Effects of cholesterol in chylomicron remnant models of lipid emulsions on apoE-mediated uptake and cytotoxicity of macrophages

Atsushi Sakurai, Shin-ya Morita, Kyoko Wakita, Yuko Deharu, Minoru Nakano, and Tetsurou Handa[1]

Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

Abstract **Chylomicron remnants have been suggested to be involved in the development of atherosclerosis. To investigate the mechanisms of chylomicron remnant-induced atherosclerosis, we prepared cholesterol (Chol)-containing emulsion particles as models for chylomicron remnants. Chol markedly increased the apolipoprotein E (apoE) binding maximum of emulsions without changing the binding affinity and thereby promoted emulsion uptake by J774 macrophages. Fluorescence measurements showed that Chol increased acyl chain order and head group hydration of the surface phospholipid (PL) layer of emulsions. The binding maximum of apoE was closely correlated with the hydration and the increase in the PL head group separation at the emulsion surface. From experiments using inhibitors for lipoprotein receptors, heparan sulfate proteoglycans and low density lipoprotein receptor-related protein were found to be the major contributors to the uptake of Chol-containing emulsions. Trypan blue dye exclusion revealed that the uptake of Chol-containing emulsions induced cytotoxicity to J774 macrophages. This study proposes a mechanism of atherosclerosis induced by chylomicron remnants.**—Sakurai, A., S. Morita, K. Wakita, Y. Deharu, M. Nakano, and T. Handa. **Effects of cholesterol in chylomicron remnant models of lipid emulsions on apoE-mediated uptake and cytotoxicity of macrophages.** ***J. Lipid Res.*** **2005.** 46: **2214–2220.**

Supplementary key words apolipoprotein E • J774 macrophages • phosphatidylcholine head group separation

In the postprandial state, nascent chylomicrons are secreted into the lymphatic system and enter the circulation via the thoracic duct. In the capillary beds of peripheral tissues, chylomicron remnants are formed after the lipoprotein lipase-mediated hydrolysis of triglycerides (1, 2). It has been suggested that chylomicron remnants are related to the development of atherosclerosis for the following reasons: hyperchylomicronemia leads to premature atherosclerosis, although cases show low levels of LDL (3), and remnant-like particles containing apolipoprotein E (apoE) have been isolated from human aortic intima and atherosclerotic plaque (4, 5). It has also been demonstrated that chylomicron remnants are able to penetrate to the arterial wall, are retained in the subendothelial space (6), and induce the foam cell formation of macrophages (7) and apoptosis in endothelial cells (8). However, the mechanisms of chylomicron remnant-promoted atherosclerosis are not clear.

Chylomicron remnants are rich in apoE, which plays a crucial role in lipoprotein metabolism through specific interactions with cell membrane receptors (9, 10). Monocyte-derived macrophages play an important role in the formation of atherosclerotic lesions. The lesions are initiated by the formation of fatty streaks in the artery, when macrophages in the vessel wall take up lipoproteins from the subendothelial space (11, 12). Chylomicron remnants are metabolized by monocyte-derived macrophages (13, 14). Free cholesterol (Chol) is a major surface lipid of chylomicron remnants (15). Macrophages accumulate Chol in advanced atherosclerotic lesions, resulting in macrophage apoptosis and the progression of the lesions (16). Chol loading induces downstream apoptotic events in cultured macrophages (17, 18). It has been demonstrated that chylomicron remnants are cytotoxic to macrophages and smooth muscle cells (7, 19), but no studies have reported the effect of Chol in chylomicron remnants on the viability of macrophages.

Several studies have shown that the lipid composition of lipoproteins and lipid emulsions influences their meta-

Manuscript received 28 April 2005 and in revised form 26 May 2005.

Published, JLR Papers in Press, June 1, 2005.
DOI 10.1194/jlr.M500167-JLR200

Abbreviations: apoE, apolipoprotein E; Chol, cholesterol; dansyl-PE, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)dipalmitoylphosphatidylethanolamine; DPH-PC, 1-palmitoyl-2-[3-(diphenylhexatrienyl)propionyl]-*sn*-3-phosphatidylcholine; HSPG, heparan sulfate proteoglycan; LRP, low density lipoprotein receptor-related protein; PC, phosphatidylcholine; PL, phospholipid; PMC-oleate, 1-pyrenemethyl 3β-(*cis*-9-octadecenoyloxy)-22,23-bisnor-5-cholenate; SM, sphingomyelin; TO, triolein.

[1] To whom correspondence should be addressed.
e-mail: handatsr@pharm.kyoto-u.ac.jp

bolic properties (20–22). The amount of Chol plays an important role in chylomicron metabolism. The regulation of the metabolic behavior is thought to arise from Chol's effect on the binding of apolipoproteins to the particle surface (23). Our previous study demonstrated that surface Chol in triglyceride-phosphatidylcholine (PC) emulsions decreased the binding capacity of apoA-I without changing the binding affinity (24).

In this study, to investigate the mechanisms of chylomicron remnant-induced atherosclerosis, we used Chol-containing emulsion particles as models for chylomicron remnants and evaluated the effects of Chol enrichment on apoE-mediated emulsion uptake by J774 macrophages and the resulting cytotoxicity. We also examined the influence of Chol enrichment on the physical state of phospholipid (PL) layers at the emulsion surface by fluorescence measurements.

EXPERIMENTAL PROCEDURES

Materials

Recombinant human apoE (isoform E3) was provided by Pepro Tech EC Ltd. (London, UK). Egg yolk PC was generously provided by Asahi Kasei Co. (Tokyo, Japan). Triolein (TO), heparin, Chol, egg yolk sphingomyelin (SM), and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Pyrenemethyl 3β-(*cis*-9-octadecenoyloxy)-22,23-bisnor-5-cholenate (PMC-oleate) and 1-palmitoyl-2-[3-(diphenylhexatrienyl)propionyl]*sn*-3-phosphatidylcholine (DPH-PC) were obtained from Molecular Probes (Eugene, OR). *N*-(5-Dimethylaminonaphthalene-1-sulfonyl)dipalmitoylphosphatidylethanolamine (dansyl-PE) was obtained from Avanti Polar Lipids (Alabaster, AL). Bovine lactoferrin and trypan blue were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals used were of the highest reagent grade.

Preparation of emulsions

Two types of emulsions, Chol-free (TO-PC) emulsion and Chol-containing (TO-PC/Chol, with a molar ratio of PC/Chol = 3:2) emulsion, were prepared using a high-pressure emulsifier (Nanomizer System YSNM-2000AR; Yoshida Kikai Co., Nagoya, Japan) as described previously (25). Briefly, a mixture of TO, PC, and Chol was suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 0.01% NaN_3 and successively emulsified under 100 MPa of pressure at 40–60°C. After the removal of contaminating vesicles by ultracentrifugation, homogenous emulsion particles were obtained. The mean particle diameter of TO-PC and TO-PC/Chol emulsions was ~120 nm, determined from dynamic light-scattering measurements using Photal LPA-3000/3100 (Otsuka Electronic Co., Osaka, Japan). The concentrations of PL, TO, and Chol were determined using enzymatic assay kits purchased from Wako Pure Chemicals. We confirmed that the surface PC and Chol composition of isolated emulsions was the same as that of the starting lipid mixtures (PC/Chol = 3:2) and that isolated emulsions contained the expected amount of Chol. The molar ratios of core TO to total surface PL for isolated TO-PC and TO-PC/Chol emulsions were 4.95 ± 0.07 and 5.21 ± 0.13, respectively.

Cell cultures

J774 macrophages were grown in a humidified incubator (5% CO_2) at 37°C in DMEM supplemented with 10% heat-inactivated FBS, L-glutamine, penicillin, and streptomycin. The FBS was replaced with 1% BSA 15 min before each experiment. Experiments were performed in DMEM containing 1% BSA.

Cell uptake assays

The cells were incubated with PMC-oleate-labeled emulsions (250 μM TO) at 37°C for 2 h. For experiments with apoE, PMC-oleate-labeled emulsions were preincubated with apoE at 37°C for 30 min, allowing sufficient time for equilibrium binding (26). After incubation, the cells were chilled on ice and washed twice with cold Hepes buffer containing 0.2% BSA and then washed twice with cold Hepes buffer alone. Cells were then dissolved in 0.2% Triton X-100. The fluorescence intensity of PMC-oleate (excitation, 342 nm; emission, 377 nm) measured with a Hitachi F-4500 spectrofluorometer and protein concentration were determined by the method of Lowry et al. (27) to calculate particle uptake.

Cell viability assay

Cell viability was assayed by trypan blue dye exclusion (28). The cells were incubated with emulsions (5 mM TO) at 37°C for 18 h. After incubation, emulsions were removed and replaced with 0.4% trypan blue solution. Macrophages were immediately examined for trypan blue uptake using an Olympus IX70 inverted microscope.

ApoE binding studies

ApoE binding assays were performed in Tris-HCl buffer (pH 7.4) according to the method described previously (24). Briefly, after the incubation of emulsions with recombinant apoE for 30 min at 37°C, the mixtures were subjected to ultracentrifugation to separate both emulsions and lipid-bound apoE from free apoE. The free apoE concentration was determined by measuring tryptophan fluorescence at 335 nm (excited at 280 nm) with a Hitachi F-4500 spectrofluorometer. The amount of lipid-bound apoE was calculated by subtracting the background amount of free apoE obtained in the top fraction from the amount found by centrifugation of lipid-free apoE solution (26). From the binding isotherms of apoE to emulsions, the binding parameters dissociation constant (K_d) and binding maximum (N) were obtained.

Fluorescence measurements

For fluorescence measurements, emulsions were prepared with the fluorophore to yield probe/PL ratios of 1:200 for DPH-PC and 1:100 for dansyl-PE. Measurements of steady-state fluorescence anisotropy of DPH-PC were performed on a Hitachi F-4500 spectrofluorometer with an excitation wavelength of 360 nm and an emission wavelength of 434 nm. Measurements of the fluorescence lifetime of dansyl-PE were carried out with a Horiba multichannel-TAC NAES-550 system. Dansyl-PE was excited through a Hoya U350 bandpass filter, and emission was observed through a Hoya Y48 cutoff filter.

Statistical analysis

Differences between two group means were determined using the nonpaired *t*-test, and differences among multiple group means were determined using the Bonferroni test. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of apoE on emulsion uptake by J774 macrophages

We examined the contribution of apoE to the uptake of TO-PC and TO-PC/Chol emulsions by J774 macrophages.

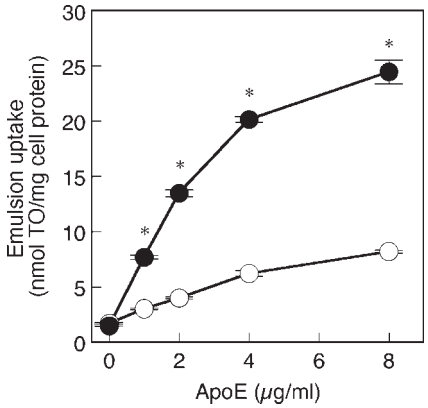

Fig. 1. Effect of apolipoprotein E (apoE) on the uptake of emulsion particles by J774 macrophages. J774 cells were incubated for 2 h at 37°C with triolein-phosphatidylcholine emulsions (TO-PC; open circles) or TO-PC/cholesterol (Chol) (3:2) emulsions (closed circles) (250 μM TO) in the absence or presence of the indicated amount of apoE. The mean particle diameter of the emulsions was ~120 nm. Values shown are means ± SEM of triplicate measurements. * Significantly different from TO-PC emulsions ($P < 0.05$).

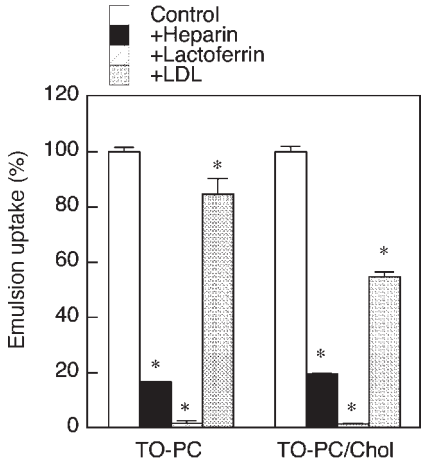

Fig. 2. Effect of inhibitors on the uptake of emulsions by J774 macrophages. J774 macrophages were incubated for 2 h at 37°C with TO-PC or TO-PC/Chol (3:2) emulsions (250 μM TO) with 4 μg/ml apoE in the absence (control; white bars) or presence of 100 μg/ml heparin (black bars), 5 mg/ml lactoferrin (light gray bars), and LDL containing 100 μg/ml apoB (dark gray bars). The mean particle diameter of the emulsions was ~120 nm. The results are expressed as percentages of the emulsion uptake measured in cells incubated with no inhibitors. Values shown are means ± SEM of triplicate measurements. * Significantly different from control ($P < 0.05$).

As shown in **Fig. 1**, the cellular uptake of both emulsions increased with the apoE concentration. In the absence of apoE, the uptake of TO-PC/Chol emulsions was similar to that of TO-PC emulsions. In the presence of apoE, the uptake of TO-PC/Chol emulsions was significantly higher than that of TO-PC emulsions.

To determine which lipoprotein receptors on J774 macrophages are involved in the interaction with emulsion particles, we performed experiments using inhibitors of lipoprotein receptors. Cell-surface heparan sulfate proteoglycans (HSPGs) and low density lipoprotein receptor-related protein (LRP) play a critical role in apoE-enriched remnant uptake. Remnants interact with HSPGs and are either transferred to the LRP for internalization or taken up directly with the HSPG-LRP complex (10). We tested whether cell surface HSPGs and LRP are involved in apoE-mediated emulsion uptake by incubating J774 macrophages with emulsions in the presence of 100 μg/ml heparin or 5 mg/ml lactoferrin (**Fig. 2**). Heparin, which is known to impede the binding of apoE-containing lipoproteins with HSPGs (29–32), significantly reduced the apoE-mediated uptake of TO-PC and TO-PC/Chol emulsions. Lactoferrin, which blocks LRP and interferes with the HSPG-mediated remnant uptake (33, 34), significantly inhibited the apoE-mediated uptake of TO-PC and TO-PC/Chol emulsions. It has been reported that the uptake of chylomicron remnants by macrophages is mediated primarily by the LDL receptor (35). We also examined whether the LDL receptor participates in apoE-mediated emulsion uptake by incubating J774 macrophages with emulsions in the presence of LDL containing 100 μg/ml apoB-100. As shown in Fig. 2, LDL reduced the apoE-mediated uptake of TO-PC and TO-PC/Chol emulsions. However, the degree of inhibition by LDL was small compared with that of heparin or lactoferrin. Consequently, the HSPG-LRP pathway was presumed to be a major contributor to the apoE-mediated uptake of TO-PC and TO-PC/Chol emulsions.

Cytotoxicity of emulsion particles to J774 macrophages

We explored J774 macrophage viability when cells were incubated with TO-PC or TO-PC/Chol emulsions. **Figure 3** shows J774 macrophage death ascertained by trypan blue dye exclusion after 18 h of incubation. There was no

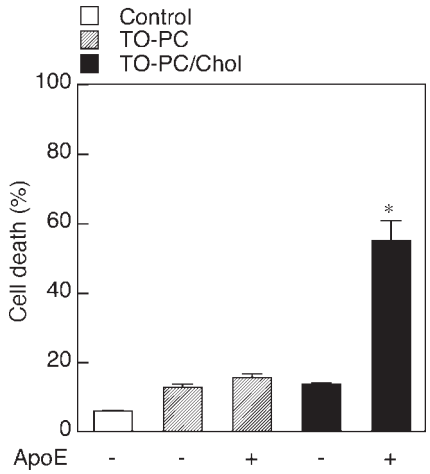

Fig. 3. J774 macrophage death in the presence of emulsion particles. J774 macrophages were incubated for 18 h at 37°C in the absence and presence of 8 μg/ml apoE without emulsions (control; open bars) or with TO-PC (hatched bars) or TO-PC/Chol (3:2) emulsions (closed bars) (5 mM TO). The cellular death (or viability) of J774 macrophages was determined using trypan blue dye exclusion. Values shown are means ± SEM of triplicate measurements. * Significantly different from control, TO-PC with or without apoE, and TO-PC/Chol without apoE ($P < 0.05$).

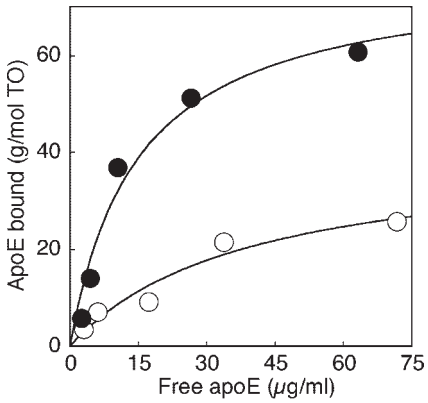

Fig. 4. Binding isotherms of apoE to TO-PC (open circles) and TO-PC/Chol (3:2) emulsions (closed circles). Amounts of bound apoE are represented as a function of the free protein concentration (n = 2).

significant change in viability for up to 18 h of exposure to TO-PC emulsions with or without apoE. On the other hand, in TO-PC/Chol emulsions, apoE markedly increased cell death: ~55% of J774 macrophages died. Incubation with only apoE did not affect J774 macrophage viability (data not shown).

ApoE binding to emulsion particles

To examine the effect of surface Chol on the binding of apoE to the emulsion surface, we performed binding analysis of apoE using a centrifugation assay. **Figure 4** shows the binding isotherms of apoE to TO-PC and TO-PC/Chol emulsions. Although apoE evidently bound to both particles in a saturable manner, replacement of surface PC by Chol markedly increased the amount of apoE binding. From the binding data, the dissociation constant K_d and the binding maximum N were obtained (**Table 1**). Chol in the emulsion surface increased the binding capacity of apoE significantly without changing the affinity. Based on our previous finding on the relationship between cell uptake and the amount of bound apoE in lipid particles (22), it is suggested that the increased uptake of Chol-containing emulsions was attributable to the increase in bound apoE.

Effects of Chol on acyl chain order and head group hydration at the emulsion surface

To investigate the effects of Chol on the surface properties of lipid emulsions governing apoE binding, we performed fluorescence measurements with DPH-PC and dansyl-PE. **Table 2** shows the results of steady-state fluorescence anisotropy of DPH-PC and the mean fluorescence lifetime of dansyl-PE. The anisotropy value at the TO-PC/Chol emulsion surface was much higher than that at the TO-PC emulsion surface, reflecting the fact that the acyl chain region of the TO-PC/Chol emulsion surface layer is more rigid. The mean fluorescence lifetime of dansyl-PE was used to evaluate PC head group hydration at the lipid particle surface. Dansyl fluorophores are located at the PC head group region in membranes (36, 37), and the lifetime of the excited state reflects their local polarity, to which water is the major contributing factor (38). The mean fluorescence lifetime in TO-PC/Chol emulsions is shorter than that in TO-PC emulsions, indicative of an increase in head group hydration at the TO-PC/Chol emulsion surface.

TABLE 1. Binding parameters of apoE to emulsions at 37°C

Emulsions	K_d	N
	μg/ml	*g/mol TO*
TO-PC	38.6 ± 20.1	77.3 ± 9.0
TO-PC/Chol	14.9 ± 4.7	40.5 ± 10.2

apoE, apolipoprotein E; Chol, cholesterol; K_d, dissociation constant; N, binding maximum; PC, phosphatidylcholine; TO, triolein. Errors for K_d and N values were estimated from regression analysis.

TABLE 2. Fluorescence parameters of emulsions at 37°C

Emulsions	Mean Fluorescence Lifetime of Dansyl-PE	Steady-State Fluorescence Anisotropy of DPH-PC
	ns	
TO-PC	13.45 ± 0.09	0.156 ± 0.001
TO-PC/Chol	12.52 ± 0.06	0.203 ± 0.004

Dansyl-PE, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)dipalmitoylphosphatidylethanolamine; DPH-PC, 1-palmitoyl-2-[3-(diphenylhexatrienyl)propionyl]-*sn*-3-phosphatidylcholine. Values shown are means ± SEM from three independent experiments.

Correlation between emulsion surface structure and apoE binding maximum

We next investigated the correlation between the emulsion surface structure and the apoE binding maximum in TO-PC, TO-PC/Chol, and TO-PC/SM emulsions. We have shown that incorporation of one of the major lipids of lipoprotein, SM, into the emulsion surface reduces the apoE binding maximum (21). We first examined the relationship of apoE binding maximum with steady-state fluorescence anisotropy of DPH-PC, but there was no appreciable correlation (**Fig. 5A**). Next, we investigated the correlation with the mean fluorescence lifetime of dansyl-PE. The lifetime reflects local polarity or hydration between PC head groups at the surface layer (21). The negative correlation between the apoE binding maximum and the lifetime (Fig. 5B) indicated that the amount of hydration between PC polar head groups [i.e., separation between the head groups (39)] plays a crucial role in apoE binding.

DISCUSSION

The Chol/PC ratio of rat lymph chylomicrons is 0.15–0.3. As has been reported, if the Chol/PC ratio is >0.15–0.3, the emulsion behaves as remnant-like particles (23) and mimics the action of chylomicron remnants. Windler, Preyer, and Greten (40) have shown that the Chol/PL ratio of the remnant fraction (d = 1.009–1.016 g/ml) of human serum in the postprandial state is close to 1. In the present study, the Chol/PC ratio of Chol-containing emul-

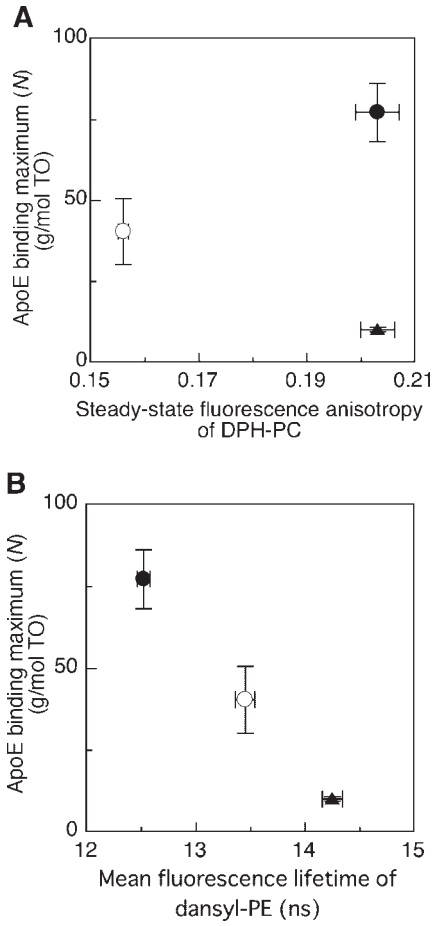

Fig. 5. Relationship of apoE binding maximum (*N*) with steady-state fluorescence anisotropy of 1-palmitoyl-2-[3-(diphenylhexatrienyl)propionyl]-*sn*-3-phosphatidylcholine (DPH-PC; A) or mean fluorescence lifetime of *N*-(5-dimethylaminonaphthalene-1-sulfonyl)dipalmitoylphosphatidylethanolamine (dansyl-PE; B). Open circles, TO-PC emulsions; closed circles, TO-PC/Chol emulsions; closed triangles, TO-PC/sphingomyelin emulsions. Error bars refer to Tables 1 and 2.

sion is 2:3 and can be applied as a chylomicron remnant model. Our results showed that Chol enrichment on the particle surface leads to PC head group separation and hydration (Table 2). Similar effects of Chol have been reported for PC vesicles (24, 39). We showed a correlation between the degree of PC head group hydration and the apoE binding maximum (Fig. 5B). We have also reported a close relationship between the degree of PC head group hydration or separation at the lipid particle surface and the apoA-I binding maximum (39). Both apoE and apoA-I have several amphipathic helical segments that are thought to be responsible for lipid binding. It has been shown that lipid binding of apoE induces conformational opening in the N-terminal domain, which exposes the hydrophobic faces of four helices, permitting their direct contact with the lipid surface (41). Such a conformation of apoE would lead to large packing stress at the lipid surface layers, and it is believed that the inverse wedge-shaped lipid, Chol, at the emulsion surface releases the stress and increases the number of binding sites for apoE. In addition, our data demonstrated that the acyl chain packing is not a determining factor in apoE binding (Fig. 5A). It is suggested that apoE binds to TO-PC/Chol emulsions through insertion between the PC head groups but does not penetrate deeply into the hydrocarbon interior.

The major change in protein composition during the conversion of chylomicrons to chylomicron remnants is the acquisition of apoE. It has been reported that the Chol/triglyceride ratio is increased by 17-fold in chylomicron remnants compared with chylomicrons (8). Therefore, chylomicron remnants are Chol- and apoE-enriched particles created by lipolysis. The Chol enrichment on the chylomicron remnant surface may contribute to the acquisition of apoE by increasing separation between the PC head groups.

Chylomicrons are too large to infiltrate into the vessel wall across the endothelial layer, but the diameter of chylomicron remnants is small enough to penetrate the endothelium (6). One of the important events in the development of atherosclerosis is the uptake of chylomicron remnants by macrophages. When macrophages take up chylomicron remnants, the cells are transformed to foam cells and secrete toxic oxidizing agents (7). Our data demonstrate that apoE promotes the J774 macrophage uptake of TO-PC/Chol emulsions as a result of the increased number of apoE bindings.

The uptake of chylomicron remnants by monocytes and macrophages could be mediated through several pathways. These include the LDL receptor (35), other members of the LDL receptor gene family, including the LRP and the very low density lipoprotein receptor (42), the scavenger receptor family, and the human triglyceride-rich lipoprotein receptor (43). It is also reported that the degradation of chylomicron remnants by macrophages occurs via phagocytosis (7). Recently, a macrophage receptor for apoB-48 was cloned (44). Our findings in this work demonstrate that the HSPG-LRP pathway plays an important role in apoE-mediated emulsion uptake by J774 macrophages. HSPGs act as potential receptors for atherogenic lipoproteins or facilitate their uptake by ligand transfer to LRP (45). LRP is a multiligand receptor that binds apoE-containing remnant lipoproteins and other proteins and is less strictly regulated by intracellular lipid level than the LDL receptor (46). Therefore, it is suggested that HSPG-LRP routes perform a major role in chylomicron remnant-induced atherosclerosis.

ApoE is expressed within atherosclerotic lesions. Although it can enter the artery wall from the periphery, a large fraction of the protein is synthesized locally by resident macrophages, as indicated by the abundant apoE mRNA detected in lesions of both humans and rabbits (47). The role of apoE in atherosclerosis is still controversial: apoE is proatherogenic by promoting macrophage uptake and the degradation of Chol-rich lipoproteins (48), or atheroprotective by facilitating cellular Chol efflux (49) and inhibiting lipoprotein aggregation induced by sphingomyelinase (50). Because J774 macrophages do not synthesize apoE (51), it is not necessary to take account of the influence of endogenous apoE. Our findings

indicate that apoE increases the uptake of atherogenic Chol-rich particles by macrophages and that these particles possibly play a role as a proatherogenic factor.

In more advanced lesions, the death of cells occurs, and this produces necrotic cores of atherosclerotic plaque (52). Several studies have suggested that remnants of postprandial lipoproteins are cytotoxic to macrophages and smooth muscle cells. Chung et al. (19) demonstrated that large quantities of nonesterified fatty acids liberated after lipolysis are the principal cytotoxic component of remnant lipoproteins. Yu and Mamo (7) found that in macrophages, chylomicron remnants are internalized via a phagocytosis-like process and that a consequence of this pathway is a respiratory burst and free radical production. These free radicals are conjectured to bring about the cytotoxicity of chylomicron remnants. On the other hand, it has been established that excess free Chol is toxic to cells (53). Cells, including macrophages, have several mechanisms to prevent the accumulation of excess free Chol, such as Chol esterification by ACAT (54) and cellular efflux of Chol (55). When one or more of these mechanisms do not work well, cell death follows, as seen in advanced atherosclerotic lesions. Feng et al. (18) have reported that excess cellular Chol induces apoptosis in macrophages by unfolded protein response induction in the endoplasmic reticulum. Hence, a possible explanation for TO-PC/Chol emulsion-induced cytotoxicity may be the induction of apoptosis in macrophages. Other mechanisms of cellular toxicity associated with excess Chol accumulation include intracellular Chol crystallization (56). Intracellular Chol crystals can probably damage cells by physically disrupting the integrity of intracellular structures. An alternative mechanism of TO-PC/Chol emulsion-induced cytotoxicity may be this crystallization in macrophages.

In conclusion, an enhancement in the apoE binding capacity of Chol-containing emulsions, caused by an increase in PC head group separation at the emulsion surface, promotes HSPG- and LRP-mediated uptake by macrophages. Furthermore, this apoE-mediated uptake of Chol-containing emulsion induces cellular toxicity to macrophages. We presume that, by a mechanism similar to that of Chol-rich emulsions, chylomicron remnants play a role in the development of atherosclerosis.

This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (Grant 17390011) and by the Program for the Promotion of Fundamental Studies in Health Science of the Pharmaceutical and Medical Devices Agency (Grant 04-8).

REFERENCES

1. Redgrave, T. G. 2004. Chylomicron metabolism. *Biochem. Soc. Trans.* **32:** 79–82.
2. Goldberg, I. J., Y. Kako, and E. P. Lutz. 2001. Responses to eating: lipoproteins, lipolytic products and atherosclerosis. *Curr. Opin. Lipidol.* **12:** 297–304.
3. Benlian, P., P. L. De Gennes, L. Foubert, H. Zhang, S. E. Gagne, and M. Hayden. 1996. Premature atherosclerosis in patients with familial chylomicronemia caused by mutation in the lipoprotein lipase gene. *N. Engl. J. Med.* **335:** 848–854.
4. Yla-Herttuala, S., O. Jaakkola, C. Enholm, M. J. Tikkanen, T. Solakivi, T. Sarkioja, and T. Nikkari. 1988. Characterization of two lipoprotein containing apolipoproteins B and E from lesion-free human aortic intima. *J. Lipid Res.* **29:** 563–572.
5. Rapp, J. H., A. Lespine, R. L. Hamilton, N. Colyvas, A. H. Chaumeton, J. Tweedie-Harman, L. Kotite, S. T. Kunitake, R. J. Havel, and J. P. Kane. 1994. Triglyceride-rich lipoproteins isolated by selective affinity anti-apolipoprotein B immunosorption from human atherosclerotic plaque. *Arterioscler. Thromb.* **14:** 1767–1774.
6. Proctor, S. D., D. F. Vine, and J. C. L. Mamo. 2002. Arterial retention of apolipoprotein B48- and B100-containing lipoproteins in atherogenesis. *Curr. Opin. Lipidol.* **13:** 461–470.
7. Yu, K. C., and J. C. L. Mamo. 2000. Chylomicron remnant-induced foam cell formation and cytotoxicity: a possible mechanism of cell death in atherosclerosis. *Clin. Sci.* **98:** 183–192.
8. Kawasaki, S., T. Taniguchi, Y. Fujioka, A. Takahashi, T. Takahashi, K. Domoto, M. Taguchi, Y. Ishikawa, and M. Yokoyama. 2000. Chylomicron remnant induces apoptosis in vascular endothelial cells. *Ann. N. Y. Acad. Sci.* **902:** 336–341.
9. Yamamoto, T., and H. Bujo. 1996. Close encounters with apolipoprotein E receptor. *Curr. Opin. Lipidol.* **7:** 298–302.
10. Mahley, R. W., and Z-S. Ji. 1999. Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. *J. Lipid Res.* **40:** 1–16.
11. Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* **362:** 801–809.
12. Li, A. C., and C. K. Glass. 2002. The macrophage foam cell as a target for therapeutic intervention. *Nat. Med.* **8:** 1235–1242.
13. Van Lenten, B. J., A. M. Fogelman, R. L. Jackson, S. Shapiro, and P. A. Edwards. 1985. Receptor-mediated uptake of remnant lipoproteins by cholesterol-loaded human monocyte-macrophages. *J. Biol. Chem.* **260:** 8783–8788.
14. Koo, C., M. E. Wernett-Hammond, Z. Garcia, M. J. Malloy, R. Uauy, C. East, D. Bilheimer, R. W. Mahley, and T. L. Innerarity. 1988. Uptake of cholesterol-rich remnant lipoproteins by human monocyte-derived macrophages is mediated by low density lipoprotein receptors. *J. Clin. Invest.* **81:** 1332–1340.
15. Redgrave, T. G. 1983. Formation and metabolism of chylomicrons. *Int. Rev. Physiol.* **28:** 103–130.
16. Tabas, I. 2002. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J. Clin. Invest.* **110:** 905–911.
17. Kellner-Weibel, G., W. G. Jerome, D. M. Small, G. J. Warner, J. K. Stoltenborg, M. A. Kearney, M. H. Corjay, M. C. Philips, and G. H. Rothblat. 1998. Effects of intracellular free cholesterol accumulation on macrophage viability: a model for foam cell death. *Arterioscler. Thromb. Vasc. Biol.* **18:** 423–431.
18. Feng, B., P. M. Yao, Y. Li, C. M. Devlin, D. Zhang, H. P. Harding, M. Sweeney, J. X. Rong, G. Kuriakose, E. A. Fisher, et al. 2003. The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. *Nat. Cell Biol.* **5:** 781–792.
19. Chung, B. H., G. A. Tallis, B. H. Simon Cho, J. P. Segrest, and Y. Henkin. 1995. Lipolysis-induced partitioning of free fatty acids to lipoproteins: effect on the biological properties of free fatty acids. *J. Lipid Res.* **36:** 1956–1970.
20. Arimoto, I., C. Matsumoto, M. Tanaka, K. Okuhira, H. Saito, and T. Handa. 1998. Surface composition regulates clearance from plasma and triolein lipolysis of lipid emulsions. *Lipids.* **33:** 773–779.
21. Morita, S., K. Okuhira, N. Tsuchimoto, A. Vertut-Doï, H. Saito, M. Nakano, and T. Handa. 2003. Effects of sphingomyelin on apolipoprotein E- and lipoprotein lipase-mediated cell uptake of lipid particles. *Biochim. Biophys. Acta.* **1631:** 169–176.
22. Saito, H., K. Okuhira, N. Tsuchimoto, A. Vertut-Doï, C. Matsumoto, T. Tanimoto, S. Okada, and T. Handa. 2001. Modulation of apolipoprotein E-mediated plasma clearance and cell uptake of emulsion particles by cholesteryl ester. *Lipids.* **36:** 27–33.
23. Maranhao, R. C., A. M. Tercyak, and T. G. Redgrave. 1986. Effects of cholesterol content on the metabolism of protein-free emulsion models of lipoproteins. *Biochim. Biophys. Acta.* **875:** 247–255.
24. Saito, H., Y. Miyako, T. Handa, and K. Miyajima. 1997. Effect of cholesterol on apolipoprotein A-I binding to lipid bilayers and emulsions. *J. Lipid Res.* **38:** 287–294.
25. Morita, S., M. Kawabe, A. Sakurai, K. Okuhira, A. Vertut-Doï, M. Nakano, and T. Handa. 2004. Ceramide in lipid particles enhances

heparan sulfate proteoglycan and low density lipoprotein receptor-related protein-mediated uptake by macrophages. *J. Biol. Chem.* **279:** 24355–24361.
26. Yokoyama, S., Y. Kawai, S. Tajima, and A. Yamamoto. 1985. Behavior of human apolipoprotein E in aqueous solutions and at interfaces. *J. Biol. Chem.* **260:** 16375–16382.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
28. Konopka, K., E. Pretzer, P. L. Felgner, and N. Duzgunes. 1996. Human immunodeficiency virus type-1 (HIV-1) infection increases the sensitivity of macrophages and THP-1 cells to cytotoxicity by cationic liposomes. *Biochim. Biophys. Acta.* **1312:** 186–196.
29. Goldstein, J. L., S. K. Basu, G. Y. Brunschede, and M. S. Brown. 1976. Release of low density lipoprotein from its cell surface receptor by sulfated glycosaminoglycans. *Cell.* **7:** 85–95.
30. Fuki, I. V., K. M. Kuhn, I. R. Lomazov, V. L. Rothman, G. P. Tuszynski, R. V. Iozzo, T. L. Swenson, E. A. Fisher, and K. J. Williams. 1997. The syndecan family of proteoglycans. Novel receptors mediating internalization of atherogenic lipoproteins in vitro. *J. Clin. Invest.* **100:** 1611–1622.
31. Al-Haideri, M., I. J. Goldberg, N. F. Galeano, A. Gleeson, T. Vogel, M. Gorecki, S. L. Sturley, and R. J. Deckelbaum. 1997. Heparan sulfate proteoglycan-mediated uptake of apolipoprotein E-triglyceride-rich lipoprotein particles: a major pathway at physiological particle concentrations. *Biochemistry.* **36:** 12766–12772.
32. Fuki, I. V., N. Blanchard, W. Jin, D. H. L. Marchadier, J. S. Millar, J. M. Glick, and D. J. Rader. 2003. Endogenously produced endothelial lipase enhances binding and cellular processing of plasma lipoproteins via heparan sulfate proteoglycan-mediated pathway. *J. Biol. Chem.* **278:** 34331–34338.
33. Ji, Z-S., and R. W. Mahley. 1994. Lactoferrin binding to heparan sulfate proteoglycans and the LDL receptor-related protein. Further evidence supporting the importance of direct binding of remnant lipoproteins to HSPG. *Arterioscler. Thromb.* **14:** 2025–2032.
34. Ziere, G. J., J. K. Krujit, M. K. Bijsterbosch, and T. J. C. van Berkel. 1996. Recognition of lactoferrin and aminopeptidase M-modified lactoferrin by the liver: involvement of proteoglycans and the remnant receptor. *Biochem. J.* **313:** 289–295.
35. Ellsworth, J. L., F. B. Kraemer, and A. D. Cooper. 1987. Transport of β-very low density lipoproteins and chylomicron remnants by macrophages is mediated by the low density lipoprotein receptor pathway. *J. Biol. Chem.* **262:** 2316–2325.
36. Waggoner, A. S., and L. Stryer. 1970. Fluorescent probes of biological membranes. *Proc. Natl. Acad. Sci. USA.* **67:** 579–589.
37. Asuncion-Punzalan, E., K. Kachel, and E. London. 1998. Groups with polar characteristics can locate at both shallow and deep locations in membranes: the behavior of dansyl and related probes. *Biochemistry.* **37:** 4603–4611.
38. Epand, R. M., and B. T. Leon. 1992. Hexagonal phase forming propensity detected in phospholipid bilayers with fluorescent probes. *Biochemistry.* **31:** 1550–1554.
39. Saito, H., M. Tanaka, E. Okamura, T. Kimura, M. Nakahara, and T. Handa. 2001. Interactions of phosphatidylcholine surface monolayers with triglyceride cores and enhanced apoA-I binding in lipid emulsions. *Langmuir.* **17:** 2528–2532.
40. Windler, E. E., S. Preyer, and H. Greten. 1986. Influence of lysophosphatidylcholine on the C-apolipoprotein content of rat and human triglyceride-rich lipoproteins during triglyceride hydrolysis. *J. Clin. Invest.* **78:** 658–665.
41. Saito, H., S. Lund-Katz, and M. C. Phillips. 2004. Contributions of domain structure and lipid interaction to the functionality of exchangeable human apolipoproteins. *Prog. Lipid Res.* **43:** 350–380.
42. Takahashi, S., Y. Kawarabayashi, T. Nakai, J. Sakai, and T. Yamamoto. 1992. Rabbit very low density lipoprotein receptor: a low density lipoprotein receptor-like protein with distinct ligand specificity. *Proc. Natl. Acad. Sci. USA.* **89:** 9252–9256.
43. Gianturco, S. H., A. H-Y. Lin, S-L. C. Hwang, J. Young, S. A. Brown, D. P. Via, and W. A. Bradley. 1988. Distinct murine macrophage receptor pathway for human triglyceride-rich lipoproteins. *J. Clin. Invest.* **82:** 1633–1643.
44. Brown, M. L., M. P. Ramprasad, P. K. Umeda, A. Tanaka, Y. Kobayashi, T. Watanabe, H. Shimoyamada, W-L. Kuo, R. Li, R. Song, et al. 2000. A macrophage receptor for apolipoprotein B48: cloning, expression, and atherosclerosis. *Proc. Natl. Acad. Sci. USA.* **97:** 7488–7493.
45. Llorente-Cortes, V., J. Martinez-Gonzalez, and L. Badimon. 2000. Differential role of heparan sulfate proteoglycans on aggregated LDL uptake in human vascular smooth muscle cells and mouse embryonic fibroblasts. *Arterioscler. Thromb. Vasc. Biol.* **22:** 1905–1911.
46. Krieger, M., and J. Herz. 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* **63:** 601–637.
47. Rosenfeld, M. E., S. Butler, V. Ord, B. A. Lipton, C. A. Dyer, L. K. Curtiss, W. Palinski, and J. L. Witztum. 1993. Abundant expression of apolipoprotein E by macrophages in human and rabbit atherosclerotic lesions. *Arterioscler. Thromb.* **13:** 1382–1389.
48. Ishibashi, S., N. Yamada, H. Shimano, N. Mori, H. Mokuno, T. Gotohda, M. Kawakami, T. Murase, and F. Takaku. 1990. Apolipoprotein E and lipoprotein lipase secreted from human monocyte-derived macrophages modulate very low density lipoprotein uptake. *J. Biol. Chem.* **265:** 3040–3047.
49. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223–261.
50. Morita, S., M. Nakano, A. Sakurai, Y. Deharu, A. Vertut-Doï, and T. Handa. 2005. Formation of ceramide-enriched domains in lipid particles enhances the binding of apolipoprotein E. *FEBS Lett.* **579:** 1759–1764.
51. Mazzone, T., and C. Reardon. 1994. Expression of heterologous human apolipoprotein E by J774 macrophages enhances cholesterol efflux to HDL_3. *J. Lipid Res.* **35:** 1345–1353.
52. Ball, R. Y., E. C. Stowers, J. H. Burton, N. R. B. Cary, J. N. Skepper, and M. J. Mitchinson. 1995. Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. *Atherosclerosis.* **114:** 45–54.
53. Warner, G. J., G. Stoudt, M. Bamberger, W. J. Johnson, and G. H. Rothblat. 1995. Cell toxicity induced by inhibition of acyl coenzyme A cholesterol acyltransferase and accumulation of unesterified cholesterol. *J. Biol. Chem.* **270:** 5772–5778.
54. Chang, T. Y., C. C. Chang, S. Lin, C. Yu, B. L. Li, and A. Miyazaki. 2001. Roles of acyl-coenzyme A:cholesterol acyltransferase-1 and -2. *Curr. Opin. Lipidol.* **12:** 289–296.
55. Tall, A. R., P. Costet, and N. Wang. 2002. Regulation and mechanism of macrophage cholesterol efflux. *J. Clin. Invest.* **110:** 899–904.
56. Kellner-Weibel, G., P. G. Yancey, W. G. Jerome, T. Walser, R. P. Mason, M. C. Philips, and G. H. Rothblat. 1999. Crystallization of free cholesterol in model macrophage foam cells. *Arterioscler. Thromb. Vasc. Biol.* **19:** 1891–1898.