The Very Low- and Intermediate-Density Lipoprotein Fraction Isolated from Apolipoprotein E-Knockout Mice Transforms Macrophages to Foam Cells through an Apolipoprotein E-Independent Pathway†

Hideki Hakamata,‡ Hisashi Sakaguchi,§,|| Chunni Zhang,‡ Naomi Sakashita,§ Hiroshi Suzuki,⊥ Akira Miyazaki,‡ Motohiro Takeya,§ Kiyoshi Takahashi,§ Nobuo Kitamura,|| and Seikoh Horiuchi*,‡

Department of Biochemistry, Second Department of Pathology, and First Department of Surgery, Kumamoto University School of Medicine, Kumamoto 860-0811, and Chugai Pharmaceutical Co. Ltd., Shizuoka 412-0038, Japan

Received April 6, 1998; Revised Manuscript Received June 8, 1998

ABSTRACT: Apolipoprotein E (apoE)-knockout mice develop severe atherosclerosis associated with high levels of very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) in plasma. To investigate the atherogenic role of VLDL and IDL, the lipoprotein fraction containing both VLDL and IDL (apoEko-VLDL/IDL) was isolated from plasma of apoE-knockout mice by ultracentrifugation, and its interaction with macrophages was studied. When peritoneal macrophages obtained from apoE-knockout mice were incubated with apoEko-VLDL/IDL, the level of cellular cholesteryl esters (CE) increased with the concentration of apoEko-VLDL/IDL. The level of cellular cholesteryl [^{3}H]oleate formed reached 15.1 nmol/mg of cell protein upon incubation with 50 μg/mL apoEko-VLDL/IDL for 18 h, which was an 8.4-fold increase over the corresponding level induced by low-density lipoprotein (LDL). The cellular CE mass was also significantly increased by apoEko-VLDL/IDL. Morphologically, after exposure to apoEko-VLDL/IDL, macrophages became strongly stained with Sudan black B. The total binding of [^{125}I]apoEko-VLDL/IDL to macrophages was effectively replaced by more than 80% by an excess of the unlabeled ligand. Specific binding, calculated by subtracting the nonspecific binding from the total binding, exhibited a saturation pattern. Similar results were obtained with cell association and degradation experiments. In addition, the endocytic degradation of [^{125}I]apoEko-VLDL/IDL was partially inhibited by LDL, whereas acetyl-LDL did not show any effect. These results indicated that apoEko-VLDL/IDL in its unmodified form produced significant CE accumulation in macrophages through a specific and apoE-independent pathway. This pathway may explain, in part, the mechanisms of foam cell formation in arterial walls and the subsequent development of atherosclerosis in apoE-knockout mice.

Macrophage-derived foam cells with a massive accumulation of cytoplasmic cholesteryl esters (CE)[1] are a characteristic feature of the early stage of atherosclerosis (*1*). Macrophages are known to take up chemically modified low-density lipoproteins (modified LDLs) such as acetylated LDL (acetyl-LDL) and oxidized LDL (Ox-LDL) through the macrophage scavenger receptor (MSR) pathway (*2*). These ligands undergo receptor-mediated endocytosis, followed by delivery through endosomes to lysosomes (*3*). In the lysosomes, the protein moiety of modified LDLs is subjected to proteolytic hydrolysis, whereas the cholesterol moiety, mainly CE, undergoes hydrolytic conversion to free cholesterol and fatty acids by acidic cholesteryl ester hydrolase (ACEH). Free cholesterol is then transported across lysosomal membranes to plasma membranes (*4*) and is further delivered to the endoplasmic reticulum, where free cholesterol is re-esterified to CE by acyl-coenzyme A:cholesterol acyltransferase (ACAT) (*5*). This leads eventually to accumulation of CE in the cytoplasm as lipid droplets and the transformation of macrophages to the morphologically distinct foam cells.

Among modified LDLs, Ox-LDL is emphasized as a likely atherogenic lipoprotein because of its presence in human and rabbit atherosclerotic plaques (*6, 7*). In addition to Ox-LDL, the β-migrating very low-density lipoprotein (β-VLDL), a lipoprotein fraction accumulated in the plasma of patients with the genetic disorder type III hyperlipoproteinemia and

† This work was supported in part by a Grants-in-Aid for Scientific Research (09877200, 09770789, 10671077, and 10877179) from the Ministry of Education, Science, Sports and Cultures of Japan and in part by a Japan Heart Foundation and Pfizer Pharmaceuticals Grant for Research on Coronary Artery Disease.

* Corresponding author: Department of Biochemistry, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860-0811, Japan. Phone and Fax: 81-96-364-6940. E-mail: horiuchi@gpo.kumamoto-u.ac.jp.

‡ Department of Biochemistry, Kumamoto University School of Medicine.

§ Second Department of Pathology, Kumamoto University School of Medicine.

|| First Department of Surgery, Kumamoto University School of Medicine.

⊥ Chugai Pharmaceutical Co. Ltd.

[1] Abbreviations: apoEko-VLDL/IDL, lipoprotein fraction containing both the very low-density lipoprotein and the intermediate density lipoprotein isolated from the plasma of apolipoprotein E-knockout mice; apoE, apolipoprotein E; LDL, low-density lipoprotein; acetyl-LDL, acetylated LDL; Ox-LDL, oxidized LDL; CE, cholesteryl ester(s); MSR, macrophage scavenger receptor(s); HDL, high-density lipoprotein; BSA, bovine serum albumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

S0006-2960(98)00762-4 CCC: $15.00
Published on Web 09/05/1998

in experimental animals fed a high-cholesterol diet, is another type of atherogenic lipoprotein which converts macrophages to foam cells via a pathway distinct from MSR (*8*, *9*). Further studies have shown that apolipoprotein E (apoE) is the ligand primarily responsible for the receptor-mediated uptake and lysosomal degradation of β-VLDL by macrophages, indicating that β-VLDL-induced foam cell formation is apoE-dependent (*10*, *11*).

ApoE, an important structural and functional constituent of plasma lipoproteins, plays a central role in chylomicron and VLDL remnant clearance (*12*). ApoE-knockout mice created by gene targeting, therefore, exhibit high levels of plasma VLDL and intermediate-density lipoprotein (IDL) fractions (*13*, *14*). ApoE-knockout mice are the first murine model to develop atherosclerosis spontaneously on a normal diet. This susceptibility to atherosclerosis is probably due to prolonged clearance of VLDL and IDL, which may lead to oxidative modification of these lipoproteins (*13*, *14*). To examine the role of MSR-AI/AII in the progression of atherosclerosis in apoE-knockout mice, we previously generated the double-knockout mice lacking both apoE and MSR-AI/AII. Morphological examination of these animals showed that the atherosclerotic lesions was significantly smaller (by 58%) than those of apoE single-knockout mice, indicating a direct role of MSR-AI/AII in atherogenesis (*15*). However, the double-knockout mice displayed significant atherosclerotic lesion formation despite a lack of MSR-AI/AII. This observation suggests the involvement of other scavenger receptors in the formation of atherosclerotic lesions, for example, MARCO (*16*), macrosialin (*17*), FcγRII-B2 (*18*), SR-BI (*19*), CD36 (*20*), LOX-1 (*21*), and SREC (*22*). However, another possibility is that VLDL and IDL accumulated in plasma directly interact with macrophages and cause intracellular CE accumulation. This study was therefore undertaken to examine this hypothesis. The results obtained clearly showed that the lipoprotein fraction containing both VLDL and IDL isolated from plasma of apoE-knockout mice (apoEko-VLDL/IDL) could induce significant CE accumulation in macrophages through an apoE-independent pathway.

MATERIALS AND METHODS

Materials. Na^{125}I (3.7 GBq/mL) and [9,10(*n*)-^{3}H]oleate (185 MBq/mL) were purchased from Amersham. Bovine serum albumin (BSA) was purchased from Wako (Osaka, Japan). Silica gel on aluminum sheets for thin-layer chromatography (TLC) was obtained from Merck. Cell culture media and reagents were obtained from Life Technologies. All other chemicals were of the best grade available from commercial sources.

Lipoproteins and Their Modifications. LDL ($d = 1.019-1.063$ g/mL) and high-density lipoprotein (HDL) ($d = 1.063-1.21$ g/mL) were isolated by sequential ultracentrifugation of fresh human plasma and dialyzed against 0.15 M NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4). Acetyl-LDL was prepared by chemical modification of LDL with acetic anhydride, as described previously (*23*). ApoEko-VLDL/IDL ($d < 1.019$ g/mL) was isolated by sequential ultracentrifugation from fresh plasma samples of apoE-knockout mice (*13*) after overnight fasting, followed by dialysis against 0.15 M NaCl and 1 mM EDTA (pH 7.4). This lipoprotein fraction is known to contain chylomicron remnants as a minor component (*13*). However, we referred to this fraction as "apoEko-VLDL/IDL" in accordance with the original study by Plump et al. (*14*). Iodination of apoEko-VLDL/IDL with ^{125}I was performed according to McFarlane (*24*). [^{125}I]ApoEko-VLDL/IDL was used within 6 h of labeling. The protein concentration was determined with the BCA protein assay reagent (Pierce Chemical Co.) using BSA as a standard, and expressed as milligrams of protein per milliliter (*25*). The extents of oxidation of LDL, HDL, acetyl-LDL, and apoEko-VLDL/IDL used in this study were determined by the thiobarbituric acid-reactive substances (TBARS) method as described previously (*26*). TBARS levels of LDL, HDL, acetyl-LDL, and apoEko-VLDL/IDL were 0.70, 0.37, 0.86, and 0.64 nmol of malondialdehyde (MDA)/mg of protein, respectively, which were negligibly low compared with those of conventional Cu^{2+}-oxidized LDL preparations (>10 nmol of MDA/mg of protein).

Cell Culture. Unless otherwise specified, cell cultures were performed at 37 °C in 5% CO_2. Peritoneal macrophages were collected from nonstimulated apoE-knockout mice (25−30 g) with 8 mL of ice-cold PBS, centrifuged at 200*g* for 5 min at 4 °C, and suspended in Dulbecco's modified Eagle's medium (DMEM) containing 3% BSA, streptomycin (0.1 mg/mL), and penicillin (100 units/mL) (medium A) (*27*). One milliliter of the cell suspension (2×10^6 cells) was seeded onto each plastic culture dish (22 mm diameter, Iwaki, Japan), and the dishes were incubated for 2 h. Cells were washed once with 1 mL of PBS containing 0.3% BSA and twice with 1 mL of PBS, and the monolayers thus formed were used in the following cellular experiments.

Assay for Cholesterol Esterification. The apoE-knockout macrophage monolayers formed through the above process were incubated in 1 mL of medium A with various concentrations of apoEko-VLDL/IDL for 18 h in the presence of 0.1 mM [^{3}H]oleate conjugated with BSA (107 190 dpm/nmol) (*28*). Cells were washed once with 1 mL of phosphate-buffered saline (PBS) containing 0.3% BSA and twice with 1 mL of PBS, followed by extraction of cellular lipids twice with 0.6 mL of hexane/2-propanol (v/v, 3/2). Cellular lipids extracted were dried under nitrogen flush, and the amount of radioactive cholesteryl [^{3}H]oleate was determined by TLC (*27*). After lipid extraction, cells were dissolved in 0.1 M NaOH and used for the determination of the cellular protein level (*25*).

Mass Determination of Cellular Cholesterol Contents. The apoE-knockout macrophage monolayers prepared as above were incubated in 1 mL of medium A with 50 μg/mL LDL, apoEko-VLDL/IDL, or acetyl-LDL. Cells were incubated for 18 h; cellular lipids were extracted as described above, and both the free cholesterol mass and CE mass were quantified by a modification (*27*) of the enzymatic fluorometric methods (*29*). Briefly, cellular lipids extracted were dried under nitrogen flush and dissolved in 180 μL of 2-propanol. Aliquots (30 μL) of the lipid extract was added to 0.4 mL of enzyme mixtures and incubated at 37 °C for 1 h (for free cholesterol) or for 2 h (for total cholesterol), followed by addition of 0.81 mL of 0.5 M NaOH to terminate the reaction. Enzyme mixtures were identical to those of Heider and Boyett (*29*) except that Carbowax-6000 was

Table 1: Lipid Contents of ApoEko-VLDL/IDL and LDL[a]

	lipid/protein weight ratio				
	TC[b]	FC[c]	CE[d]	TG[e]	PL[f]
LDL	1.51	0.41	1.10	0.42	0.92
apoEko-VLDL/IDL	3.83	1.11	2.72	0.38	1.50

[a] ApoEko-VLDL/IDL (d < 1.019 g/mL) was isolated from the plasma of apoE-knockout mice, and LDL (d = 1.019−1.063 g/mL) was isolated from fresh human plasma by sequential ultracentrifugation. The lipid contents of these lipoproteins were determined as described in Materials and Methods. Experimental errors in lipid determination were within 5%. [b] TC, total cholesterol. [c] FC, free cholesterol. [d] CE, cholesteryl ester. [e] TG, triglycerides. [f] PL, phospholipids.

replaced by 0.01% Triton X-100 and the enzyme concentrations were 2 times higher (0.16 unit/mL cholesterol oxidase and 0.16 unit/mL cholesteryl ester hydrolase). The fluorescence intensity was measured with excitation at 320 nm and emission at 407 nm. The CE level was calculated by subtracting the amount of free cholesterol from the amount of total cholesterol. Cells were dissolved in 0.1 M NaOH to determine cell protein levels using the BCA protein assay reagent.

Histochemistry. The apoE-knockout macrophage monolayers were incubated in medium A for 18 h with the lipoproteins (50 μg/mL) to be tested. Cells were washed once with 1 mL of PBS containing 0.3% BSA and twice with 1 mL of PBS and then fixed with 4% periodate-lysine-paraformaldehyde fixative at 4 °C for 20 min, followed by treatment with PBS containing 0.05% saponin (Sigma, St. Louis, MO) for 10 min. The cells were then stained by the Sudan black B (SBB) method (*30*).

Cellular Assays. For the binding experiments, the prepared macrophage monolayers (2×10^6 cells) were washed once with 1 mL of ice-cold PBS containing 0.3% BSA and twice with 1 mL of ice-cold PBS. Each well was incubated for 90 min on ice in 1 mL of medium A with the indicated concentrations of [^{125}I]apoEko-VLDL/IDL (453 cpm/ng of protein). The cells were washed once with 1 mL of ice-cold PBS containing 0.3% BSA and twice with 1 mL of ice-cold PBS. Cells were dissolved with 1 mL of 0.1 M NaOH, and the cell-bound radioactivity was determined as described previously (*31*). Nonspecific binding was measured by parallel incubation with an excess amount of the same unlabeled ligand. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

To determine the cell association of [^{125}I]apoEko-VLDL/IDL, and its endocytic degradation by macrophages, the apoE-knockout macrophage monolayers (2×10^6 cells) prepared as above were incubated for 5 h at 37 °C in 1 mL of medium A with various amounts of [^{125}I]apoEko-VLDL/IDL. To determine endocytic degradation, 0.75 mL of the culture medium was taken from each well and added to 0.25 mL of ice-cold 40% trichloroacetic acid and 0.2 mL of 0.7 M $AgNO_3$, and the mixture was incubated at room temperature for 30 min to precipitate free iodine. After centrifugation at 700*g* for 10 min, 0.6 mL of the resulting supernatant was used for determination of radioactivity using a γ-counter. The remaining cells in each well were washed once with 1 mL of PBS containing 0.3% BSA and twice with 1 mL of PBS, dissolved in 1 mL of 0.1 M NaOH, and the cell-associated radioactivity was determined as described previously (*31*, *32*).

Lipid Analysis. The lipid contents of lipoproteins were determined on a Hitachi 7450 automatic analyzer using standard enzymatic methods as described previously (*33*−*35*).

Electrophoresis. Agarose gel electrophoresis using a Universal Gel/8 electrophoresis kit (Ciba-Corning Diagnostic Corp., Tokyo, Japan) and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using a 4 to 20% gradient gel containing 1% SDS (Daiichi Pure Chemical Co., Tokyo, Japan) were performed as described previously (*27*).

Statistical Analysis. Data were evaluated by the Student's *t* test. Differences were considered significant when P was less than 0.05.

RESULTS

Physicochemical Properties of ApoEko-VLDL/IDL. We characterized the physicochemical properties of apoEko-VLDL/IDL isolated by ultracentrifugation. Lipid analyses showed that the ratios of CE, free cholesterol, and phospholipid to the protein of apoEko-VLDL/IDL were higher than those of LDL, whereas the triglyceride ratio did not differ from that of LDL (Table 1). Agarose gel electrophoresis showed apoEko-VLDL/IDL migrated to a pre-β-position (*36*) between HDL and LDL (Figure 1A, lane c). Gradient SDS−PAGE (4 to 20%) showed that apoEko-VLDL/IDL was composed of a main band of 220 kDa and minor bands of 28, 43, and <10 kDa, the former corresponding to apoB48 with the latter bands probably corresponding to apoA-I, apoA-IV, and apoCs, respectively (Figure 1B, lane c) (*37*), confirming the lack of apoE in apoEko-VLDL/IDL in our preparation.

ApoEko-VLDL/IDL-Induced CE Accumulation in Macrophages Obtained from ApoE-Knockout Mice. To test the possibility that apoEko-VLDL/IDL induces CE accumulation in macrophages, peritoneal macrophages obtained from apoE-knockout mice were incubated with apoEko-VLDL/IDL in the presence of [^{3}H]oleate. As shown in Figure 2, the incorporation of [^{3}H]oleate into cholesteryl [^{3}H]oleate increased in a saturation pattern with the concentration of apoEko-VLDL/IDL, and reached 15.1 nmol/mg of cell protein at 50 μg/mL apoEko-VLDL/IDL. Levels of cholesteryl [^{3}H]oleate were 39- and 8.4-fold greater than and $^1/_3$ of those after exposure of macrophages to medium alone (nonloaded), LDL, and acetyl-LDL, respectively.

In the next step, we examined whether the apoEko-VLDL/IDL-induced increase in the esterification rate of cholesterol (Figure 2) was accompanied by an increase in cellular CE mass. Macrophages from apoE-knockout mice were incubated with 50 μg/mL apoEko-VLDL/IDL, and free cholesterol and CE masses were determined. As shown in Figure 3, the level of CE mass in macrophages after incubation with medium alone was negligibly low, whereas the cellular CE mass was significantly increased when the cells were incubated with apoEko-VLDL/IDL. The level of cellular CE mass induced by apoEko-VLDL/IDL was significantly higher (~8-fold) than that for parallel incubation with LDL. These findings indicated that apoEko-VLDL/IDL could induce a significant CE mass accumulation in apoE-knockout macrophages.

Morphological Observation of Macrophage-Derived Foam Cells. To examine whether cellular CE accumulation

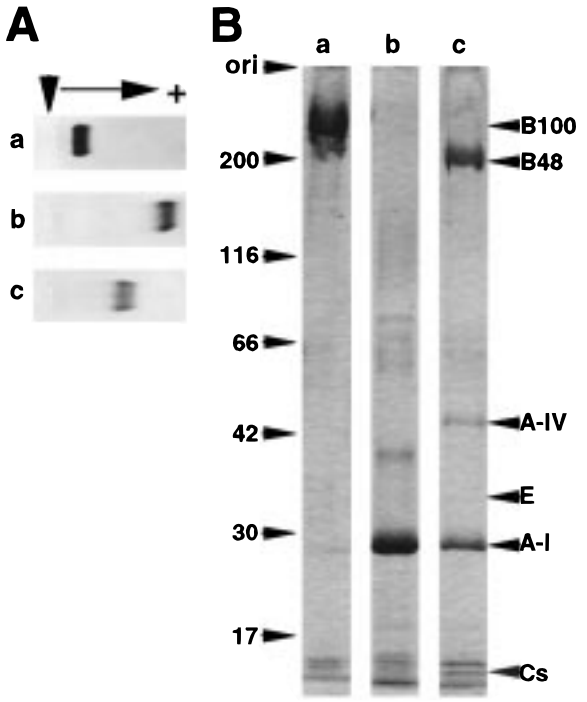

FIGURE 1: Agarose gel electrophoretic mobility (A) and SDS−polyacrylamide gel electrophoresis (B) of apoEko-VLDL/IDL. (A) LDL (lane a), HDL (lane b), and apoEko-VLDL/IDL (lane c) (10 μg/lane) were electrophoresed on a 1% agarose gel and stained with Coomassie Brilliant Blue. The arrowhead shows the origin and the arrow the direction. (B) LDL (lane a), HDL (lane b), and apoEko-VLDL/IDL (lane c) (5 μg/lane) were electrophoresed on gradient SDS−PAGE (4 to 20%) under reducing conditions and stained with Coomassie Brilliant Blue. The molecular mass (arrowheads) is indicated in kilodaltons.

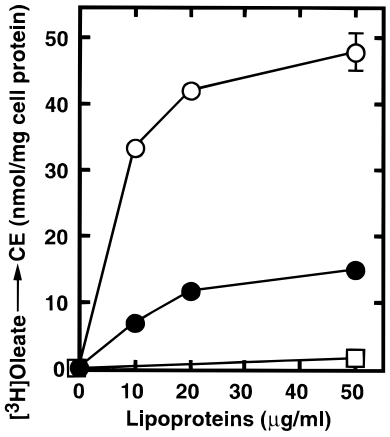

FIGURE 2: Effect of apoEko-VLDL/IDL on CE accumulation in macrophages obtained from apoE-knockout mice. Macrophages from apoE-knockout mice (2×10^6) were incubated for 18 h with the indicated concentrations of apoEko-VLDL/IDL (●), acetyl-LDL (○), or LDL (□) in the presence of 0.1 mM [^{3}H]oleate. Cellular lipids were extracted, and the radioactivity of cholesteryl [^{3}H]oleate was determined as described in Materials and Methods. Data are representative of two separate experiments with triplicate wells. Bars represent the standard deviation (SD).

transformed macrophages to foam cells, we stained these cells with Sudan black B (SBB). Histochemically, lipid accumulation was negligible in nonloaded or LDL-treated peritoneal macrophages obtained from apoE-knockout mice (panels A and B of Figure 4). In sharp contrast, all of the macrophages treated with acetyl-LDL were positive for SBB

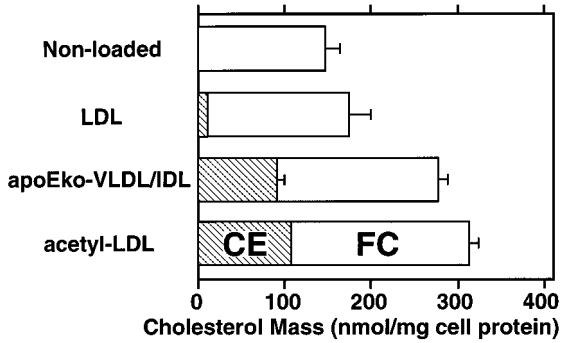

FIGURE 3: ApoEko-VLDL/IDL-induced CE mass accumulation in macrophages from apoE-knockout mice. Macrophages from apoE-knockout mice (2×10^6) were incubated for 18 h with 50 μg/mL LDL, apoEko-VLDL/IDL, or acetyl-LDL. Cellular lipids were extracted, and the masses of cellular cholesteryl esters (CE) and free cholesterol (FC) were determined as described in Materials and Methods. Each value is the mean ± SD of triplicate experiments.

(Figure 4D). Similarly, upon incubation with apoEko-VLDL/IDL, macrophages were positive for SBB (Figure 4C). However, the SBB-positive macrophages were about 30% of the total cells exposed to apoEko-VLDL/IDL. The staining intensity of the apoEko-VLDL/IDL-treated macrophages was more marked, compared with that of the acetyl-LDL-treated macrophages.

Interaction of ApoEko-VLDL/IDL with Macrophages Obtained from ApoE-Knockout Mice. In the next step, the interaction of apoEko-VLDL/IDL with apoE-knockout macrophages was determined by the level of cellular binding, cell association, and intracellular degradation. Figure 5 shows the level of binding of [^{125}I]apoEko-VLDL/IDL to macrophages from apoE-knockout mice at 0 °C. The level of total binding was effectively replaced by more than 80% by an excess of the unlabeled ligand. The level of specific binding, calculated by subtracting the level of nonspecific binding from the level of total binding, exhibited a saturation pattern. The Scatchard analysis of this specific binding of apoEko-VLDL/IDL to macrophages disclosed two binding sites. One site had an apparent dissociation constant (K_d) of 11.8 μg/mL and a maximal surface binding (B_{max}) of 101 ng/mg of cell protein, while the other site had an apparent K_d of 66.7 μg/mL and a B_{max} of 209 ng/mg of cell protein. Figure 6 shows the results of cell association and degradation experiments. The total degradation of [^{125}I]apoEko-VLDL/IDL by macrophages increased for 5 h in a dose-dependent manner, and was effectively replaced by more than 80% by an excess of the unlabeled ligand (Figure 6B). Specific degradation, calculated by subtracting the level of nonspecific degradation from the level of total degradation, apparently exhibited a saturation pattern. Similar results were obtained in cell association of [^{125}I]apoEko-VLDL/IDL with apoE-knockout macrophages (Figure 6A). Because our incubation medium contained only BSA as a protein component, these results clearly indicated the presence of high-affinity binding sites on the apoE-knockout macrophage cell surface which mediated the uptake and lysosomal degradation of apoEko-VLDL/IDL in an apoE-independent manner.

Effect of Acetyl-LDL and LDL on the Uptake of ApoEko-VLDL/IDL by Macrophages Obtained from ApoE-Knockout Mice. To further characterize the pathway of apoEko-VLDL/

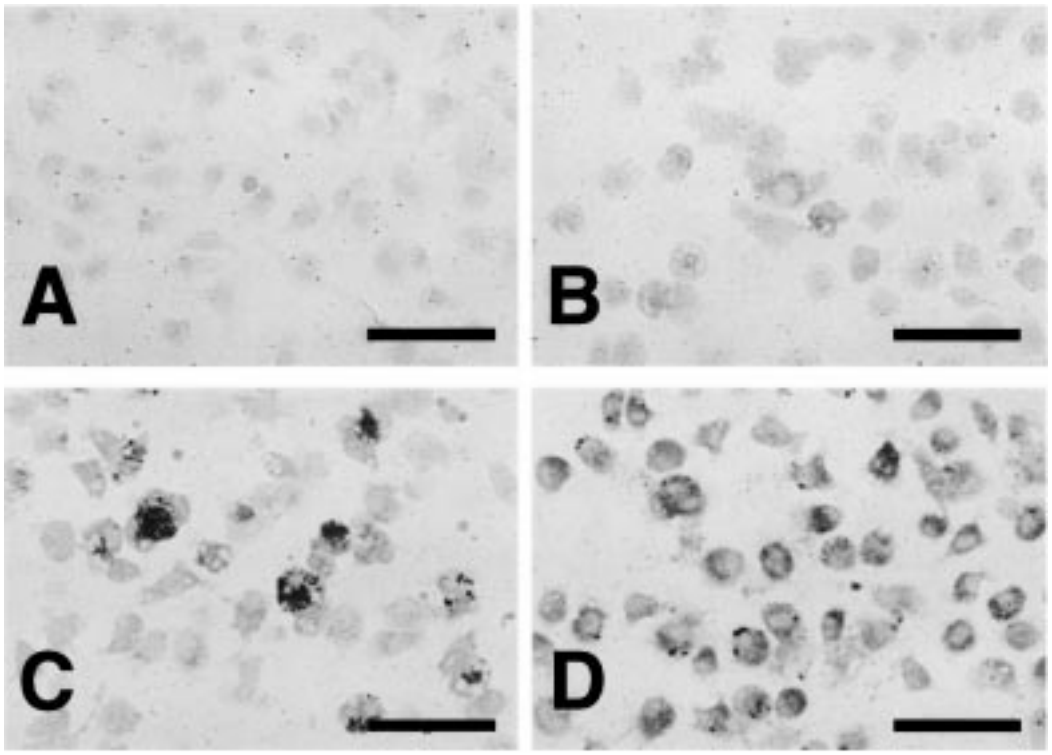

FIGURE 4: Histochemical demonstration of lipid accumulation in lipoprotein-loaded apoE-knockout peritoneal macrophages by the Sudan black B method. Macrophages from apoE-knockout mice were incubated for 18 h with 50 μg/mL LDL, apoEko-VLDL/IDL, or acetyl-LDL. Intracytoplasmic lipid accumulation is demonstrated as gray-black fine granules: (A) nonloaded macrophages, (B) LDL-treated macrophages, (C) apoEko-VLDL/IDL-treated macrophages, and (D) acetyl-LDL-treated macrophages. The scale bar in each micrograph indicates 50 μm.

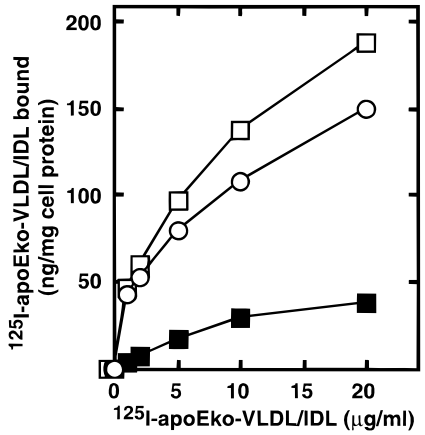

FIGURE 5: Binding of [^{125}I]apoEko-VLDL/IDL to macrophages from apoE-knockout mice. Macrophages from apoE-knockout mice (2×10^6) were incubated for 90 min on ice with increasing concentrations of [^{125}I]apoEko-VLDL/IDL with or without a 20-fold excess of unlabeled apoEko-VLDL/IDL. The cells were washed, and the amount of cell-bound [^{125}I]apoEko-VLDL/IDL was determined as described in Materials and Methods. The level of specific binding (○) was calculated by subtracting the level of nonspecific binding (■) from the level of total binding (□). Data are representative of two separate experiments with triplicate wells. Bars represent the SD, which is very small and within the symbol in data without the error bars.

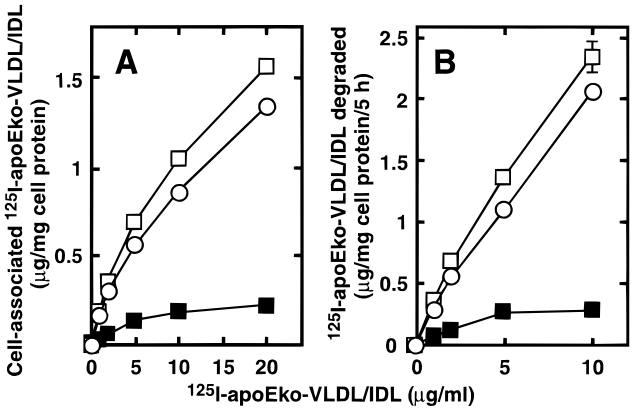

FIGURE 6: Cell association of [^{125}I]apoEko-VLDL/IDL with and subsequent endocytic degradation by macrophages from apoE-knockout mice. Macrophages from apoE-knockout mice (2×10^6) were incubated for 5 h with the indicated concentrations of [^{125}I]-apoEko-VLDL/IDL with or without a 20-fold excess of unlabeled apoEko-VLDL/IDL. The amounts of cell-associated [^{125}I]apoEko-VLDL/IDL (A) and that degraded by the cells (B) were determined as described in Materials and Methods. The levels of specific cell association and degradation (○) were calculated by subtracting the level of nonspecific cell association and degradation (■) from the level of total cell association and degradation (□), respectively. Data are representative of two separate experiments with triplicate wells. SD values are small and within the symbols.

IDL degradation by macrophages, we incubated apoE-knockout macrophages with [^{125}I]apoEko-VLDL/IDL in the presence of acetyl-LDL and LDL. As shown in Figure 7B, the inhibition of degradation of [^{125}I]apoEko-VLDL/IDL by unlabeled apoEko-VLDL/IDL amounted to a maximum of 61%. In contrast, acetyl-LDL showed no inhibitory effect. On the other hand, under these conditions, LDL partly inhibited the degradation of [^{125}I]apoEko-VLDL/IDL by up to 32%. Similar results were obtained for cell association of [^{125}I]apoEko-VLDL/IDL (Figure 7A). These results showed that the uptake pathway of apoEko-VLDL/IDL is independent of that of acetyl-LDL and is partly inhibited by LDL, suggesting the presence of a MSR-independent lysosomal pathway which is partly shared with LDL.

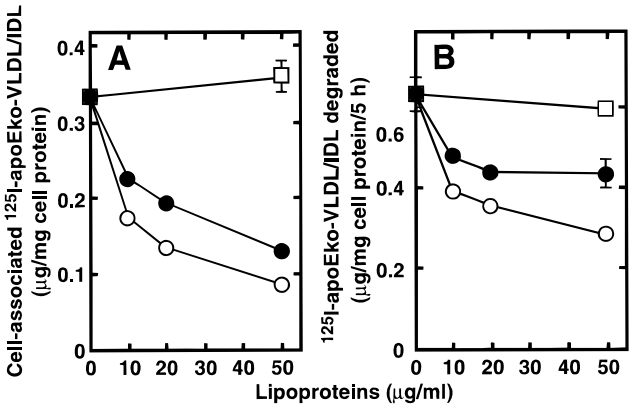

FIGURE 7: Effect of LDL and acetyl-LDL on cell association and degradation of [^{125}I]apoEko-VLDL/IDL by macrophages from apoE-knockout mice. Macrophages from apoE-knockout mice (2×10^6) were incubated for 5 h with 2 μg/mL [^{125}I]apoEko-VLDL/IDL in the presence of the indicated concentrations of acetyl-LDL (□), LDL (●), and apoEko-VLDL/IDL (○). The levels of cell association (A) and subsequent endocytic degradation (B) were determined as described in Materials and Methods. Data are representative of two separate experiments with triplicate wells. Bars represent the SD, which is very small and within the symbol in data points where there are no error bars.

DISCUSSION

The results of this study can be grouped under two major findings. First, apoEko-VLDL/IDL isolated from the plasma of apoE-knockout mice induced significant CE accumulation in peritoneal macrophages obtained from the same mice (Figures 2 and 3). Morphological examination showed that after incubation with apoEko-VLDL/IDL, macrophages exhibited a phenotype typical of lipid-laden foam cells (Figure 4C). Second, binding (Figure 5), cell association (Figure 6A), and degradation (Figure 6B) experiments clearly indicated the presence of high-affinity binding sites on the macrophage cell surface which mediated the uptake and lysosomal degradation of apoEko-VLDL/IDL in an apoE-independent manner. These findings suggest that apoEko-VLDL/IDL may interact with macrophages and transform these cells to foam cells in vivo, thus acting as an atherogenic lipoprotein.

In our previous study, we examined the role of MSR-AI/AII, a representative receptor for acetyl-LDL, in apoE-rich VLDL/IDL-induced macrophage foam cell formation (*38*). For this purpose, macrophages from MSR-AI/AII-knockout mice and their wild-type littermates were incubated with VLDL/IDL isolated from plasma of cholesterol-fed LDL receptor-knockout mice. The results clearly showed that cellular CE accumulation occurred in macrophages from MSR-AI/AII-knockout mice as well as in those from their wild-type littermates, indicating that MSR-AI/AII are not significantly involved in apoE-rich VLDL/IDL-induced foam cell formation. In a parallel experiment, the effect of apoEko-VLDL/IDL was also examined. In a similar fashion, cellular CE accumulation also occurred in macrophages from MSR-AI/AII-knockout mice (data not shown). Thus, it is unlikely that MSR-AI/AII play a significant role in apoEko-VLDL/IDL-induced macrophage foam cell formation.

Recent studies from several laboratories have demonstrated that several membrane proteins function as receptors for triglyceride-rich lipoprotein remnants such as VLDL remnant (IDL) and chylomicron remnant in macrophages (*39*). First, the LDL receptor has been considered as a candidate responsible for the uptake of β-VLDL by mouse peritoneal macrophages (*40*, *41*). Second, the LDL receptor-related protein (LRP) which recognizes apoE-containing lipoproteins as effective ligands (*42*) is expressed by cultured human monocyte-derived macrophages (*43*). The ligand binding of remnant particles to LRP is enhanced by lipoprotein lipase (LPL) in which cell surface heparan sulfate proteoglycans play a significant role (*39*). Third, the VLDL receptor is expressed by macrophages in rabbit and human atherosclerotic lesions (*44*, *45*). These receptors are structurally categorized as members of the LDL receptor gene family, which recognize apoE as an effective ligand. However, because apoEko-VLDL/IDL lacks apoE and peritoneal macrophages obtained from apoE-knockout mice do not produce apoE, the contribution of apoE to our experimental system seems to be negligible. Moreover, apoEko-VLDL/IDL is not rich in triglyceride relative to LDL (Table 1), and its protein components are characteristically rich in apoB48, apoA-I, and apoA-IV (Figure 1B) (*13*, *14*, *37*), indicating that apoEko-VLDL/IDL is physicochemically different from remnant lipoproteins rich in apoE. Taken together, it is unlikely that apoEko-VLDL/IDL directly binds to the LDL receptor, LRP, or the VLDL receptor. However, in fact, endocytic degradation of apoEko-VLDL/IDL was partially inhibited by LDL (Figure 7B). Therefore, we cannot exclude the possibility that a member of the LDL receptor gene family or a receptor closely related to the receptor family is involved in the interaction of apoEko-VLDL/IDL with apoE-knockout macrophages. Further studies are needed to clarify this issue.

Our results demonstrated macrophage uptake of apoEko-VLDL/IDL in an apoE-independent manner. Consistent with this notion, apoE-independent pathways for uptake of remnant particles by macrophages have been proposed by Gianturco et al. (*46*). They found an apoE- and LPL-independent uptake pathway for triglyceride-rich lipoproteins which causes CE accumulation in murine P388D_1 macrophages. Further extensive studies by Ramprasad and coworkers (*47*) showed two major binding proteins with molecular masses of 200 and 235 kDa (MBP 200 and MBP 235, respectively) by using ligand blot analysis of trypsinized VLDL which lacks the ligand activity of apoE. It is therefore possible that these proteins play a role in the uptake of apoEko-VLDL/IDL by macrophages. Another candidate for the receptor for apoEko-VLDL/IDL is the lipolysis-stimulated receptor (LSR) proposed by Yen et al. (*48*). The LSR, activated by oleate and expressed by hepatocytes and LDL receptor-deficient fibroblasts, recognizes VLDL, chylomicron, and LDL as effective ligands. In this study, cell association and subsequent degradation of apoEko-VLDL/IDL were partly inhibited by LDL (Figure 7). Although the expression of LSR in macrophages has not yet been shown, it is expected that the LSR may play a role in apoEko-VLDL/IDL uptake by macrophages. However, because the primary structures of all these candidates (MBP 200, MBP 235, and LSR) have not been identified, further studies would help solve the molecular role of these receptors in apoEko-VLDL/IDL metabolism in macrophages.

One interesting observation in this study would be the heterogeneous response of apoE-knockout macrophages to apoEko-VLDL/IDL (Figure 4C). When the macrophages

were incubated with apoEko-VLDL/IDL, only one-third of the total macrophages were positively stained with Sudan black B, suggesting that one-third of apoE-knockout macrophage subpopulations express functional receptor(s) for apoEko-VLDL/IDL. It is known that tissue macrophages are classified into two different subpopulations according to their origins (for review, see ref *49*): blood monocyte-derived macrophages which are called "exudate macrophages" and "resident macrophages" which are originally derived from hematopoietic stem cells and myeloid precursor cells (GM-CFC) that transmigrated to peripheral tissues in the early stage of bone marrow differentiation. Resident macrophages are characterized by their capacity to proliferate by themselves in peripheral tissues to supply local macrophages (*49*). On the basis of the cytochemical localization pattern of peroxidase activity, these cells are categorized into three differentiation stages (immature macrophages, resident macrophages, and peroxidase-negative macrophages) (*49*). According to these concepts, the restricted expression of the putative apoEko-VLDL/IDL receptor(s) among apoE-knockout macrophages could be explained either by macrophage subpopulations or by a difference in the stage of differentiation. This notion raises an interesting possibility that the expression of the apoEko-VLDL/IDL receptor(s) is used as a marker of a subpopulation or the differentiation stage of macrophages.

Recently, Hendricks et al. (*50*) examined the interaction between VLDL ($d < 1.006$) isolated from the plasma of apoE-knockout mice and murine J774 macrophages. The motivation for their study was quite similar to that for this study; however, the results obtained in the two studies differ considerably. In their study, incubation of J774 cells with 50 μg/mL apoEko-VLDL for 24 h resulted in a 3-fold increase in cellular CE content, compared to a nonloaded control. In contrast, when determined by [^{3}H]oleate incorporation, the cellular CE content in macrophages from apoE-knockout mice was increased 39-fold by incubation with 50 μg/mL apoEko-VLDL/IDL for 18 h (Figure 2). These cells were positively stained with Sudan black B (Figure 4C). Thus, the increase in cellular CE was more dramatic in our study. This difference might be due to differences in cell types, culture conditions, or both. Since both the macrophages and apoEko-VLDL/IDL in our study were prepared from the same mouse strain, it is possible that our results may more closely reflect an in vivo situation. Although the mechanisms leading to the spontaneous development of atherosclerosis in the apoE-knockout mice fed a normal diet are unclear, the results of this study suggest that accumulated VLDL/IDL in their plasma may directly interact with macrophages and transform these cells to foam cells in arterial wall lesions. Further studies are needed to identify the molecular structure of the putative apoEko-VLDL/IDL receptor(s).

ACKNOWLEDGMENT

We are grateful to Drs. Hirofumi Matsuda, Takashi Kawano, Takeshi Biwa, Takashi Kawasaki, and Yuko Hasunuma for their collaborative endeavors during the course of this study. We also thank Dr. F. G. Issa from the Department of Medicine (University of Sydney, Sydney, Australia) for careful reading and editing of the manuscript.

REFERENCES

1. Ross, R. (1993) *Nature 362*, 801−809.
2. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) *N. Engl. J. Med. 320*, 915−924.
3. Mori, T., Takahashi, K., Naito, M., Kodama, T., Hakamata, H., Sakai, M., Miyazaki, A., Horiuchi, S., and Ando, M. (1994) *Lab. Invest. 71*, 409−416.
4. Liscum, L., and Underwood, K. W. (1995) *J. Biol. Chem. 270*, 15443−15446.
5. Chang, T. Y., Chang, C. C. Y., and Cheng, D. (1997) *Annu. Rev. Biochem. 66*, 613−638.
6. Palinski, W., Rosenfeld, M. E., Ylä-Herttuala, S., Gurtner, G. C., Socher, S. S., Butler, S. W., Parthasarathy, S., Carew, T. E., Steinberg, D., and Witztum, J. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1372−1376.
7. Ylä-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L., and Steinberg, D. (1989) *J. Clin. Invest. 84*, 1086−1095.
8. Goldstein, J. L., Ho, Y. K., Brown, M. S., Innerarity, T. L., and Mahley, R. W. (1980) *J. Biol. Chem. 255*, 1839−1848.
9. Mahley, R. W., Innerarity, T. L., Brown, M. S., Ho, Y. K., and Goldstein, J. L. (1980) *J. Lipid Res. 21*, 970−980.
10. Innerarity, T. L., Arnold, K. S., Weisgraber, K. H., and Mahley, R. W. (1986) *Arteriosclerosis 6*, 114−122.
11. Bates, S. R., Coughlin, B. A., Mazzone, T., Borensztajn, J., and Getz, G. S. (1987) *J. Lipid Res. 28*, 787−797.
12. Mahley, R. W. (1988) *Science 240*, 622−630.
13. Zhang, S. H., Reddick, R. L., Piedrahita, J. A., and Maeda, N. (1992) *Science 258*, 486−471.
14. Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setälä, K., Walsh, A., Verstuyft, J. G., Rubin, E. M., and Breslow, J. L. (1992) *Cell 71*, 343−353.
15. Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T., Takashima, Y., Kawabe, Y., Cynshi, O., Wada, Y., Honda, M., Kurihara, H., Aburatani, H., Doi, T., Matsumoto, A., Azuma, S., Noda, T., Toyoda, Y., Itakura, H., Yazaki, Y., Horiuchi, S., Takahashi, K., Kruijt, J. K., van Berkel, T. J. C., Steinbrecher, U. P., Ishibashi, S., Maeda, N., Gordon, S., and Kodama, T. (1997) *Nature 386*, 292−296.
16. Elomaa, O., Kangas, M., Sahlberg, C., Tuukkanen, J., Sormunen, R., Liakka, A., Thesleff, I., Kraal, G., and Tryggvason, K. (1995) *Cell 80*, 603−609.
17. Ramprasad, M. P., Fischer, W., Witztum, J. L., Sambrano, G. R., Quehenberger, O., and Steinberg, D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9580−9584.
18. Stanton, L. W., Tyler White, R., Bryant, C. M., Protter, A. A., and Endemann, G. (1992) *J. Biol. Chem. 267*, 22446−22451.
19. Acton, S. L., Scherer, P. E., Lodish, H. F., and Krieger, M. (1994) *J. Biol. Chem. 269*, 21003−21009.
20. Endemann, G., Stanton, L. W., Madden, K. S., Bryant, C. M., Tyler White, R., and Protter, A. A. (1993) *J. Biol. Chem. 268*, 11811−11816.
21. Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H., Hoshikawa, H., Aiba, Y., Tanaka, T., Miwa, S., Katsura, Y., Kita, T., and Masaki, T. (1997) *Nature 386*, 73−77.
22. Adachi, H., Tsujimoto, M., Arai, H., and Inoue, K. (1997) *J. Biol. Chem. 272*, 31217−31220.
23. Murakami, M., Horiuchi, S., Takata, K., and Morino, Y. (1987) *J. Biochem. (Tokyo) 101*, 729−741.
24. McFarlane, A. S. (1958) *Nature 182*, 53.
25. Miyazaki, A., Sakai, M., Suginohara, Y., Hakamata, H., Sakamoto, Y., Morikawa, W., and Horiuchi, S. (1994) *J. Biol. Chem. 269*, 5264−5269.
26. Ohta, T., Takata, K., Horiuchi, S., Morino, Y., and Matsuda, I. (1989) *FEBS Lett. 257*, 435−438.
27. Kawano, T., Hakamata, H., Ohta, T., Ding, Y., Yoshida, M., Ueda, S., and Horiuchi, S. (1997) *Biochemistry 36*, 9816−9825.
28. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) *Methods Enzymol. 98*, 241−260.
29. Heider, J. G., and Boyett, R. L. (1978) *J. Lipid Res. 19*, 514−518.

30. Pearse, A. G. E. (1985) in *Histochemistry: Theoretical and Applied*, 4th ed., Vol. 2, pp 786−849, Churchill Livingstone, London.
31. Hakamata, H., Miyazaki, A., Sakai, M., Matsuda, H., Suzuki, H., Kodama, T., and Horiuchi, S. (1998) *J. Lipid Res. 39*, 482−494.
32. Sakai, M., Miyazaki, A., Hakamata, H., Kodama, T., Suzuki, H., Kobori, S., Shichiti, M., and Horiuchi, S. (1996) *J. Biol. Chem. 271*, 27346−27352.
33. Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W., and Fu, P. C. (1974) *Clin. Chem. 20*, 470−475.
34. Takayama, M., Itoh, S., Nagasaki, T., and Tanimizu, I. (1977) *Clin. Chim. Acta 79*, 93−98.
35. Spayd, R. W., Bruschi, B., Burdick, B. A., Dappen, G. M., Eikenberry, J. N., Esders, T. W., Figueras, J., Goodhue, C. T., LaRossa, D. D., Nelson, R. W., Rand, N. R., and Wu, T.-W. (1978) *Clin. Chem. 24*, 1343−1350.
36. Ishibashi, S., Perrey, S., Chen, Z., Osuga, J., Shimada, M., Ohashi, K., Harada, K., Yazaki, Y., and Yamada, N. (1996) *J. Biol. Chem. 271*, 22422−22427.
37. Ishibashi, S., Herz, J., Maeda, N., Goldstein, J. L., and Brown, M. S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4431−4435.
38. Sakaguchi, H., Takeya, M., Suzuki, H., Hakamata, H., Kodama, T., Horiuchi, S., Gordon, S., van der Laan, J. W., Kraal, G., Ishibashi, S., Kitamura, N., and Takahashi, K. (1998) *Lab. Invest. 78*, 423−434.
39. Beisiegel, U. (1995) *Curr. Opin. Lipidol. 6*, 117−122.
40. Koo, C., Wernette-Hammond, E. M., and Innerarity, T. L. (1986) *J. Biol. Chem. 261*, 11194−11201.
41. Ellsworth, J. L., Kraemer, F. B., and Cooper, A. D. (1987) *J. Biol. Chem. 262*, 2316−2325.
42. Beisiegel, U., Weber, W., Ihrke, G., Herz, J., and Stanley, K. K. (1989) *Nature 341*, 162−164.
43. Watanabe, Y., Inaba, T., Shimano, H., Gotoda, T., Yamamoto, K., Mokuno, H., Sato, H., Yazaki, Y., and Yamada, N. (1994) *Arterioscler. Thromb. 14*, 1000−1006.
44. Nakazato, K., Ishibashi, T., Shindo, J., Shiomi, M., and Maruyama, Y. (1996) *Am. J. Pathol. 149*, 1831−1838.
45. Multhaupt, H. A., Gafvels, M. E., Kariko, K., Jin, H., Arenas-Elliot, C., Goldman, B. I., Strauss, J. F., III, Angelin, B., Warhol, M. J., and McCrae, K. R. (1996) *Am. J. Pathol. 148*, 1985−1997.
46. Gianturco, S. H., Brown, S. A., Via, D. P., and Bradley, W. A. (1986) *J. Lipid Res. 27*, 412−420.
47. Ramprasad, M. P., William, R. L., Bradley, A., and Gianturco, S. H. (1995) *Biochemistry 34*, 9126−9135.
48. Yen, F. T., Mann, C. J., Guermani, L. M., Hanouche, N. F., Hubert, N., Hornick, C. A., Bordeau, V. N., Agnani, G., and Bihain, B. (1994) *Biochemistry 33*, 1172−1180.
49. Takahashi, K., Naito, M., and Takeya, M. (1996) *Pathol. Int. 46*, 473−485.
50. Hendriks, W. L., van der Sman-de, B., van Vlijimen, B. J. M., van Vark, L. C., Hofker, M. H., and Havekes, L. M. (1997) *Arterioscler. Thromb. Vasc. Biol. 17*, 498−504.

BI980762V