Goldstein and co-workers (3). After incubation for 24 h at 37°C (day 2), the medium was replaced with 10 ml of RPMI 1640 medium. After further incubation for 24 h at 37°C (day 3), the monolayers were then placed on ice, the medium was replaced with 5 ml of PBS containing 4 mM EDTA. The cells were scraped with a rubber policeman without noticeable cell disruption and collected by centrifugation. In experiments using DMSO as a solvent for EAB-309, an ACAT inhibitor (19), control cultures received equivalent amounts of DMSO which was always < 0.05% (vol/vol).

In experiments using 25-hydroxy cholesterol (25-OH cholesterol) or maleylated BSA (Mal-BSA), the dishes received 10 ml of RPMI medium containing 25-OH cholesterol or Mal-BSA. After 24 h of incubation at 37°C (day 2), the cells were collected. In experiments using ethanol as a solvent for 25-OH cholesterol, control dishes received equivalent amounts of ethanol which was always < 0.2%. Mal-BSA was prepared according to the method of Bulter et al. (20).

After washing with 40 ml of PBS, aliquots of cell suspension were removed for protein assay and cholesterol assay. Then the cells were harvested in 0.8 ml of PBS containing protease inhibitors (2 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (p-APMSF), 2.5 μg/ml of pepstatin A, 2.5 μg/ml of antipain, 2.5 μg/ml of chymostatin, and 2.5 μg/ml of leupeptin) and homogenized at 4°C with 40 strokes of a Potter Elvehjem homogenizer with a tight-fitting pestle according to the method of Tabas et al. (5). Cell debris was then removed from the cell homogenate by centrifugation at 300 *g* for 10 min. The resulting supernatant fraction of whole homogenate was stored in liquid nitrogen until the determination of SCP_2 by enzyme immunoassay (EIA) and immunoblot analysis. The cells were also collected after washing twice with the ice-cold PBS and total RNA was purified from these cells.

SDS-PAGE and immunoblot procedure. SDS-PAGE and immunoblot analysis were performed using previously reported methods (11). The samples were treated with 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 7% glycerol, and 0.01% bromophenol blue for 10 min at 85°C. After electrophoresis the proteins were electrophoretically transferred from gel to nitrocellulose membranes (0.45 μm pore size). The membranes were subjected to treatment with anti-SCP_2 IgG and secondary, [^{125}I] anti–rabbit Ig F(ab′)$_2$ fragment from donkey. Then the membranes were exposed to x-ray film at −80°C with an intensifying screens for 48 h.

Preparation of rat SCP_2 cDNA probe. cDNA was prepared from rat liver poly(A)$^+$ RNA using a cDNA Synthesis Kit. A rat SCP_2 cDNA fragment was obtained using PCR (21). Rat liver cDNA was used as template, and the following oligonucleotides were used as primers: CACGAATTCGGAAAGTGGGTCATAAACCCTAG and CTCGAATTCTCATCTTGATGTCCTGAGG. Isolation and sequencing of positive clones were with standard methods (22, 23). The positive clone (664 bp) coded the 502–1165 nucleotide segment of rat SCP_2 cDNA reported by Billheimar et al. (24) and contained the full-length coding region for the rat SCP_2 and had the sequence reported (25).

mRNA extraction and hybridization. Total RNA was isolated from rat peritoneal macrophages and rat liver according to the method of Auffray and Rougen (26). mRNA was purified from total RNA using an mRNA Purification Kit. For hybridization experiments, mRNA was separated on a denaturing 1.0% agarose gel containing 2.0% formaldehyde and transferred to a Hybond-N$^+$ nylon membrane by capillary blotting. After fixing the RNA blot by a FUNA-UV-LINKER (Funakoshi Co. Ltd., Tokyo, Japan) at $1{,}200\times 10^2$ μJ/cm^2, the blot was prehybridized overnight in 5× SSPE (1× SSPE contains 0.15 M NaCl, 0.01 M NaH_2PO_4 H_2O, 1 mM EDTA, pH 7.5), 5× Denhardt's solution [1× Denhardt's solution contains 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinyl pyrolidone, 0.02% (wt/vol) BSA], 0.1% SDS, 20 μg salmon sperm DNA/ml, and 50% (vol/vol) formamide at 42°C. Hybridization was performed at 42°C overnight in 5× SSPE, 2× Denhardt's solution, 0.1% SDS, 10 μg salmon sperm DNA/ml and 50% (vol/vol) formamide to which a [α-^{32}P] dCTP-labeled 664 bp SCP_2 cDNA probe was added (random prime labeling, specific activity > 1×10^9 cpm/μg). After hybridization, the membrane filter was washed twice with 6× SSPE containing 0.2% SDS at room temperature for 15 min, twice with 1× SSPE containing 0.1% SDS at 37°C for 15 min , once with 1× SSPE containing 0.1% SDS at 65°C for 30 min. Autoradiography was performed overnight at −80°C. Densitometric analysis of Northern blot of SCP_2 was performed by NIH Image 1.44 software (27) using the actin blot as denominator.

EIA of SCP_2. The supernatant fraction of whole homogenate of rat peritoneal macrophages was diluted in PBS containing 0.1% (wt/vol) BSA, protease inhibitors (2 mM p-APMSF, 2.5 μg/ml of pepstatin A, 2.5 μg/ml of antipain, 2.5 μg/ml of chymostatin, and 2.5 μg/ml of leupeptin) to a sample protein concentration of 1 mg/ml. After heat treatment, levels of SCP_2 in the sample was determined by use of EIA according to the method as reported previously (11).

Cholesteryl esterification in rat peritoneal macrophages. Rat peritoneal macrophages were plated in 35-mm dishes at a density of 1×10^7 cells/dish in RPMI 1640 medium containing 20% FCS and 40 mg/ml gentamicin. After 24 h incubation at 37°C, the monolayers were washed with PBS and then incubated for 24 h in RPMI 1640 medium in the absence or presence of 50 μg/ml of AcLDL. After that, medium was replaced to RPMI 1640 medium and incubated for 24 h. During the last 2 h of each incubation point, the cells were pulsed with [^{14}C]oleic acid–albumin complex (0.2 mM oleic acid, 84 nBq/nmol of [^{14}C]oleic acid) at 37°C (5). Lipids of these cells were extracted by the method of Bligh and Dyer (28). Cholesteryl [^{14}C]oleate formed was separated by thin layer chromatography and the radioactivity of the corresponding band was counted by liquid scintillation counting.

Chemical assays. Protein concentrations were determined by the method of Bradford (29) with bovine γ-globulin as a standard. Free cholesterol and cholesteryl esters were assayed according to the method of Saitoh et al. (30).

Chemicals. [^{125}I] anti–rabbit Ig F(ab′)$_2$ fragment from donkey, nitrocellulose hybridization transfer membranes (0.45 mm pore size), multiprime DNA labeling system, [γ-^{32}P] dCTP, Hybond-N$^+$ nylon membrane, and dried milk-membrane blocking agent were purchased from Amersham (Cardiff, U.K.). [^{14}C]oleic acid (2.1 GBq/mmol), [4-^{14}C]cholesterol (2.05 GBq/mmol) and [^{125}I] molecular weight markers

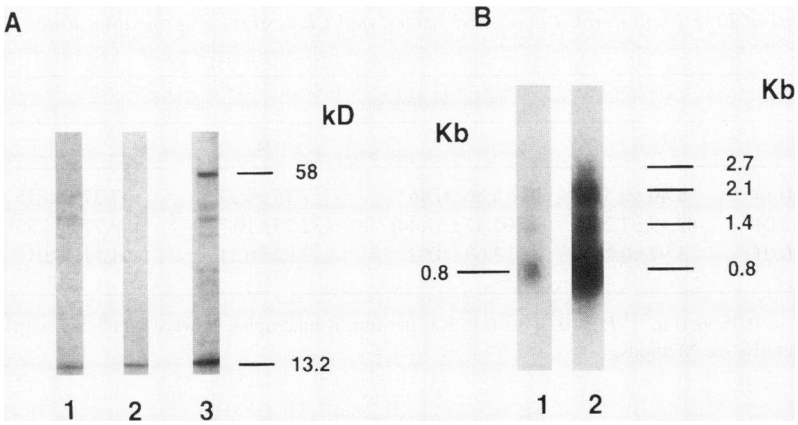

Figure 1. The result of an immunoblot (*A*) and a Northern blot (*B*) analysis of rat peritoneal macrophages. (*A*) For immunoblot analysis, whole homogenate of rat peritoneal macrophages (50 μg), rat liver cytosol (10 μg), and purified SCP_2 (5 ng) were applied to the gel. Lane *1*, purified SCP_2; lane *2*, whole homogenate of rat peritoneal macrophages; lane *3*, rat liver cytosol. (*B*) For Northern blot analysis, mRNA was purified from 2×10^8 cells of rat peritoneal macrophages and 4 μg of mRNA was applied to the gel. 2 μg of mRNA purified from rat liver was applied to the gel. Lane *1*, rat peritoneal macrophages; lane *2*, rat liver. For further experimental details, see Methods.

set (1.85 MBq/mg) were obtained from New England Nuclear (Boston, MA). RPMI 1640 medium and FCS was obtained from GIBCO BRL Life Technologies Inc. (Gaithersburg, MD). 100-mm dish and 35-mm dishes were obtained from Corning (Corning, MA). Electrophoresis calibration kit, Protein G–Sepharose 4 FF gel and CNBr-activated Sepharose 4B were obtained from Pharmacia AB (Upsala, Sweden). Cholesterol, oleic acid, oleyl-CoA, 25-OH cholesterol, *p*-nitrophenyl phosphate, pepstatin A, antipain, chymostatin and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). ATP was obtained from Boehringer Mannheim GmbH (Mannheim, F.R.G.). Bio-Rad protein dye reagent was obtained from Bio-Rad Laboratories (Richmond, CA). SDS-PAGE plate (10–20%) was obtained from Daiichi Pure Chemicals Co., Ltd.(Tokyo, Japan). The x-ray film (Rxo-H) and the intensifying screen (HR-8) were purchased from Fuji Photo Film Co., Ltd. (Kanagawa, Japan). The microtiter plates were purchased from C.A. Greiner and Sohne (Nurtingen, F.R.G.). The phosphatase-labeled affinity-purified antibody to rabbit IgG (H+L) (goat) was obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). TLC plates (LK5D) was obtained from Whatman Chemical Separation Inc. (Clifton, NJ). p-APMSF was purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). Time Saver cDNA Synthesis Kit and mRNA Purification Kit were purchased from Pharmacia LKB (Piscataway, NJ). Rat liver poly(A)$^+$ RNA was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). GeneAmp PCR reagent kit was purchased from Perkin Elmer Cetus (Norwalk, CT). EAB-309 was kindly provided from Dr. Kazuo Suzuki, Mitsubishi-Kasei Co., Ltd. (Machida, Tokyo, Japan). Other chemicals and solvents were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan) and were of analytical reagent grade.

Data analysis. Data analysis was performed using "Manual of Pharmacologic Calculations with Computer Programs" software (31) on a Toshiba J 3100 SGT personal computer. Statistical significance was determined with procedure 39 of "t-Test I: Grouped Data" (31). A rejection level of $P < 0.05$ was considered significant.

Results

Presence of SCP_2 protein and mRNA in rat peritoneal macrophages. Fig. 1 *A* shows the result of an immunoblot analysis of whole homogenate of rat peritoneal macrophages, rat liver cytosolic fraction, and SCP_2 purified from rat liver using an affinity-purified rabbit antibody against SCP_2 as a probe. In the Western blot analysis, only one protein was recognized and located at the same molecular weight (13.2 kD) as an authentic liver SCP_2 (Fig. 1 *A*). Another immunoreactive protein which can be detected in rat liver cytosolic fraction exhibited a molecular mass of 58 kD, though this protein was undetectable in rat peritoneal macrophages.

The levels of SCP_2 mRNA in rat liver and rat peritoneal macrophages were analyzed. The 664-bp SCP_2 cDNA fragment, which was used as a probe, recognized mRNA species of 0.8, 1.4, 2.1, and 2.7 kb in rat liver (Fig. 1 *B*). The predominant transcript was 0.8 kb. In rat peritoneal macrophages, the 0.8-kb transcript was observed. After exposure for longer period, two additional transcripts, 1.4 and 2.1 kb, could be observed (data not shown).

Effect of AcLDL on the cholesterol metabolism and the expression of both SCP_2 and its mRNA in rat peritoneal macrophages. Table I shows the time course in the accumulation of cholesteryl esters and free cholesterol in rat peritoneal macrophages incubated with 50 μg/ml of AcLDL. Accumulation of cholesteryl esters occurred rapidly during first 12 h of incubation and then gradually up to 48 h of incubation. The content of free cholesterol in macrophages incubated with 50 μg/ml of AcLDL increased gradually and reached maximum at 24 h. On the other hand, contents of free cholesterol at 36 and 48 h were decreased compared with that at 12 h. Table II shows the effect of incubation with various concentrations of AcLDL for 24 h on the accumulation of cholesteryl esters and free cholesterol in rat peritoneal macrophages. AcLDL increased concentrations of cholesteryl esters and free cholesterol in rat peritoneal macrophages dose-dependently and reached maximum at 50 μg/ml.

Table I shows the time course of cholesterol esterification in rat peritoneal macrophages in the presence of 50 μg/ml of AcLDL. In the presence of AcLDL, cholesterol esterification in macrophages increased rapidly and reached a maximum level from 6 to 12 h. However, cholesterol esterification in macrophages decreased gradually after this period but did not return to control level. In the absence of AcLDL, no significant increase in cholesterol esterification was observed at any indicated points. Table II shows the effect of the incubation with various concentrations of AcLDL on cholesterol esterification in rat peritoneal macrophages. AcLDL also increased cholesterol esterification in rat peritoneal macrophages dose-dependently.

Fig. 2 *A* shows the time course of the changes in SCP_2 content in whole homogenate of rat peritoneal macrophages in the presence or the absence of 50 μg/ml of AcLDL. In the presence of AcLDL, SCP_2 content increased gradually up to 48 h. A significant increase of SCP_2 content was observed at points of both 36 and 48 h. On the other hand, in the absence of AcLDL, SCP_2 content increased slightly at 48 h, though statistically not significant. Fig. 2 *B* shows the dose effect of AcLDL on the changes of SCP_2 in whole homogenate of rat peritoneal macrophages for 48 h. AcLDL increased SCP_2 content in whole

Table I. Time Course of the Changes in Cellular Contents of Free Cholesterol, Cholesteryl Esters, and Cholesterol Esterification Rate in Rat Peritoneal Macrophages Incubated with AcLDL

	Incubation time (h)				
	0	6	12	24	48
Free cholesterol (μg/mg protein)	3.20±0.14	6.40±0.51*	5.86±0.86*	7.75±0.17‡	7.37±0.61‡
Cholesteryl esters (μg/mg protein)	24.60±3.01	82.49±7.21‡	105.47±37.44‡	137.25±10.71‡	178.77±24.55‡
Cholesterol esterification (nmol/mg protein/h)	0.05±0.01	3.97±0.28‡	3.67±0.03‡	2.92±0.11‡	1.17±0.01‡

The results represent means±SD of the mean. $n = 3$. * $P < 0.05$ vs 0 h, ‡ $P < 0.01$ vs 0 h. Rat peritoneal macrophages were incubated with or without 50 μg/ml of AcLDL. For further experimental details, see Methods.

homogenate of rat peritoneal macrophages in a dose-dependent manner.

Fig. 3 *A* (lanes *1* and *2*) shows the result of an immunoblot analysis of whole homogenate of rat peritoneal macrophages, when incubated with or without 50 μg/ml of AcLDL for 48 h. The level of SCP_2 represented as 13.2 kD was enhanced by 50 μg/ml of AcLDL. Fig. 3 *B* (lanes *1* and *2*) shows the changes of SCP_2 mRNA level in rat peritoneal macrophages, when incubated with or without 50 μg/ml of AcLDL for 18 h. An increase in 0.8 kb SCP_2 transcript expression was observed when incubated with 50 μg/ml of AcLDL.

Effect of EAB-309, an inhibitor of ACAT, on AcLDL-induced SCP_2 gene expression. Fig. 4 shows the changes of both free cholesterol and cholesteryl esters in rat peritoneal macrophages incubated with 50 μg/ml of AcLDL in the presence or absence of 100 nM EAB-309 for 48 h. Incubation with 100 nM EAB-309 markedly reduced cholesteryl esters accumulation produced by 50 μg/ml AcLDL. On the other hand, cellular free cholesterol showed a fourfold increase. Incubation with 100 nM EAB-309 completely inhibited the threefold increase in macrophage cholesteryl ester synthesis produced by AcLDL at a concentration of 50 μg/ml (data not shown). Fig. 5 shows the effect of EAB-309 on the changes in SCP_2 content. Incubation with 100 nM EAB-309 significantly enhanced SCP_2 response produced by 50 μg/ml AcLDL, while incubation with EAB-309 alone had no effect (Fig. 5). Fig. 3 *A* (lanes *2* and *3*) shows the result of an immunoblot analysis of whole homogenate of rat peritoneal macrophages, when incubated with 50 μg/ml of AcLDL in the presence or absence of 100 nM EAB-309 for 48 h. Incubation with 100 nM EAB-309 enhanced an increase in SCP_2 level produced by 50 μg/ml AcLDL. Fig. 3 *B* (lanes *2* and *3*) shows the changes of SCP_2 mRNA level in rat peritoneal macrophages, when incubated with 50 mg/ml of AcLDL in the presence or absence of 100 nM EAB-309 for 18 h. The ratios of SCP_2 blot (0.8 kb) to actin blot in control, AcLDL and AcLDL + EAB-309 were 0.361, 0.581, and 0.670, respectively. An increase in 0.8-kb SCP_2 transcript expression was observed in the presence of 100 nM EAB-309.

Effect of 25-OH cholesterol and Mal-BSA on the expression of both SCP_2 and its mRNA. Fig. 6 shows the time course of SCP_2 response produced by 5 μg/ml of 25-OH cholesterol. In the presence of 25-OH cholesterol, SCP_2 level in macrophages increased to a maximum at 12 h. No further increase was observed until 24 h. In the absence of 25-OH cholesterol, no significant change in SCP_2 level in macrophages was observed at any time point during experimental period. Fig. 7 shows the dose effect of 25-OH cholesterol on SCP_2 content in rat peritoneal macrophages. As shown in Fig. 7, incubation with 25-OH cholesterol resulted in a dose-dependent increase of SCP_2 level in macrophages. SCP_2 level in macrophages increased to a maximum of 2.5-fold over the basal value at 5 μg/ml of 25-OH cholesterol.

Fig. 8 shows the effect of 25-OH cholesterol and Mal-BSA on SCP_2 content in rat peritoneal macrophages. As shown in Fig. 8, incubation with 25-OH cholesterol (5 μg/ml) for 24 h resulted in 2.5-fold increase in SCP_2 content, while Mal-BSA (300 μg/ml) had no significant effect. Fig. 9 *A* shows the result of an immunoblot analysis of whole homogenate of rat peritoneal macrophages, when incubated with 25-OH cholesterol or Mal-BSA for 24 h. Incubation with 25-OH cholesterol (5 μg/ml) resulted in an increase in the level of SCP_2. Incubation with Mal-BSA (300 μg/ml) had no significant effect on SCP_2. Fig. 9 *B* shows the effect of 25-OH cholesterol and Mal-BSA on SCP_2 mRNA level in rat peritoneal macrophages. The ratios of SCP_2 blot (0.8 kb) to actin blot in control, 25-OH cholesterol and Mal-BSA were 0.355, 0.620, and 0.391, respectively. As

Table II. Dose Effect of AcLDL on Cellular Contents of Free Cholesterol, Cholesteryl, Esters, and Cholesterol Esterification Rate in Rat Peritoneal Macrophages

	Concentration of AcLDL (μg/ml medium)				
	0	6.25	12.5	25	50
Free cholesterol (μg/mg protein)	3.59±0.97	3.87±0.66	3.81±0..40	4.18±0.46	5.37±0.50*
Cholesteryl esters (μg/mg protein)	20.32±5.52	40.35±6.21‡	49.27±6.62‡	90.83±10.92‡	124.90±9.17‡
Cholesterol esterification (nmol/mg protein/h)	0.21±0.01	0.66±0.02*	0.87±0.10‡	0.98±0.05‡	1.16±0.18‡

The results represent means±SD of the mean. $n = 3$. * $P < 0.05$ vs 0 μg/ml AcLDL, ‡ $P < 0.01$ vs 0 μg/ml AcLDL. Rat peritoneal macrophages were incubated with the indicated concentration of AcLDL for 48 h. For further experimental details, see Methods.

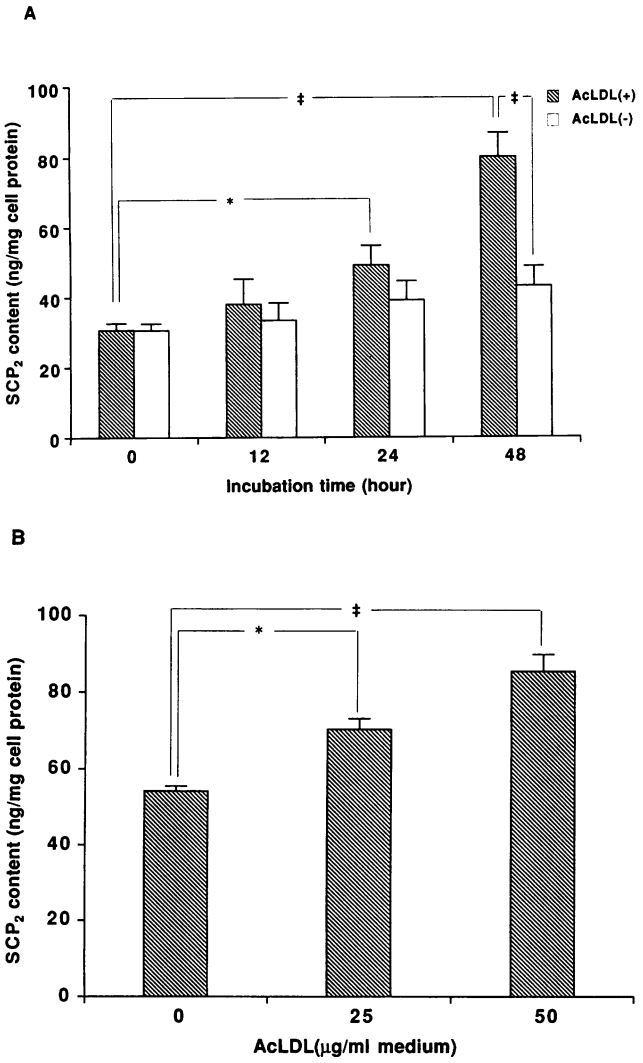

Figure 2. Effect of AcLDL on SCP_2 content in whole homogenate of rat peritoneal macrophages measured by EIA. For the time dependence study (*A*), macrophages were incubated with or without 50 μg/ml of AcLDL. For the concentration dependence study (*B*), rat peritoneal macrophages were incubated with the indicated concentration of AcLDL. (n = 3, mean±SEM, * $P < 0.05$, ‡ $P < 0.01$) For further experimental details, see Methods.

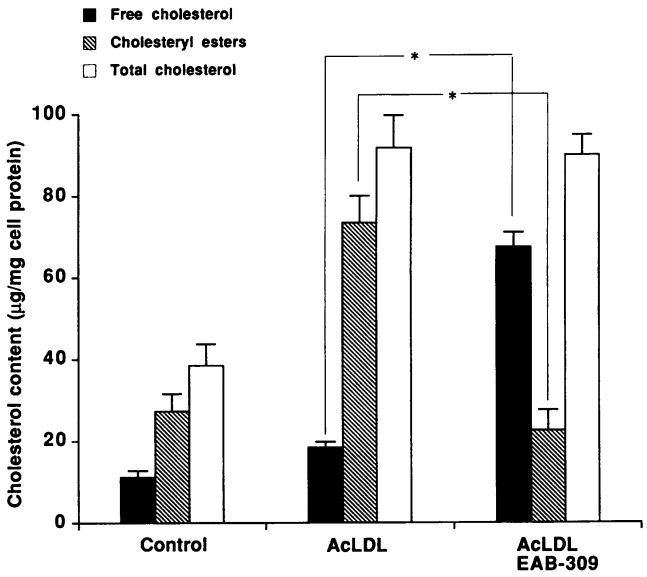

Figure 4. Effect of EAB-309 on cellular contents of free cholesterol, cholesteryl esters and total cholesterol in rat peritoneal macrophages when incubated with AcLDL. Rat peritoneal macrophages were incubated with 50 μg/ml of AcLDL in the presence or the absence of 100 nM EAB-309 for 48 h. (n = 3, mean±SEM, * $P < 0.01$) For further experimental details, see Methods.

shown, incubation with 25-OH cholesterol (5 μg/ml) for 8 h clearly increased SCP_2 mRNA level in macrophages at the 1 μg/ml concentration, while Mal-BSA (300 μg/ml) had no significant effect.

Discussion

The purpose of the present investigation was to explore the hypothesis that SCP_2 may play an important role in the formation of foam cells from macrophages, which should be one of the most important steps in the pathogenesis of atherosclerosis. For this purpose, we first studied whether SCP_2 is present in rat peritoneal macrophages or not, and then determined the regulation of SCP_2 and its mRNA in macrophages during foam cell formation induced by the incubation with AcLDL.

Western blot analysis of the whole homogenate of rat peritoneal macrophages using the specific antibody against SCP_2 dem-

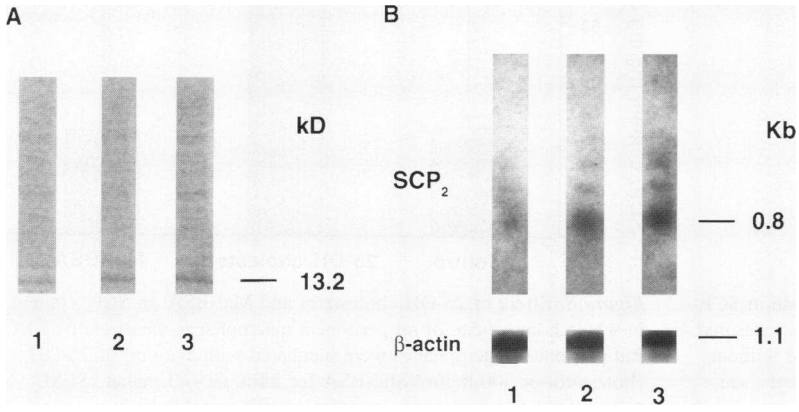

Figure 3. The result of an immunoblot (*A*) and a Northern blot (*B*) analysis of rat peritoneal macrophages incubated with or without AcLDL in the presence or the absence of EAB-309. For an immunoblot analysis (*A*), rat peritoneal macrophages were incubated with or without 50 μg/ml of AcLDL in the presence or the absence of 100 nM EAB-309 for 48 h. For a Northern blot analysis (*B*), macrophages were incubated with or without 50 μg/ml of AcLDL in the presence or the absence of 100 nM EAB-309 for 18 h. 4 μg of mRNA purified from macrophages was applied to the gel. Rat β-actin was also hybridized as control. Lane *1*, rat peritoneal macrophages incubated without AcLDL; lane *2*, rat peritoneal macrophages incubated with AcLDL; lane *3*, rat peritoneal macrophages incubated with AcLDL in the presence of EAB-309. For further experimental details, see Methods.

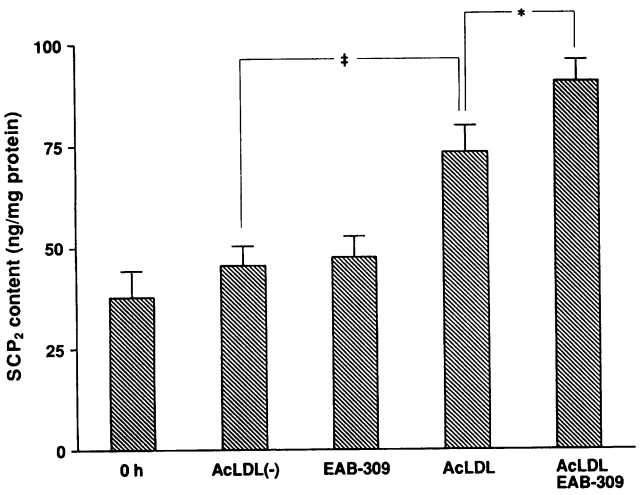

Figure 5. Effect of EAB-309 on SCP_2 content in rat peritoneal macrophages when incubated with AcLDL. Rat peritoneal macrophages were incubated with or without 50 μg/ml of AcLDL in the presence or the absence of 100 nM EAB-309 for 48 h. (n = 3, mean±SEM, * P < 0.05, ‡ P < 0.01) For further experimental details, see Methods.

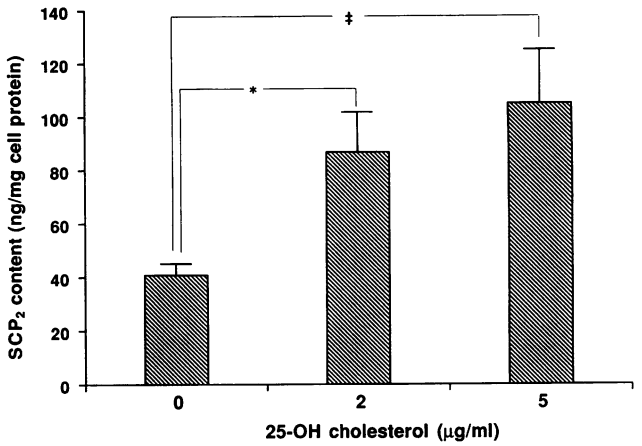

Figure 7. Effect of 25-OH cholesterol on SCP_2 content in rat peritoneal macrophages. Rat peritoneal macrophages were incubated with the indicated concentration of 25-OH cholesterol for 24 h. (n = 3, mean±SEM, * P < 0.05, ‡ P < 0.01) For further experimental details, see Methods.

onstrated the presence of one protein showing the same molecular mass of 13.2 kD as authentic SCP_2 purified from rat liver cytosol. Another cross-reactive protein with a molecular mass of 58 kD was observed in rat liver, not in rat peritoneal macrophages. van Amerongen et al. (32), van Heusden et al. (33) and Ban et al. (11) reported the presence of similar cross-reactive 58-kD protein in rat adrenocortical cells, Leydig cells, and aorta, respectively. This 58-kD protein has been designated as sterol carrier protein x (SCPx), because it contains the complete sequence of SCP_2 at the COOH-terminal region (24, 34, 35). SCPx has been reported to be predominantly present in peroxisomes in the liver (36–38), though the functions of SCPx still remain unclear.

The levels of SCP_2 mRNA were analyzed in rat liver and rat peritoneal macrophages. In rat liver, four mRNA species of 0.8, 1.4, 2.1, and 2.7 kb were detected. The 0.8- and 1.4-kb transcripts have been reported to encode a 15-kD protein (pre-SCP_2) with a 20–amino acid prepeptide that is posttranslationally removed to form mature SCP_2 (39, 40). The 2.1- and 2.7-kb transcripts are shown to encode SCPx (25). Sheedorf and Assmann (25) and Ossendorp et al. (41) demonstrated that the four mRNA species are generated from one gene, first by differential splicing and then by alternative adenylation. In rat peritoneal macrophages, a mRNA transcript of 0.8 kb was observed, while those of 1.4, 2.1, and 2.7 kb are expressed only in trace amounts. This result clearly demonstrated that mRNA encoding SCP_2 was present in rat peritoneal macrophages. Ossendorp et al. (41) described that the 2.1-kb mRNA is substantially expressed only in liver and kidney, and to a lesser extent in lung and intestine. Seedorf and Assmann (25) also described that with the exception of liver, the 2.1-kb mRNA is expressed only in trace amounts in all tissues analyzed. These results are in agreement with the present findings that the 0.8-kb mRNA is substantially expressed in rat peritoneal macrophages, while

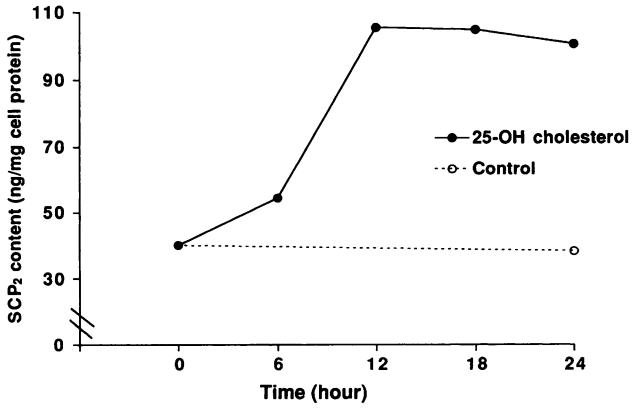

Figure 6. Time course of 25-OH cholesterol-induced increase in SCP_2 content in whole homogenate of rat peritoneal macrophages measured by EIA. Rat peritoneal macrophages were incubated with or without 5 μg/ml of 25-OH cholesterol. For further experimental details, see Methods.

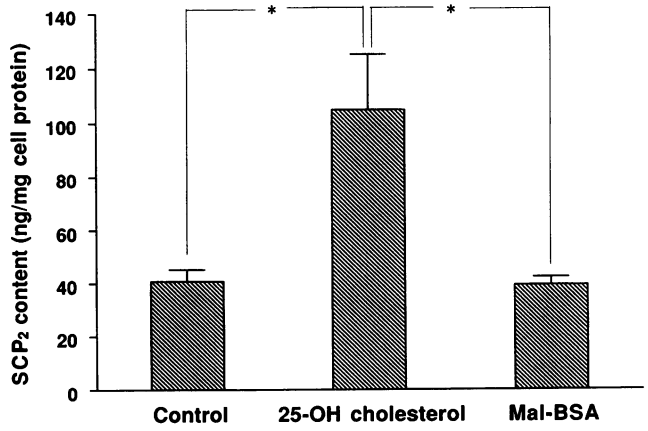

Figure 8. Effect of 25-OH cholesterol and Mal-BSA on SCP_2 content in whole homogenate of rat peritoneal macrophages measured by EIA. Rat peritoneal macrophages were incubated with 5 μg/ml of 25-OH cholesterol or 300 mg/ml Mal-BSA for 24 h. (n = 3, mean±SEM, * P < 0.01) For further experimental details, see Methods.

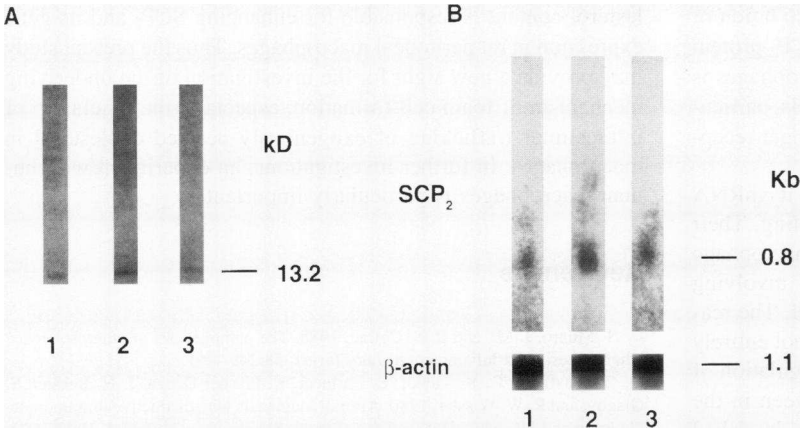

Figure 9. The result of an immunoblot (*A*) and a Northern blot (*B*) analysis of rat peritoneal macrophages incubated with 25-OH cholesterol or Mal-BSA. For an immunoblot analysis (*A*), rat peritoneal macrophages were incubated with 5 μg/ml of 25-OH cholesterol or 300 μg/ml Mal-BSA for 24 h. For Northern blot analysis (*B*), macrophages were incubated with 5 μg/ml of 25-OH cholesterol or 300 μg/ml Mal-BSA for 12 h. 4 μg of mRNA purified from macrophages was applied to the gel. Rat β-actin was also hybridized as control. Lane *1*, control; lane *2*, 25-OH cholesterol; lane *3*, Mal-BSA. For further experimental details, see Methods.

the 2.1-kb mRNA is expressed only in trace amounts. These data clearly demonstrate that SCP_2 mRNA was transcribed and translated, and that SCP_2 protein was present in the rat peritoneal macrophages.

When rat peritoneal macrophages were incubated with AcLDL, the content of SCP_2 in whole homogenate was increased in a time- and dose-dependent fashion. Furthermore, SCP_2 mRNA level was also increased by AcLDL treatment. These findings suggest that AcLDL stimulates SCP_2 synthesis probably through the increasing transcription of SCP_2 mRNA in macrophages, although decreased degradation of SCP_2 protein and its mRNA could also be one of contributing factors.

In the present study, Western blot analysis of macrophage proteins showed that the SCP_2 protein increased in response to AcLDL for 48 h (Fig. 3). Of note is the fact that SCPx was undetectable in rat peritoneal macrophages, indicating a selective accumulation of SCP_2 in macrophages in response to AcLDL. The present finding is in good agreement with those reported by Trzeciak et al. (40), who described that ACTH or dibutyryl cAMP stimulated synthesis of SCP_2 in rat adrenal cells with little apparent increase in SCPx. Rennert et al. (42) also reported that the SCP_2 protein in rat ovary increased in response to 8-bromo-cAMP treatment, while SCPx was not detected, despite the increase in mRNAs which encode SCPx. Taken together, there may be a selective accumulation of SCP_2 in these cells. Differential regulation of the mRNAs between SCP_2 and SCPx under certain circumstances has been reported (42, 43). Fujiki et al. (39) described that the mRNAs encoding SCP_2 and SCPx, which have been shown to be in a single gene, may be presumably transcribed with different promotor(s).

In the present study, AcLDL increased cellular contents of cholesteryl esters and SCP_2 in macrophages in dose- and time-dependent manner. In addition, the coincubation of EAB-309 with AcLDL inhibited the accumulation of cholesteryl esters and markedly increased intracellular free cholesterol content associated with the enhancement of SCP_2 gene expression. These data suggested that cholesteryl esters accumulation was not necessary for the enhancement of SCP_2 gene expression. It was also concluded that a cellular free cholesterol, not cholesterolyl esters, positively regulates SCP_2 gene expression in macrophages.

It is well established that mono-oxygenated metabolites of cholesterol, i.e., oxysterols, are potent mediators of cellular expression of cholesterol-sensitive proteins such as LDL receptor and 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase (44). One of these oxysterols, 25-OH cholesterol has been identified in various mammalian tissues and has been shown to be one of the most potent suppressers of LDL receptor and HMG-CoA reductase (45–47). To get further insight into the regulation of SCP_2 gene expression by cholesterol, we investigated the possibility that 25-OH cholesterol is of importance for the up-regulation of SCP_2 in rat peritoneal macrophages induced by AcLDL. Incubation with 25-OH cholesterol dose- and time-dependently increased SCP_2 content. In addition, incubation with 25-OH cholesterol clearly increased SCP_2 mRNA level in macrophages. These findings reveal that SCP_2 gene expression in macrophages is regulated by cholesterol and/or by oxysterol derivatives, and we presume, therefore, that AcLDL-induced accumulation of SCP_2 in macrophages is due to at least in part, to the action of cholesterol. The present findings can provide a new concept that SCP_2 gene is one of the gene family which is regulated by cellular cholesterol. However the molecular mechanism of regulation of SCP_2 mRNA by cholesterol is unknown.

The molecular mechanisms accounting for sterol-mediated regulation of gene expression have been examined in great detail in the LDL receptor and HMG-CoA reductase genes (48, 49). For these genes, sterol repression is associated with a sterol responsive element located in the 5′-flanking region of these genes (44). In addition, the identification of proteins that bind to the sterol regulatory element in the promoter of these genes has been reported (50). These reports provide a significant step in the study of transcriptional regulation of SCP_2 by cholesterol and its oxysterol derivatives in macrophages. Therefore identification of the *cis*-elements that are responsible for SCP_2 gene responsiveness to cholesterol seems to be essential in order to clarify the mechanism by which cholesterol alters SCP_2 gene expression.

It is well established that macrophages uptake AcLDL via scavenger receptor (3). Murata et al. (51) reported that in addition to AcLDL, Mal-BSA, which was internalized by scavenger receptor-mediated endocytosis, stimulated the secretion of lipoprotein lipase without the change of the content of intracellular cholesterol. This suggests that the receptor-mediated endocytosis of ligands via the scavenger receptor might play a key role in the stimulation of lipoprotein lipase in macrophages. To clarify the possible involvement of an activation of scavenger receptors in AcLDL-induced enhancement of SCP_2 gene expression in rat peritoneal macrophages, we studied the effect of Mal-BSA on SCP_2 gene expression in peritoneal macrophages. Since treatment of macrophages with Mal-BSA does not increase

SCP_2 content and its mRNA, it is unlikely that an activation of scavenger receptors also causes the accumulation SCP_2 protein and its mRNA. Thus, SCP_2 gene expression in macrophages is regulated by mechanisms involving cellular lipid levels, particularly free cholesterol level that are unrelated to scavenger receptor and to endocytosis in general.

Recently, Baum et al. (52) reported that SCP_2 and its mRNA level in rat liver were unaffected by cholesterol feeding. Their results are in contrast to the present results that macrophage SCP_2 gene expression is regulated by mechanisms involving cellular lipid levels, particularly free cholesterol level. The reasons for the discrepancy between the two studies are not entirely clear. Possible explanations might be the different regulation of SCP_2 gene expression by free cholesterol level between in the liver and in other tissues. Scott Simonet et al. (53) described that the human apoprotein E (apo E) and apoprotein C genes are expressed at high levels in the liver in contrast to lower levels of expression in selected other tissues. They also reported that hepatic control region was located in promotor region and that hepatic control region could direct high level liver expression of a heterologus promotor/gene construct. Although analysis of promotor region of SCP_2 gene has not been reported yet, there is a possibility that similar liver-specific enhancer region might be present in promoter region of SCP_2 gene. Further study seems to be needed.

The role of SCP_2 in macrophages, especially during foam cell formation, has just begun to be explored but a number of interesting questions are raised by the present studies. It is well known that the uptake of AcLDL caused a markedly accelerated cholesterol esterification catalyzed by ACAT resulting in large accumulation of esterified cholesterol within the macrophages (3, 54). It has been shown that SCP_2 enhanced ACAT-catalyzed cholesterol esterification in microsomes of liver, adrenocortical cells most likely by increasing the availability of exogenous cholesterol to the enzyme, ACAT in microsomes (11, 55, 56). We found the same effect of SCP_2 on ACAT-catalyzed cholesterol esterification in microsomes of rat peritoneal macrophages (data not shown). Thus it raises a possibility that SCP_2 might be involved in an accelerated cholesterol esterification in AcLDL-loaded macrophages. In the present study, maximal whole cell cholesterol esterification in rat peritoneal macrophages was observed at 12 h. On the other hand, the level of SCP_2 in macrophages at 12 h showed no significant increase. The levels of SCP_2 in nonstimulated rat peritoneal macrophages might be sufficient for intracellular trafficking of cholesterol to ACAT during foam cell formation. It is well known that AcLDL treatment enhanced apo E gene expression in macrophages (57). Apo E is shown to play an important role in reverse cholesterol transport from peripheral cells such as macrophages to liver (58, 59). Mazzone et al. (57) reported that maximal and significant increase in apoE mRNA gene expression in THP-1 cells was observed 48 h after the addition of AcLDL. In the present study, maximal increase in the levels of SCP_2 content was also observed at 48 h. From these evidences, it is inferred that SCP_2 may be involved in cholesterol efflux from cholesterol-loaded macrophages. Further studies seem to be required to clarify this question.

In summary, we first demonstrated that SCP_2 exists in rat peritoneal macrophages and that AcLDL treatment of rat peritoneal macrophages resulted in the increase in SCP_2 and its mRNA in macrophages associated with the accumulation of lipids. It is also suggested that increased macrophage-free cholesterol content is responsible for enhancing SCP_2 and its gene expression in rat peritoneal macrophages. Thus the present study may provide a new sight for the investigation of the underlying mechanism of foam cell formation, especially the regulation of intracellular trafficking of exogenously derived cholesterol in macrophages. In further investigations, an experiment with human macrophages is particularly important.

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