Paradoxical effect on atherosclerosis of hormone-sensitive lipase overexpression in macrophages

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Abstract Foam cells formed from receptor-mediated uptake of lipoprotein cholesterol by macrophages in the arterial intima are critical in the initiation, progression, and stability of atherosclerotic lesions. Macrophages accumulate cholesterol when conditions favor esterification by acyl-CoA:cholesterol acyltransferase (ACAT) over cholesteryl-ester hydrolysis by a neutral cholesteryl-ester hydrolase, such as hormone-sensitive lipase (HSL), and subsequent cholesterol efflux mediated by extracellular acceptors. We recently made stable transfectants of a murine macrophage cell line, RAW 264.7, that overexpressed a rat HSL cDNA and had a 5-fold higher rate of cholesteryl-ester hydrolysis than control cells. The current study examined the effect of macrophage-specific HSL overexpression on susceptibility to diet-induced atherosclerosis in mice. A transgenic line overexpressing the rat HSL cDNA regulated with a macrophage-specific scavenger receptor promoter-enhancer was established by breeding with C57BL/6J mice. Transgenic peritoneal macrophages exhibited macrophage-specific 7-fold overexpression of HSL cholesterol esterase activity. Total plasma cholesterol levels in transgenic mice fed a chow diet were modestly elevated 16% compared to control littersmates. After 14 weeks on a high-fat, high-cholesterol diet, total cholesterol increased 3-fold, with no difference between transgenics and controls. However, HSL overexpression resulted in thicker aortic fatty lesions that were 2.5-times larger in transgenic mice. HSL expression in the aortic lesions was shown by immunocytochemistry. Atherosclerosis was more advanced in transgenic mice exhibiting raised lesions involving the aortic wall, along with lipid accumulation in coronary arteries occurring only in transgenics. Thus, increasing cholesteryl-ester hydrolysis, without concomitantly decreasing ACAT activity or increasing cholesterol efflux, is not sufficient to protect against atherosclerosis. — Escary, J-L., H. A. Choy, K. Reue, X-P. Wang, L. W. Castellani, C. K. Glass, A. J. Lusis, and M. C. Schotz. Paradoxical effect on atherosclerosis of hormone-sensitive lipase overexpression in macrophages. J. Lipid Res. 1999. 40: 397–404.

Supplementary key words cholesteryl-ester hydrolysis • acyl-CoA:cholesterol acyltransferase • foam cells • aortic lesions • transgenic mice

Atherogenesis involves receptor-mediated uptake of lipoproteins by macrophages in the arterial intima, leading to cellular accumulation of cholesterol and formation of the foam cells characteristic of aortic fatty lesions (1, 2). The intracellular deposition of cholesteryl esters is thought to result from an imbalance favoring cholesterol esterification by acyl-CoA:cholesterol acyltransferase (ACAT) in the endoplasmic reticulum over its release by a neutral cholesteryl-ester hydrolase (nCEH) and subsequent efflux via the plasma membrane mediated by extracellular acceptors, such as high density lipoprotein (HDL) (3–5). Inadequate hydrolysis of cholesteryl esters could contribute not only to the development of atherosclerotic lesions but also to the instability of advanced plaques, increasing the risk for thrombosis (6). In addition, free cholesterol stimulates ACAT activity (7, 8), and its accumulation contributes to crystal formation in advanced lesions (9).

In studies of model foam cells using cholesterol-loaded mouse peritoneal macrophages in culture, the net hydrolysis of accumulated cholesteryl esters can be accomplished by inhibiting ACAT or by removing its substrate, free cholesterol, with extracellular acceptors (3). The hydrolysis reaction itself, however, is not readily modulated to increase the net degradation of cholesteryl esters. Modest 2-fold stimulation of the hydrolysis reaction by cAMP treatment has been observed in the J774 macrophage cell line (10). The mechanism of activation was presumably by cAMP-dependent protein kinase-mediated phosphorylation of hormone-sensitive lipase (HSL), the principal nCEH identified in mouse macrophages (11–14). We have further demonstrated the role of HSL in macrophage cholesteryl-ester hydrolysis by producing a RAW 264.7 murine

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; HDL, high density lipoprotein; HSL, hormone-sensitive lipase; LDL, low density lipoprotein; nCEH, neutral cholesteryl-ester hydrolase; VLDL, very low density lipoprotein.

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macrophage cell line that stably overexpresses rat HSL (14). These cells exhibited a 5-fold higher rate of hydrolysis with cAMP activation compared to control RAW cells, and a dramatic reduction in cholesteryl-ester accumulation (14).

Inhibitors of ACAT activity have been used to reduce atherosclerosis in animal models (e.g., 15). The mode of action, however, has been thought to be primarily the inhibition of intestinal cholesterol absorption, and not directly of cholesterol esterification in arterial cells (16). In the current study, we examined the effect of increasing macrophage-specific cholesteryl-ester hydrolysis in vivo on the susceptibility of mice to diet-induced atherosclerosis. Transgenic mice were made with a rat HSL transgene driven by a macrophage-specific scavenger receptor promoter-enhancer (17). The transgenic mice expressed a macrophage-specific 7-fold increase in HSL activity, and exhibited a modest 16% elevation in plasma cholesterol levels on a chow diet compared to control littermates. When fed an atherogenic diet, transgenic and control mice showed similar increases in total cholesterol levels, but paradoxically, the transgenic mice had increased incidence and severity of aortic fatty lesions. We propose that increased levels of free cholesterol in macrophages from enhanced cholesteryl-ester hydrolysis may stimulate cholesterol esterification, resulting in greater accumulation of cellular cholesterol.

MATERIALS AND METHODS

Generation of transgenic mice

The pAL1-HSL plasmid Xhol-NotI fragment containing a rat HSL cDNA and a human macrophage-specific scavenger receptor promoter-enhancer (17) was injected into fertilized B6/C3H × B6) oocytes. Embryos were implanted in pseudopregnant Swiss-Webster mice at the UCLA Transgenic Facility. Thirteen founder animals were obtained and screened for genomic incorporation of the transgene by PCR analysis of tail-tip DNA as previously described (14). Transgene-positive founder males were mated with C57BL/6 females to establish transgenic lines.

Diet induction of atherosclerosis

Individually housed mice were maintained on a standard mouse chow containing 4% (w/w) fat. To examine susceptibility to diet-induced atherosclerosis, 3-month-old F3 female littermates were fed ad libitum an atherogenic diet (Harlan Teklad TD 90221) containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid for 14 weeks.

Macrophage and tissue samples

Peritoneal macrophages were elicited with thioglycollate and harvested using established procedures (18). Cells were resuspended in DMEM (containing 10% fetal bovine serum, 2 mm l-glutamine, 1 mm sodium pyruvate, 0.1 mm non-essential amino acids, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.25 μg/ml fungizone) and cultured at 4×10⁶ cells in 6-well cell-culture plate (for nCEH assays and Western analysis) or 1×10⁶ cells in a 12-well plate (cholesteryl-ester turnover assays) for 24 h at 37°C in 5% CO₂ before use. Whole-cell extracts of macrophages were prepared by sonication and protein content was determined (14).

Mouse tissues (brain, heart, lung, liver, kidney, spleen, adipose, testis) collected from a transgenic and one control F1 adult male were homogenized in cold buffer (25 mm Tris-HCl, pH 7.4, 1 mm EDTA, 20% glycerol, 10 μg/ml leupeptin, 0.1 mm benzamidine) using a Tissumizer (Tekmar). Supernatants from a 5-min, 16,000 g centrifugation at 4°C were frozen at −80°C before assay.

Neutral cholesteryl-ester hydrolyase assay

Extracts (5–10 μg protein) of macrophages and tissues were assayed for nCEH activity at pH 7.0 and 37°C with an emulsion of cholesteryl[1-14C]oleate and phosphatidylcholine-phosphatidyl-choline (14). A milliunit of enzyme hydrolyzes 1 nmol of cholesteryl ester in one minute.

Antibody titration of nCEH activity

nCEH activity in 10 μg extract was titrated with a neutralizing antibody, chicken anti-rat HSL IgG (provided by Dr. Cecilia Holm, Lund University). Preincubation of extract with antibody or chicken nonimmune IgG (equivalent protein) at 37°C for 1 h was followed by assay for surviving nCEH activity (14).

Western analysis of HSL

Western blotting followed a procedure described previously for comparing levels of transgenic HSL (14). One hundred μg of macrophage or tissue extract (10 μg adipose from F3 mice was resolved by SDS-PAGE, blotted, and probed with rabbit anti-rat HSL IgG (1:3000 dilution; antisera provided by Dr. Fredric B. Kraemer, Stanford University; 19) or rabbit nonimmune IgG (1:1000 dilution). Visualization of proteins used capture of enhanced chemiluminescence (Pierce) with film. HSL was identified by comparison with a recombinant rat HSL standard (provided by Dr. Cecilia Holm), HSL in adipose extracts, and protein molecular weight markers. Proteins other than 84-kDa HSL were also detected with both antibody and nonimmune IgG. However, nCEH activity was titrated only by HSL antibody (see Results; 14), indicating that the other proteins are unrelated to HSL.

Cholesteryl-ester turnover

Peritoneal macrophages were loaded with cholesterol by incubation with acetylated low density lipoprotein (LDL) and cholesteryl-ester hydrolysis was measured by pulse-chase of cholesteryl[3H]oleate (14). Briefly, cells were cultured for 24 h with 150 μg/ml acetylated-LDL and 0.2 mm [3H]oleate (200,000 dpm/mm). After 24 h equilibration of cellular cholesterol pools in the absence of lipoprotein, [3H]oleate was removed (0 h) and the absolute rate of cholesteryl-ester hydrolysis was determined in the presence of an ACAT inhibitor (10 μm CI-976; provided by Dr. Roger Newton, Parke-Davis). Dibutylryc cAMP (1 mm) was also added at 0 h to stimulate activity by overexpressed HSL (14). At 3, 6, and 9 h, cultures were extracted to measure [3H]cholesteryl ester and protein content. A semi-logarithmic plot of percentage of [3H]cholesteryl oleate present at 0 h as a function of time yielded a rate constant to derive β, the time in hours for hydrolysis of half the cholesteryl oleate in cells.

Plasma cholesterol

Mice were fasted overnight and blood was collected by retro-orbital bleeding under isoflurane anesthesia for enzymatic determination of cholesterol in plasma from individual animals (20). Cholesterol distribution was also measured in plasma lipoproteins resolved by gel filtration of pooled samples (3-4 mice each in three pools each of controls and transgenics) using fast performance liquid chromatography (21).

Quantitation of aortic lesions

Transgenic and control littermates fed an atherogenic diet were examined for the incidence and severity of atherosclerosis.
(20). Mice were killed and the heart and proximal aorta were dissected, embedded, and sectioned. Selected serial sections were stained with oil red O and hematoxylin, examined by light microscopy, and quantitated. Some adjacent sections were examined by immunocytochemistry (20), using rabbit anti-rat HSL IgG (19) or MO-MA-2, a rat monoclonal antibody for mouse macrophages (22). Specificity of the HSL antibody was previously shown by Western blotting (19), and checked here by positive immunocytochemical staining of mouse adipose tissue and no staining of liver.

Statistical analysis

Results are presented as mean ± SEM, and statistical significance was determined with Student’s t test.

RESULTS

Production of HSL-overexpressing transgenic mice

To examine the effect of HSL overexpression in macrophages on cholesterol metabolism and susceptibility to atherosclerosis, transgenic mice were designed to express a rat HSL cDNA regulated by a human macrophage scavenger receptor promoter-enhancer. We have described this chimeric HSL gene, pAL1-HSL, and its overexpression in stable transfectants of the RAW macrophage cell line (14). In addition, this scavenger receptor promoter-enhancer has been shown to direct growth hormone expression to peritoneal macrophages as well as macrophage foam cells in atherosclerotic lesions of transgenic mice (17). Injection of pAL1-HSL in (C57BL/6J × C3H/HeJ)F1 oocytes fertilized with C57BL/6J sperm yielded three male founder mice with genomic incorporation of the transgene. F1 offspring of founders crossed to C57BL/6J females were examined for the presence of the transgene with PCR and assayed for overexpression of nCEH activity as described below. Mice found to overexpress HSL were backcrossed to C57BL/6J females, in order to increase the atherosclerosis-susceptible C57BL/6J genetic background (20). F2 offspring were manipulated in the same fashion to obtain the F3 generation with >95% C57BL/6J background for study of diet-induced atherosclerosis.

**Fig. 1.** HSL overexpression in macrophages from transgenic mice. (A) nCEH activity was measured in extracts of peritoneal macrophages elicited from chow-fed female mice (four F3 controls and two each of F1 and F3 transgenics) as described in Materials and Methods. HSL transgenic mice showed 7-fold overexpression compared to control littermates (P < 0.0003). The mean and SEM for four individuals is presented for each group. (B) A Western blot of extracts (100 µg) of control and nCEH-overexpressing peritoneal macrophages was probed with anti-rat HSL IgG and chemiluminescent signals were captured on film as described in Materials and Methods. Transgenic cells (tg) showed a large increase in HSL at 84 kDa (arrow). The prominent protein band below HSL in control and transgenic extracts was also detected with nonimmune IgG and is unrelated to HSL.

**Fig. 2.** Antibody titration of HSL in transgenic macrophages. A representative extract of nCEH-overexpressing peritoneal macrophages was preincubated with anti-HSL or nonimmune IgG before assaying for enzyme activity as described in Materials and Methods. Essentially all activity was titrated specifically by the HSL antibody.
Macrophage-specific overexpression of HSL

Transgene-containing and control F1 offspring were examined for increased expression of HSL by assaying cell-free extracts of elicited peritoneal macrophages for nCEH activity. One of the three founders produced offspring overexpressing nCEH activity in macrophages: 1.6 versus 0.3 mU/mg for two transgenics and two controls, respectively. Overall, 7-fold overexpression of nCEH activity (1.9 ± 0.2 mU/mg for transgenics) was maintained in peritoneal macrophages through the F3 generation of this founder (Fig. 1A). The level of nCEH activity in cells from control littersmates (0.27 ± 0.01 mU/mg) was comparable to that measured in peritoneal macrophages from C57BL/6j mice (14). Western blot analysis also showed a dramatic increase of 84-kDa HSL protein in peritoneal macrophages from transgenic mice compared to controls (Fig. 1B). Virtually all of the nCEH activity in macrophages from transgenic mice was titrated with a chicken anti-rat HSL antibody previously shown to be specific for HSL (14). As shown in Fig. 2, more than 90% of nCEH activity was inhibited by small amounts of HSL antibody, while no activity was affected by nonimmune chicken IgG. Antibody specificity was also confirmed by high-affinity titration of nCEH activity in mouse adipose tissue (23; data not shown).

A comparison of peritoneal macrophages and other tissues from a transgenic mouse to those from a control littermate by Western analysis with rabbit anti-rat HSL IgG (19) indicated that HSL overexpression in the transgenic mouse was macrophage specific. In contrast to the overexpression in peritoneal macrophages (Fig. 1B), there was no discernible increase of HSL protein in homogenates of other tissues that normally express HSL (adipose, testis, and heart; 19, 24) prepared from the transgenic mouse compared to its control littermate (data not shown). No HSL was detected by Western blotting of non-HSL expressing tissues, including brain, lung, liver, kidney, and spleen (19, 24). In addition, macrophage-specific overexpression was demonstrated by comparing nCEH activity in tissue extracts (Fig. 3). HSL transgenic peritoneal macrophages were unique in exhibiting a 5-fold increase in nCEH activity. The much smaller differences (=2-fold) between transgenic and control mice observed for other tissues likely reflect normal animal-to-animal variation of non-HSL cholesterol esterases.

Increased cholesteryl-ester hydrolysis by HSL-overexpressing macrophages

To examine the consequence of the macrophage-specific increase in HSL expression on foam cell formation, cholesteryl-ester hydrolysis was measured in cultures of peritoneal macrophages obtained from chow-fed mice. After loading the cells with esterified cholesterol by incubation with acetylated LDL and [3H]oleate, the rates of hydrolysis of cholesteryl[3H]oleate in transgenic and control macrophages were measured in the presence of an ACAT inhibitor to prevent re-esterification (see Materials and Methods). The HSL-overexpressing cells degraded cholesteryl esters about twice as fast as control cells (t1/2 = 8.1 and 14 h, respectively; Fig. 4).

![Figure 3](image-url)

**Fig. 3.** Macrophage-specific overexpression of HSL activity. nCEH activity was measured in extracts of peritoneal macrophages (pool of two mice each) and tissues from chow-fed littermates. Except for the overexpression in transgenic peritoneal macrophages, there was no significant difference between control and transgenic mice in HSL-expressing tissues (adipose, testis, heart). Non-expressing tissues (brain, lung, liver, kidney, spleen) showed some variation in activity from non-HSL cholesterol esterases. Averages and ranges of duplicate assays are shown.
Increased atherosclerosis in HSL transgenic mice

Clear differences in the incidence and severity of atherosclerosis in control and transgenic mice were observed after 14 weeks on the atherogenic diet. Surprisingly, HSL transgenic mice had aortic fatty lesions with a mean area 2.5-times larger than the lesions in control animals, 11741 versus 4708 \( \mu \text{m}^2 \) (Table 2). Aortic disease was more advanced in HSL transgenic than control mice (Table 2). Whereas all 11 control and 10 transgenic animals exhibited type I lesions (20) involving the aortic valve and attachments only, 80\% (8 of 10) of these in the transgenic mice had progressed from flat to raised lesions compared to only 55\% (6 of 11) in the controls. In addition, 80\% (8 of 10) of HSL transgenic mice had type II lesions (20) involving the aortic wall, with all reaching the raised stage. In contrast, only 18\% (2 of 11) of control animals developed type II lesions. Coronary artery involvement was also more advanced in transgenic mice, with the accumulation of oil red O-stained lipid occurring exclusively in these mice.

A representative section of control aortas shows small type I lesions with little oil red O-staining lipid at the aortic valve attachments only (Fig. 5A). In contrast, a typical lesion in transgenic mice was extensively stained with oil red O, including the free aortic wall plus valve attachments (Fig. 5B), characteristic of type II lesions. Lipid accumulation in coronary arteries exclusively in transgenic mice is shown in Fig. 5C. HSL was expressed in the aortic lesions, which contained predominantly macrophage foam cells, as shown by adjacent serial sections stained with oil red O (Fig. 5D, F), HSL-specific IgG (Fig. 5E, G), or a macrophage-specific antibody (Fig. 5H).

The accumulation of more oil red O-staining cholesteryl esters by transgenic compared to non-transgenic arterial macrophages in vivo was corroborated in vitro. Peritoneal macrophages were cultured for 24 h in the continuous presence of acetylated LDL and cholesterol mass was measured as previously described (14). Cholesterol-loaded macrophages from transgenic mice contained about two times more cholesteryl esters than cells from non-transgenic mice (456 versus 241 nmol/mg protein). Little difference in free cholesterol was detected (about 10\% more in transgenic macrophages).

### DISCUSSION

HSL overexpression by stable transfectants of the RAW 264.7 murine macrophage cell line was previously shown
to enhance the hydrolysis of intracellular cholesteryl esters and consequently retard their accumulation, thus providing a model for controlling foam cell development (14). To test the effect of macrophage-specific HSL overproduction on the development of atherosclerosis, we have generated a transgenic mouse line with macrophage-specific HSL overexpression using the same macrophage scavenger receptor promoter-enhancer applied in the RAW cell studies. HSL overexpression was demonstrated by increased nCEH activity in cell-free extracts of peritoneal macrophages from transgenic mice, and confirmed by titration of the enzyme activity with an HSL-specific antibody. Macrophage specificity was shown by a direct comparison of control and transgenic littermates in a survey of tissues. In both Western analysis of HSL protein and assay for nCEH activity, only peritoneal macrophages exhibited

<table>
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<th>Mice</th>
<th>Lesion Size ( \mu m^2/) Section</th>
<th>Aortic Valve (Type I)</th>
<th>Aortic Wall (Type II)</th>
<th>Coronary Artery</th>
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<tr>
<td>Control</td>
<td>4708 ± 1482 (11)</td>
<td>11/11 (100%)</td>
<td>2/11 (18%)</td>
<td>0/11 (0%)</td>
</tr>
<tr>
<td>Transgenic</td>
<td>11741 ± 2005 (10)</td>
<td>10/10 (100%)</td>
<td>8/10 (80%)</td>
<td>5/10 (50%)</td>
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Incidences of aortic fatty lesions and lipid accumulation in coronary arteries after 14 weeks on an atherogenic diet are shown. Lesion sizes are means with SEM and (n).

\( ^a P < 0.005 \) compared to controls.

Fig. 5. Increased atherosclerosis in transgenic mice and HSL expression in aortic lesions. Aortic and coronary sections made after 14 weeks on an atherogenic diet were examined for fatty lesions and HSL expression. (A) Small aortic lesions in control mouse stained with oil red O. (B) Large aortic lesion in a transgenic mouse stained with oil red O. (C) Coronary artery lesions in the same transgenic mouse as in B stained with oil red O. (D, E) HSL expression in aortic lesion demonstrated with adjacent sections of a second transgenic mouse stained with oil red O and anti-HSL IgG, respectively. (F, G, H) HSL expression in macrophage foam cells shown with adjacent sections of a third transgenic mouse stained with oil red O, HSL antibody, and a macrophage antibody, respectively. All sections were also stained with hematoxylin, and intimal and underlying media cells can be distinguished by a bluish stain. Immunocytochemistry controls without primary antibodies gave clean backgrounds. The apparent stain for HSL in the aortic valve also occurred in sections of control mice, and could be due to nonspecific sticking of the antibody to connective tissue. Photomicrographs were taken at original magnification of 150×.
greatly enhanced expression in transgenic cells. This is consistent with the cell specificity previously found in transgenic mice for growth hormone expression controlled by the macrophage scavenger receptor promoter-enhancer (17). In addition, the absence of increased hepatic activity from potential transgenic expression in macrophage-lineage Kupffer cells also agrees with the previous study using this promoter-enhancer (17). This could reflect developmental differences despite common ontogeny between macrophages derived from monocytes recruited to peripheral tissues, including foam cell precursors in the arterial intima, and tissue-specific cells of macrophage lineage (26). Alternatively, perhaps HSL was not detectable in the whole-organ extracts because macrophages did not constitute a large enough proportion of the tissue mass.

Cultures of transgenic peritoneal macrophages exhibited increased capacity for cholesteryl-ester hydrolysis; they degraded cholesteryl esters twice as fast as control cells. The interesting and surprising outcome of enhanced HSL expression in macrophages in vivo was the accompanying increase in the incidence and severity of atherosclerosis found in mice after 14 weeks on an atherogenic diet. Although the transgenic mice did exhibit slightly elevated levels of plasma cholesterol with a chow diet compared to control littermates, both groups had similar cholesterol levels on the atherogenic diet. Thus, the increased atherosclerosis cannot be explained by altered plasma lipid levels. The higher aortic lesion scores for the transgenic mice are due to greater accumulation of neutral lipids as displayed by the oil red O stain, even though HSL expression in aortic lesions was verified by immunocytochemistry. This is a seemingly paradoxical result, as the increases in HSL expression and cholesteryl ester hydrolysis would be expected to result in reduced cholesteryl-ester accumulation, and hence, fewer lesions. However, the transgenic mice had larger and more advanced aortic fatty lesions, including increased involvement of the aortic wall and coronary arteries. The atherogenic diet elicited aortic valve (type I) lesions in all transgenic and control animals studied, but the transgenic mice had more advanced, raised (versus flat) type I lesions. In addition, whereas 80% of the transgenic mice developed lesions also in the aortic wall (type II), only 18% did so in the control group. The greater severity of atherosclerosis in the transgenic mice was also evident in lipid accumulation in the coronary arteries of this group exclusively.

Given our recent demonstration that increasing the capacity of RAW macrophages to hydrolyze cholesteryl esters by overexpression of HSL can dramatically decrease lipid accumulation in cultured foam cells (14), the current finding that macrophage-specific HSL overexpression in transgenic mice leads instead to greater susceptibility to developing atherosclerosis is surprising. A possible result of increased cholesteryl-ester hydrolysis in vivo is the accumulation of free cholesterol, especially if efflux is inadequate in removing the higher level of cholesterol generated in transgenic compared to control macrophages. The end result would be increased cholesterol esterifica-

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