

## Apolipoprotein A-I-stimulated Apolipoprotein E Secretion from Human Macrophages Is Independent of Cholesterol Efflux\*

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Apolipoprotein A-I (apoA-I)-mediated cholesterol efflux involves the binding of apoA-I to the plasma membrane via its C terminus and requires cellular ATP-binding cassette transporter (ABCA1) activity. ApoA-I also stimulates secretion of apolipoprotein E (apoE) from macrophage foam cells, although the mechanism of this process is not understood. In this study, we demonstrate that apoA-I stimulates secretion of apoE independently of both ABCA1-mediated cholesterol efflux and of lipid binding by its C terminus. Pulse-chase experiments using <sup>35</sup>S-labeled cellular apoE demonstrate that macrophage apoE exists in both relatively mobile (E<sub>m</sub>) and stable (E<sub>s</sub>) pools, that apoA-I diverts apoE from degradation to secretion, and that only a small proportion of apoA-I-mobilized apoE is derived from the cell surface. The structural requirements for induction of apoE secretion and cholesterol efflux are clearly dissociated, as C-terminal deletions in recombinant apoA-I reduce cholesterol efflux but increase apoE secretion, and deletion of central helices 5 and 6 decreases apoE secretion without perturbing cholesterol efflux. Moreover, a range of 11- and 22-mer  $\alpha$ -helical peptides representing amphipathic  $\alpha$ -helical segments of apoA-I stimulate apoE secretion whereas only the C-terminal  $\alpha$ -helix (domains 220–241) stimulates cholesterol efflux. Other  $\alpha$ -helix-containing apolipoproteins (apoA-II, apoA-IV, apoE2, apoE3, apoE4) also stimulate apoE secretion, implying a positive feedback autocrine loop for apoE secretion, although apoE4 is less effective. Finally, apoA-I stimulates apoE secretion normally from macrophages of two unrelated subjects with genetically confirmed Tangier Disease (mutations C733R and c.5220–5222delTCT; and mu-

tations A1046D and c.4629–4630insA), despite severely inhibited cholesterol efflux. We conclude that apoA-I stimulates secretion of apoE independently of cholesterol efflux, and that this represents a novel, ABCA1-independent, positive feedback pathway for stimulation of potentially anti-atherogenic apoE secretion by  $\alpha$ -helix-containing molecules including apoA-I and apoE.

The anti-atherogenic effects of high density lipoprotein (HDL)<sup>1</sup> (1) are at least in part attributed to the ability of HDL to stimulate cholesterol and phospholipid efflux from lipid-loaded macrophages, providing the initial step of reverse cholesterol transport (RCT). Apolipoprotein A-I (apoA-I), the major protein component of HDL, is understood to play an important role in this process (1).

Lipid removal by apoA-I involves a cAMP-inducible active transport pathway (2) and is known to be mediated by the ATP-binding cassette transporter-1 (ABCA1/ABC-1) (3). These transporters are membrane proteins that utilize ATP hydrolysis to transport substrates across membranes (4). Mutations in ABCA1/ABC-1 cause Tangier disease (TD), a severe HDL deficiency syndrome characterized by very low plasma levels of HDL and apoA-I, accumulation of cholesterol in tissue macrophages, and a predisposition to atherosclerosis (5–7). Fibroblasts from subjects with Tangier disease show impaired cholesterol and phospholipid release to lipid-free apolipoproteins (8). In addition, inhibition of ABCA1 expression or activity inhibits lipid efflux to apoA-I in normal fibroblasts (3, 6).

Properties additional to induction of cholesterol efflux may contribute to the anti-atherogenic effect of apoA-I. Human atherosclerotic lesions contain apoE protein and mRNA, especially in association with macrophage foam cells (9). Secretion

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<sup>1</sup> The abbreviations used are: HDL, high density lipoprotein; PLV, phospholipid vesicles; apoA, apolipoprotein; ABCA1, ATP-binding cassette transporter; SPM, sphingomyelin; PC, phosphatidylcholine; TD, Tangier disease; LDL, low density lipoprotein; apo, apolipoprotein; ABCA1, ATP-binding cassette transporter A1; RCT, reverse cholesterol transport; AcLDL, acetylated LDL; HMDM, human primary monocyte-derived macrophages; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; SPM, egg yolk sphingomyelin; BCA, bichononic acid; PBS, phosphate-buffered saline; AU, arbitrary units; RT-PCR, reverse transcriptase-PCR.

of apoE by macrophages may protect against atherosclerosis, as indicated by the effects of transplantation of apoE-expressing bone marrow into apoE-null mice (10, 11). This may depend upon enhanced local clearance of cellular lipids and/or inhibition of local inflammatory responses, in addition to lowering plasma concentrations of atherogenic plasma lipoproteins. Recent studies demonstrated that apoA-I stimulates both apoE secretion and cholesterol efflux from human and mouse foam cell macrophages (12, 13). Stimulation of apoE secretion by apoA-I and by HDL is most likely post-transcriptionally regulated and is observed for both endogenous apoE (macrophages) and recycled apoE (hepatocytes) (12–15). Recent studies indicate that ABCA1 may be involved in basal apoE secretion (16). However, the requirement for ABCA1 activity in apoA-I-induced apoE secretion has never been reported.

In this study we provide evidence that apoA-I-stimulated apoE secretion is mechanistically distinct from its stimulation of ABCA1-mediated cholesterol efflux. We demonstrate that apoA-I promotes secretion of a mobile pool of apoE, which is otherwise destined for intracellular degradation, that this process does not require interactions between the C terminus of apoA-I and the plasma membrane and occurs normally in macrophages of patients with Tangier disease, implying independence from ABCA1 activity.

#### EXPERIMENTAL PROCEDURES

**Reagents**—All solvents were high performance liquid chromatography (HPLC) grade (Mallinckrodt). Polyclonal goat anti-human antibodies to human apoE were obtained from Chemicon International Inc. For immunoblotting of nitrocellulose membranes after non-denaturing gel electrophoresis, polyclonal goat antibodies to human apoA-I and polyclonal sheep antibodies to human apoA-II (Roche Applied Science) were used. Secondary rabbit polyclonal anti-goat or anti-sheep horseradish peroxidase-linked antibodies, nitrocellulose membranes (0.45  $\mu\text{m}$ ), and enhanced chemiluminescence (ECL) reagents and Hyperfilm were obtained from Amersham Biosciences. Human apoE standard was from Biotest. [ $1\alpha,2\beta(n)$ - $^3\text{H}$ ]cholesterol and [*methyl*- $^3\text{H}$ ]choline chloride were supplied by Amersham Biosciences (specific activity: 44 Ci/mmol and 79 Ci/mmol, respectively). LDL, acetylated LDL (AcLDL), [ $^3\text{H}$ ]cholesterol-labeled AcLDL were prepared as described (17, 18). 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC), egg yolk sphingomyelin (SPM), and sodium cholate were purchased from Sigma. Bicinchoninic acid (BCA) reagent for protein determination was also supplied by Sigma-Aldrich. For pulse-chase studies, methionine-free medium was supplied by Invitrogen and [ $^{35}\text{S}$ ]TRAN-label (1175 Ci/mmol) from ICN.

**Apolipoproteins**—Human apoA-I and apoA-II were isolated from HDL by ultracentrifugation and anion exchange chromatography (17, 19). Human apoA-IV was isolated from the density  $>1.25$  g/ml fraction of plasma by adsorption onto synthetic triglyceride-rich microemulsions. The microemulsions were delipidated and the apoA-IV purified by anion exchange chromatography on a Q-Sepharose fast flow column (20, 21). Human apoE2, apoE3, and apoE4 were expressed by BL21 *Escherichia coli* (provided by Dr. Karl Weisgraber, Gladstone Institute) and purified by gel filtration chromatography.

**ApoA-I Peptides and Recombinant Proteins**—Pure peptides representing peptides of apoA-I were synthesized using an automated solid phase peptide synthesizer as described previously (22–24). To promote the  $\alpha$ -helical stability of the peptide molecules, the N and C termini were acetylated and amidated (except for peptide 1–33) respectively (25). Truncated apoA-I propeptide mutants were expressed in an *E. coli* PGEX expression system (26). Cholesterol efflux to these proteins has previously been published (27). ApoA-I deletion mutants were constructed as described previously (28, 29). Wild-type apoA-I and engineered variants of apoA-I were expressed in expression host *E. coli* strain BL21-DE3 and isolated by gel filtration and anion exchange chromatography (29). Identical stimulation of apoE secretion from human macrophage foam cells was observed in control experiments using recombinant wild-type apoA-I and human apoA-I prepared from human plasma. In previous studies we established that 5–10  $\mu\text{g}/\text{ml}$  apoA-I was saturating for cholesterol efflux and apoE secretion from human and murine macrophages (13). In the present studies, peptides and proteins were added to cells at a final concentration of 25  $\mu\text{g}/\text{ml}$  culture medium after first establishing that this was in excess of saturating concentrations for induction of apoE secretion for all molecules.

**Preparation of Phospholipid ApoA-I and Phospholipid ApoA-II Discs**—Discs were prepared as described previously (19) using the cholate dialysis method (30). After preparation, all discs were dialyzed extensively against 0.01 M Tris-buffered saline containing 0.15 M NaCl, 0.005% (w/v) EDTA- $\text{Na}_2$ , 0.006% (w/v)  $\text{NaN}_3$ , and stored under argon. Lipid-free apoA-I and apoA-II were not present in the preparations as judged by electrophoresis on non-denaturing 3–40% gradient gels. Phospholipid composition was varied by altering the proportions of phospholipids added at the time of preparation. All chemical analyses of reconstituted particles were carried out on a Roche/Hitachi 902 analyzer (Roche Diagnostics, Zurich, Switzerland). Phospholipid concentrations were determined enzymatically (31, 32). The Stokes' diameter and surface charge of the particles was measured by non-denaturing PAGE and agarose gel electrophoresis (19, 33). In some experiments, after cell efflux incubations, media containing apoA-I phospholipid or apoA-II phospholipid discs underwent non-denaturing polyacrylamide gel electrophoresis, followed by electrophoretic transfer nitrocellulose membranes and immunoblotting for apoE, apoA-I, or apoA-II to investigate possible association of apoE with phospholipid-containing particles.

**Isolation and Culture of Human Monocyte-derived Macrophages (HMDM)**—Human monocytes (apoE3/E3 phenotype) were isolated from white cell concentrates from healthy donors using centrifugal elutriation as described (13). Genotyping was completed by *Taq* polymerase chain reaction amplification of a 232-base pair fragment of the *apoE* gene followed by cutting with the restriction endonuclease *Cfo*1 by the Department of Biochemistry, Royal Prince Alfred Hospital. White cell buffy coat concentrates ( $<24$  h, *ex vivo*) and human serum were provided by the New South Wales Red Cross blood transfusion service, Sydney, Australia. Purified monocytes ( $>95\%$  purity by nonspecific esterase staining) were differentiated by plating at  $1.5 \times 10^6$  cells per 22-mm diameter culture dish (Costar) in RPMI 1640 containing penicillin G and streptomycin (50 units/ml and 50  $\mu\text{g}/\text{ml}$ , respectively), L-glutamine (2 mM), and 10% (v/v) heat-inactivated whole human serum for 6 days. Following differentiation, the cells were washed and enriched with unesterified cholesterol (FC) and cholesteryl ester (CE) by incubation in RPMI 1640 containing 10% LPDS (v/v) and AcLDL (50  $\mu\text{g}$  protein/ml) for 4 days (13).

Mononuclear cells from individual donors (Tangier disease subjects and their controls) were isolated by minor modification of a previously described density separation method (34, 35) using OptiPrep™ solution of density 1.068 g/ml and a solution of density 1.063 g/ml to remove platelets and lymphocytes, respectively. Total cells were plated at a density of  $2 \times 10^6$  cells/22-mm diameter culture well (Falcon). Cells were differentiated to macrophages in RPMI 1640-containing antibiotics, glutamine, 50 ng/ml M-CSF (36), and heat-inactivated whole human serum (13, 37) for 6 days. At this time cells were washed prior to cholesterol enrichment (see above). Direct comparisons between HMDM isolated from normal donors by density centrifugation and centrifugal elutriation confirmed identical cholesterol efflux and apoE secretion by either method.

**Lipid Efflux and ApoE Secretion**—To achieve cholesterol loading of macrophages, 2  $\mu\text{Ci}/\text{ml}$  [ $^3\text{H}$ ]cholesterol in ethanol was incorporated into AcLDL before incubating with cells for 4 days as described (18). Triplicate cultures were harvested after loading to confirm efficient enrichment with FC and CE by HPLC. Cells were subsequently washed, equilibrated overnight in RPMI 1640 containing 0.1% (w/v) bovine serum albumin, to allow equilibration of [ $^3\text{H}$ ]cholesterol in FC and CE pools. In some experiments, cells were loaded with AcLDL without [ $^3\text{H}$ ]cholesterol, and phospholipids were labeled at the end of the 4-day cholesterol-loading period by incubating cells with RPMI 1640 containing 3  $\mu\text{Ci}/\text{ml}$  [ $^3\text{H}$ ]choline chloride in ethanol (final 2% (v/v)) and 0.1% bovine serum albumin (w/v) for 22 h, followed by equilibration for 1 h in RPMI 1640 containing 0.1% bovine serum albumin.

Equilibrated, cholesterol-enriched cells were washed with warm PBS and incubated in 1.0 ml of efflux medium comprising RPMI 1640 with or without 25  $\mu\text{g}/\text{ml}$  apoA-I or the appropriate additions. After 1–8 h, media were removed, mixed with Complete® protease inhibitor (Roche Applied Science) and 0.02 TIU of aprotinin (Sigma) and spun for 2 min at  $16,000 \times g$  to remove any detached cells. The cultures were washed twice with ice-cold PBS and then scraped in 0.8 ml of cold PBS containing Complete® protease inhibitor and aprotinin.

**Quantitation of Cholesterol and Cholesteryl Ester in Cells and Media**—40- $\mu\text{l}$  media aliquots and 5- $\mu\text{l}$  cell aliquots were analyzed by scintillation counting to quantify efflux of [ $^3\text{H}$ ]cholesterol. The proportions of label in cell [ $^3\text{H}$ ]FC and [ $^3\text{H}$ ]CE were determined after separation by TLC in heptane/ethyl acetate (1:1, v/v). Standards of FC and CE were identified by charring at 100 °C in 10%  $\text{CuSO}_4$ , 8.5%  $\text{H}_3\text{PO}_4$ . The masses of FC and CE in cells and media were determined by reverse-

phase HPLC after extraction into methanol/hexane (17, 38). Radiochemical equilibrium was determined by calculating the specific activity of free and esterified cholesterol (dpm per nmol) (18) and was reproducibly achieved after 4 days of cholesterol enrichment and overnight equilibration.

**Quantitation of Phospholipids in Cells and Media**—Phospholipids were extracted from 150- $\mu$ l aliquots of cell lysates and media samples using the method of Blich and Dyer including two backwashes with methanol/water (10:9, v/v) as described previously (39). After evaporation of the chloroform phase, lipids were dissolved in 200  $\mu$ l of chloroform/methanol (2:1, v/v), and 50- $\mu$ l aliquots were counted by scintillation counting to determine phospholipid efflux. Total phospholipid mass was determined using a modification of the Bartlett assay as described (40) and was used to determine specific activity (dpm per nmol phospholipid).

**Quantitation of ApoE in Cell Lysates and Efflux Media by Western Blot and by ELISA**—Aliquots (55  $\mu$ l) of cell culture medium were mixed with sample buffer (27.5  $\mu$ l) containing 10 mM dithiothreitol, heated to 100 °C for 5 min, and separated by SDS-PAGE using a 4% stacking gel, and 12.5% polyacrylamide resolving gel under reducing conditions in Tris-glycine buffer as described (13). Electrophoretic Western blot transfer onto 0.45- $\mu$ m nitrocellulose membranes was performed in Tris-glycine buffer for 1 h at 25–30 V, blocked before incubating with primary antibody to apoE (goat anti-human polyclonal, 1:5000 dilution), and washing and incubating with 1:5000 dilution of secondary antibody (rabbit anti-goat IgG) conjugated with horseradish peroxidase (13). Membrane chemiluminescence signal was quantified using Kodak Digital Science 1D and expressed as arbitrary units (AU) per mg of cell protein. In most experiments, a serial dilution of authentic human apoE standard in sample buffer was separated and blotted in parallel to allow calculation of secreted apoE mass as microgram per culture or  $\mu$ g per mg of cell protein. The linear response range of chemiluminescence signal to mass of authentic human apoE standard was defined for each Western blot in each experiment.

In addition, apoE secretion was measured in some experiments using ELISA. NUNC 96 well immunoplates were coated with 1.5  $\mu$ g/ml mouse monoclonal anti-human apoE antibody (Biosdesign) in 0.2 M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> buffer. Standards were prepared from purified human apoE (Biosdesign), whereas antibodies used in Western analysis were used as tagging antibodies (goat anti-human polyclonal, 1:3750 and rabbit anti-goat horseradish peroxidase, 1:2500).

**Metabolic Labeling of Cell Proteins with [<sup>35</sup>S]Methionine/Cysteine**—To study the fate of newly synthesized apoE, cholesterol-enriched HMDM were labeled in methionine-free medium (Invitrogen) containing 250  $\mu$ Ci/ml [<sup>35</sup>S]TRAN-label (1175 Ci/mmol, ICN) for 3 h, then washed and chased in RPMI 1640 media containing 2 mM methionine (Sigma) and cysteine (Sigma). <sup>35</sup>S-labeled apoE in cell lysates and culture medium were determined by immunoprecipitation using 1:10,000 dilution of the polyclonal goat antibody to human apoE and protein A-Sepharose (Amersham Biosciences) after separation by SDS-PAGE, quantified by phosphorimaging (Photostimulated Luminescence, Fujix BAS 1000) and expressed as AU of apoE/mg of cell protein (13). Where indicated, in experiments detecting small amounts of secreted apoE requiring increased sensitivity, immunoprecipitated <sup>35</sup>S-labeled apoE was directly quantified by radiometric detection, after confirming a single band on SDS-PAGE and phosphorimaging. [<sup>35</sup>S]Methionine/cysteine labeling was also used to discriminate between cell derived (secreted) and exogenous apoE in experiments investigating the secretion of apoE by recombinant apoE2, E3, and E4.

**Biotinylation and Isolation of Biotinylated ApoE**—Cholesterol-enriched HMDM were incubated with 200  $\mu$ Ci/ml [<sup>35</sup>S]TRAN-label in methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 10% DMEM for 16 h. Surface proteins were then biotinylated by placing the cells on ice for 5 min, followed by incubation with 1 mg/ml sulfo-NHS-SS-Biotin (Pierce) in PBS at 4 °C for 45 min. Cells were washed twice with ice-cold PBS and subsequently incubated with or without 25  $\mu$ g/ml apoA-I for 30 min at 37 °C. ApoE was immunoprecipitated as described above, and biotinylated apoE separated from total immunoprecipitated apoE as described (41). In short, total apoE was released from protein A-Sepharose beads by boiling in 100  $\mu$ l of HEPES-buffered saline containing 1% SDS at 90 °C for 3 min. Biotinylated apoE was separated from unbiotinylated apoE using ImmunoPure Immobilized Streptavidin (Pierce) and both were quantified using scintillation counting and SDS-PAGE/phosphorimaging. Biotinylated apoE was quantified both as AU of apoE/mg of cell protein and as percent of total immunoprecipitated, secreted apoE. In these experiments, 3.5  $\pm$  0.5% of total cellular apoE was biotinylated, and 34.0  $\pm$  9.7% of cellular apoE protein was in plasma membrane fractions isolated by sucrose

density gradient (42). As only cell surface proteins are biotinylated at 4 °C (41), it is estimated that  $\sim$ 11.2  $\pm$  3.2% of the plasma membrane pool of apoE was biotinylated in these experiments.

**Analysis of apoE mRNA by Real Time PCR**—Cells were harvested for total RNA using Tri Reagent (Sigma) according to the manufacturer's instructions with the exception that 1-bromo-3-chloropropane (Sigma) was substituted for chloroform. For each reverse-transcription reaction, 1  $\mu$ g of total RNA was reverse-transcribed using oligo(dT) primers (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). Relative real-time RT-PCR was performed using IQ SYBR Green Supermix (Bio-Rad) on an ABI 7700 Sequence Detector (PE Biosystems) and analyzed using ABI Prism sequence detector software v1.6.3 (PE Biosystems). Gene-specific primers were used as follows: ApoE, 5'-CT-GCTCAGCTCCCAGGTCACCCAGG-3' (forward), 5'-CTTCTGCAGGT-CATCGGCATCGCGG-3' (reverse), annealing at 60 °C, 30 cycles, product size 321 base pairs. ABCA1, 5'-GCACCTGAGGAAGATGCTGAAA-3' (forward), 5'-AGTTCCTGGAAGGTCTTGTTCAC-3' (reverse), annealing at 60 °C, 28 cycles, product size 205 base pairs. Results were normalized to that of a housekeeping gene, *PBGD*, 5'-GAGTGATTCG-CGTGGGTACC-3' (forward), 5'-GGCTCCGATGGTGAAGCC-3' (reverse), annealing at 55–60 °C. Melting curve analysis was performed to confirm production of a single product in these reactions. Results after exposure to apoA-I were related to those without apoA-I (RPMI 1640 medium control) in each experiment, and the results of three experiments averaged.

**Sucrose Density Ultracentrifugation**—To determine the buoyant density of secreted apoE, media samples were subjected to sucrose density ultracentrifugation using a minor modification of a published protocol (43). Following efflux, 625- $\mu$ l aliquots of media were centrifuged at 16,000  $\times$  g for 2 min to remove cellular debris, and the supernatant underlaid. Underlay was completed using 2 ml of 1.33% sucrose solution, 1.23 ml of 35% sucrose solution, and 1.23 ml of 65% sucrose solution in 5.1 ml of Beckman quick-seal tubes, and samples were centrifuged at 100,000 rpm (543,000  $\times$  g), for 20 h at 16 °C, using a TL100.4 rotor in Beckman TLX-Ultracentrifuge. 15  $\times$  250  $\mu$ l aliquots were taken from above and analyzed for [<sup>3</sup>H]cholesterol and apoE (Western blot).

**Tangier Disease Patients (TD1 and TD2)**—A previously unreported 56-year-old male with clinical features consistent with Tangier Disease (TD1) was identified. The patient had premature coronary artery disease, very low plasma HDL-cholesterol (3.1–7.7 mg/dl, normal range 30–70 mg/dl) and apoA-I concentrations (0.002–0.006 mg/dl, normal 110–140 mg/dl), low total cholesterol (61.9–104.4 mg/dl, normal range 116–225 mg/dl) and LDL-cholesterol (19.3 mg/dl, normal range 50–190 mg/dl) level. He also had elevated plasma triglycerides (177.1–336.6 mg/dl, normal <200 mg/dl), bilateral cataracts and hepatomegaly. There was no splenomegaly, and tonsils had been removed in childhood. The patient was genotyped (S. Rust, G. Assmann, Institut für Arterioskleroseforschung an der Westfälischen Wilhelms-Universität Münster, Germany). Two novel amino acid mutations within the *ABCA1* gene were found: C733R in exon 16 (c.2197C>T) and a deletion of three nucleotides in exon 38 (c.5220–5222delTCT) on different alleles as shown by segregation analysis (numbering follows recommendations of the international nomenclature working group). Both mutations affect amino acids conserved between human and mice. Neither of the mutations has been previously described. A healthy subject with normal lipoprotein profile and no history of vascular disease was used as a source of control macrophages (HC1). Since completion of this study, TD1 has died.

A second TD patient (TD2), his healthy normal brother (HC2), and his heterozygous parents (HZ2A (mother) and HZ2B (father)) have been previously described (44, 45). The *ABCA1* mutations in this TD patient were published as a missense mutation in exon 21 (A986D) and a frameshift mutation in exon 33 (4570insA, A1484' X1492) (45). Using current nomenclature, these are respectively equivalent to a missense mutation in exon 22 (c.3137C>A; A1046D) and a frameshift mutation in exon 34 (c.4629–4630insA; A1544 fs X1552). HC2 has neither *ABCA1* mutation, and HZ2A and HZ2B contain the 4629insA and the A1046D mutations, respectively. Plasma HDL-cholesterol concentrations for HC2, TD2, HZ2A, and HZ2B were respectively 43.0, 2.7, 25.8, and 38.6 mg/dl and apoA-I levels were respectively 129, <4.5, 95.7, and 115.8 mg/dl. Plasma triglyceride concentrations were elevated in all subjects, being 507.7 mg/dl for HC2, 246.3 mg/dl for TD2, 337.6 mg/dl for HZ2A, and 396.9 mg/dl for HZ2B.

**Protein Estimation**—Protein contents of LDL samples and cell lysates were determined in duplicate or triplicate for each sample measured, using the BCA method with bovine serum albumin as standard (17).

**Cell Viability**—Cell viability following various treatments was routinely assessed by light microscopic morphology, by estimation of cell protein and by measuring leakage of lactate dehydrogenase into the medium (46). Cell viability was routinely between 85 and 100% after incubation in efflux media.

**Data Analysis**—The degradation and secretion of cellular apoE from pulse-chase experiments were simultaneously fitted to a first order rate equation with  $k_1$  and  $k_2$  describing the rate constants for secretion and degradation, respectively. The first order rate equation shown in Equation 1,

$$\frac{\partial E_M}{\partial t} = -(k_1 + k_2) \times E_M \quad (\text{Eq. 1})$$

was fitted to the experimental secretion and degradation data using a non-linear least-squares fitting program (Solver in Microsoft Excel). The quality of the fit was evaluated by an error function as previously described (42).

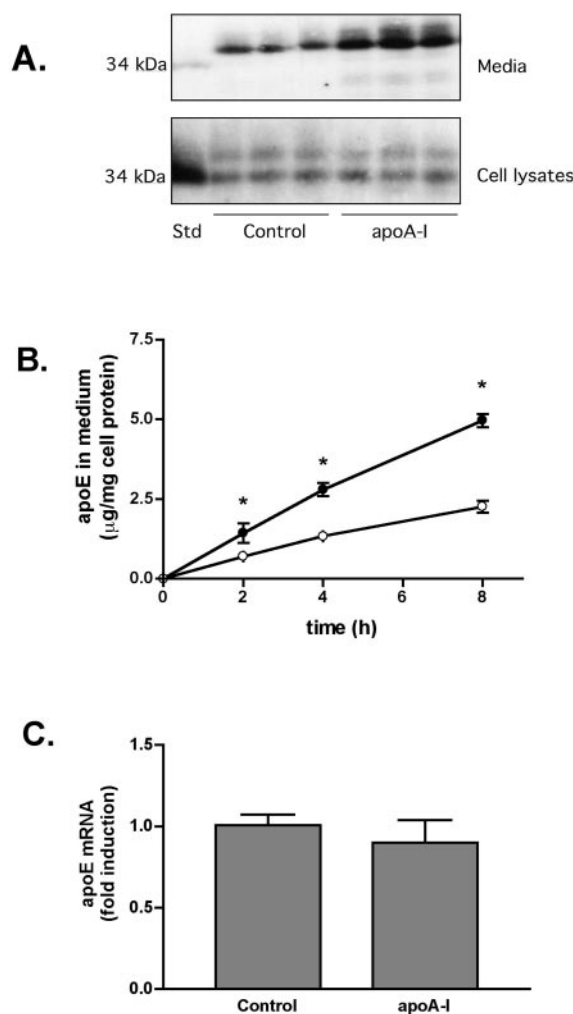
Efflux was calculated as [ $^3\text{H}$ ]lipids (*e.g.* cholesterol or phospholipid) released to the medium expressed as a percentage of the total  $^3\text{H}$  cellular lipids at  $T_0$  (17, 18). Unless otherwise stated, data presented are mean  $\pm$  S.D. of triplicate cultures from single experiments and are representative of at least 2–3 independent experiments. The significance of results was assessed by unpaired Student's *t* test for simple comparisons of two groups, and one-way analysis of variance using Tukey's test as post-hoc correction for multiple comparisons. Differences were considered significant at  $p < 0.05$ .

## RESULTS

**ApoA-I Stimulates the Secretion of ApoE**—We have previously shown that apoA-I stimulates the secretion of murine macrophage apoE in a time- and concentration-dependent manner and that this is not inhibited by the transcription inhibitor actinomycin D (13). Here we confirm that apoA-I stimulates apoE secretion from primary human macrophages in a time-dependent manner, without affecting net cell-associated apoE protein or cellular apoE mRNA levels (Fig. 1). Secreted apoE was always  $>34$  kDa, consistent with the known extensive glycosylation of secreted apoE, whereas cell-associated apoE was of multiple masses consistent with variable degrees of glycosylation during apoE processing within cells.

To determine the effect of apoA-I on secretion and degradation of preformed apoE (in the absence of ongoing *de novo* synthesis), pulse-chase experiments were performed. Pulse incubations first incorporated [ $^{35}\text{S}$ ]methionine into cellular apoE, which was then chased with or without apoA-I in the absence of label (Fig. 2). ApoA-I rapidly stimulated secretion of [ $^{35}\text{S}$ ]apoE, differences between apoA-I-containing and control media being apparent within 30 min of incubation. Analysis of residual cell  $^{35}\text{S}$ -labeled apoE during chase incubations demonstrated a rapid and nearly identical decline in cell-associated apoE with or without apoA-I. Net degradation of apoE (cells plus media) was lower in apoA-I-containing cultures relative to control conditions and was matched by increased secretion of apoE to apoA-I-containing media. The identical rate of decline of cell-associated apoE in control and apoA-I-exposed conditions suggests that apoA-I diverts apoE to secretion from a cellular pool of apoE, which is otherwise degraded.

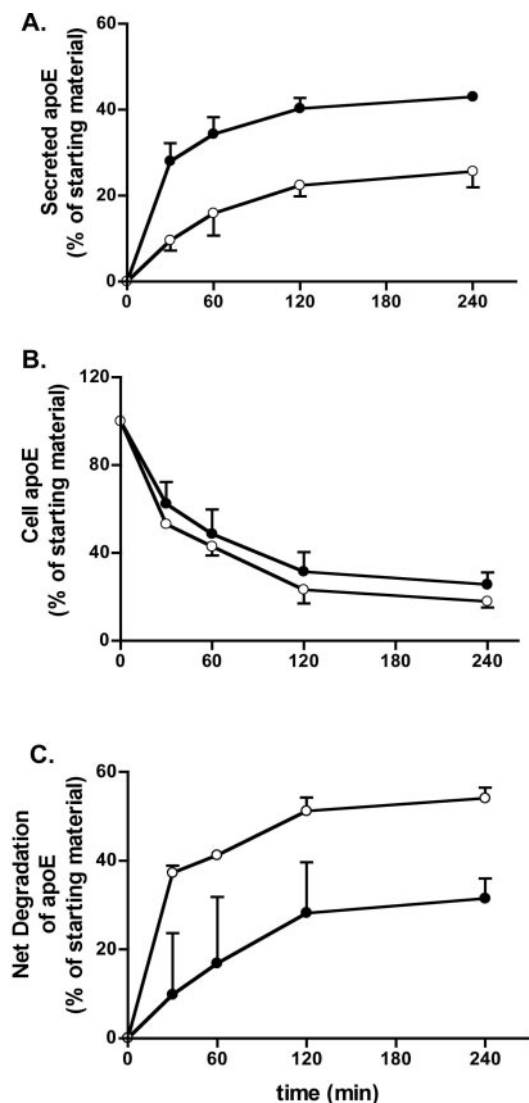
To further investigate the pool of apoE mobilized by apoA-I, the degradation and secretion of cellular apoE were simultaneously fitted to a first order rate equation, with  $k_1$  describing the rate constant for secretion and  $k_2$  that for degradation. As we have previously described, the boundary conditions for accepting modeling of experimental data were an error function (ERF)  $0.01 < \text{ERF} < 0.05$  (42). An initial model assumed all *de novo* synthesized apoE was equally susceptible to degradation or secretion, and that net degradation and secretion proceeded at constant rates and were independent of each other. However, comparison of experimental and calculated data using this model identified that ERF were excessive and did not fulfill the boundary conditions (ERF for control 0.081 and for



**FIG. 1. Post-transcriptional stimulation of apoE secretion by apoA-I.** HMDM were incubated for 4 days with 50  $\mu\text{g}/\text{ml}$  AcLDL and then washed and incubated for the times indicated in RPMI 1640 with (apoA-I, ●) or without (Control, ○) 25  $\mu\text{g}/\text{ml}$  apoA-I. ApoE protein was determined by Western blotting at 8 h (A, three independent cell cultures, media above, cell lysates below) or ELISA at variable times (B). ApoE mRNA levels (C) were determined at 8 h by real-time RT-PCR relative to the housekeeping gene *PBGD*, as described under "Experimental Procedures." \*,  $p < 0.01$  relative to control.

apoA-I 0.069, respectively). A second model simultaneously fitted the degradation and secretion of  $^{35}\text{S}$ -labeled cellular apoE to a first order rate equation as described in "Experimental Procedures." The cellular-labeled apoE at the start of the chase was divided into two pools: a larger mobile pool of apoE ( $E_M \sim 75\%$ ) and a smaller stable pool ( $E_s \sim 25\%$ ), which was neither degraded nor secreted during the duration of the experiment. The presence of  $E_s$  was apparent from the residual pool of undegraded cell-associated apoE, and the presence of such a stable pool has previously been identified on the surface of HepG2 cells (47). Using this model, secretion and degradation of apoE were mutually dependent, and the boundary conditions for goodness of fit were fulfilled (Fig. 3). ApoA-I increased the rate constant for secretion ( $k_1$ ) and decreased the rate constant for degradation ( $k_2$ ) relative to control conditions, but did not substantially alter the size of  $E_s$  and  $E_m$ . Thus apoA-I-stimulated secretion from the mobile pool of apoE, which was otherwise fated for intracellular degradation.

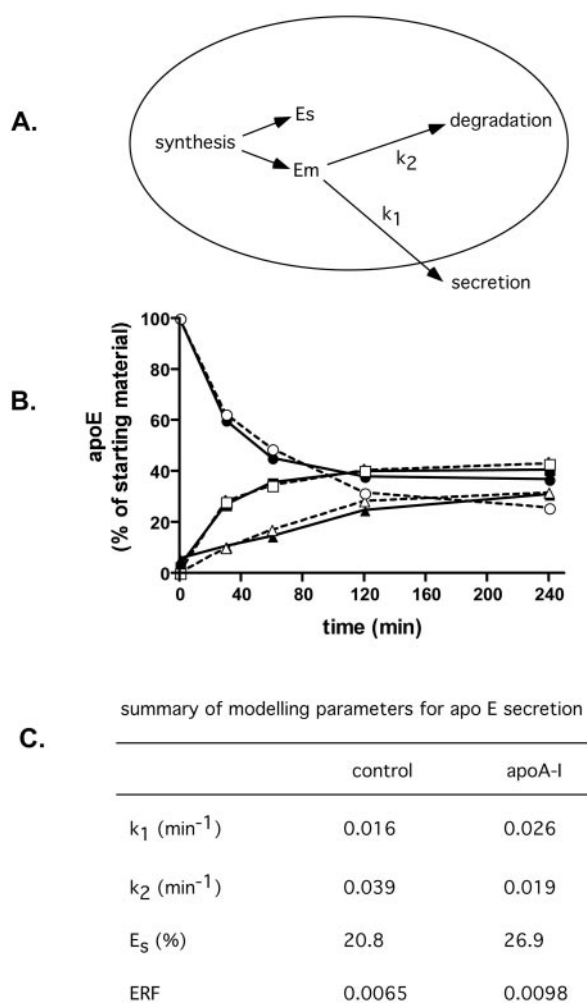
We considered that cell surface apoE might contribute to the mobile pool of apoE secreted to apoA-I, as apoE can be mobilized from the surface of cells (48). To investigate this, [ $^{35}\text{S}$ ]methionine-labeled HMDM were incubated at 4  $^\circ\text{C}$  with biotin to



**FIG. 2. ApoA-I stimulates secretion of preformed apoE and decreases net apoE degradation.** HMDM were incubated for 4 days with 50  $\mu\text{g/ml}$  AcLDL and then washed and incubated in methionine-free Dulbecco's modified Eagle's medium with 200  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine/cysteine for 3 h. Cells were then washed and chased without ( $\circ$ ) or with ( $\bullet$ ) 25  $\mu\text{g/ml}$  apoA-I. At indicated times  $^{35}\text{S}$ -labeled apoE was immunoprecipitated from media and cell lysates, separated and detected by SDS-PAGE and phosphorimaging of a single 34-kDa band, and measured by scintillation counting of the excised band, as described under "Experimental Procedures." At the start of the chase ( $T_0$ ), total apoE was  $2.78 \pm 0.21 \times 10^6$  cpm/mg cell protein. *A*, secreted apoE; *B*, cell-associated apoE; *C*, net degradation of apoE, calculated by subtracting residual apoE in cells and media from apoE in cells at  $T_0$ . All data are expressed as percent of total apoE at  $T_0$ .

label cell surface proteins, before cells were exposed to control media or apoA-I for 30 min at 37  $^{\circ}\text{C}$  to allow apoE secretion (Fig. 4A). Cell and medium samples were sequentially immunoprecipitated, first using antibodies against apoE, then against biotin. ApoA-I stimulated the secretion of biotin-labeled apoE 2-fold relative to control medium, indicating that cell surface apoE did contribute to the secreted apoE. However, biotin-labeled apoE represented <2% of secreted apoE in both control ( $0.6 \pm 0.3\%$ ) and apoA-I-containing ( $1.6 \pm 0.3\%$ ) conditions at 30 min that were much less than the estimated proportion of plasma membrane apoE, which was biotinylated ( $11.2 \pm 3.2\%$ , see "Experimental Procedures"). These data indicated that the net contribution of the cell surface pool to overall apoE secretion was minor.

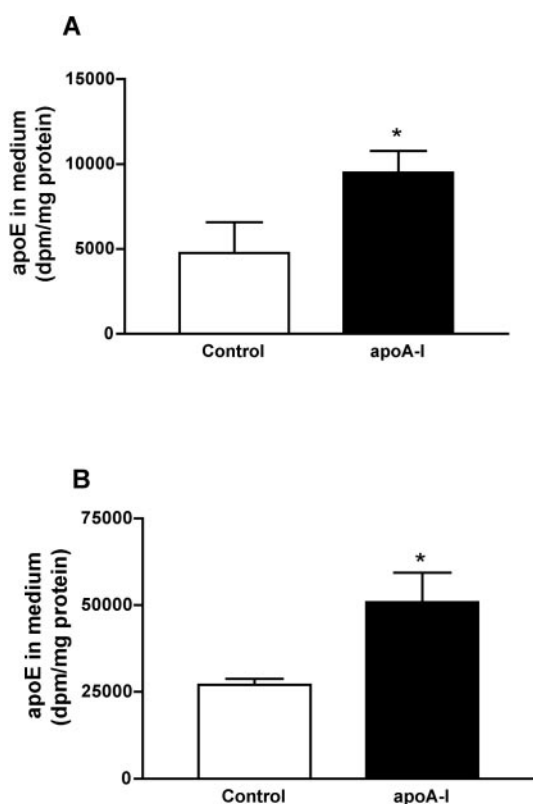
As cell surface apoE can be re-endocytosed (41), the pool of



**FIG. 3. ApoA-I diverts apoE from degradation to secretion.** The degradation and secretion of  $^{35}\text{S}$ -labeled cellular apoE pulse-chase experiments were simultaneously fitted to a first order rate equation as described under "Experimental Procedures." *A*, model for apoE turnover with  $E_s$  and  $E_m$  representing stable and mobile cellular apoE pools, respectively, and  $k_1$  and  $k_2$ , respectively, describing the rate constants for secretion and degradation. *B*, experimental (open symbols, dashed line) and calculated (closed symbols, solid line) kinetic data for secreted ( $\square$ ,  $\blacksquare$ ), cellular ( $\circ$ ,  $\bullet$ ), and degraded ( $\triangle$ ,  $\blacktriangle$ ) apoE in the presence of apoA-I. *C*, summary of modeling parameters,  $E_s$  representing the percent of cellular apoE in the stable pool, and *ERF* representing the error function of modeling parameters.

biotin-labeled apoE secreted to apoA-I could represent apoE directly mobilized from the cell surface, or apoE which has been re-internalized and re-secreted, although little recycling would be expected over 30 min. To confirm that apoA-I directly mobilizes cell surface apoE, [ $^{35}\text{S}$ ]methionine-labeled HMDM were pre-cooled to 4  $^{\circ}\text{C}$  and then incubated with control medium or apoA-I at 4  $^{\circ}\text{C}$  for 30 min (Fig. 4B). ApoA-I approximately doubled the release of cell surface apoE at 4  $^{\circ}\text{C}$  (from  $3.2 \pm 0.4\%$  and  $6.1 \pm 1.5\%$  of total cell apoE present at  $T_0$ ), indicating that cell surface apoE does contribute directly to the mobile pool of apoE released to apoA-I, although, overall, this remains a minor proportion of total apoE released.

*C-terminal Truncations of pro-ApoA-I Differentially Affect Cholesterol Efflux and ApoE Secretion*—Previous studies have indicated that the C terminus of apoA-I is particularly important for the stimulation of cholesterol efflux and binding of apoA-I to the cell surface (49), and this is related to the lipid binding ability of the C terminus (27, 50). We investigated the role of the C terminus in inducing apoE secretion from primary human foam cell macrophages using recombinant pro-apoA-I-

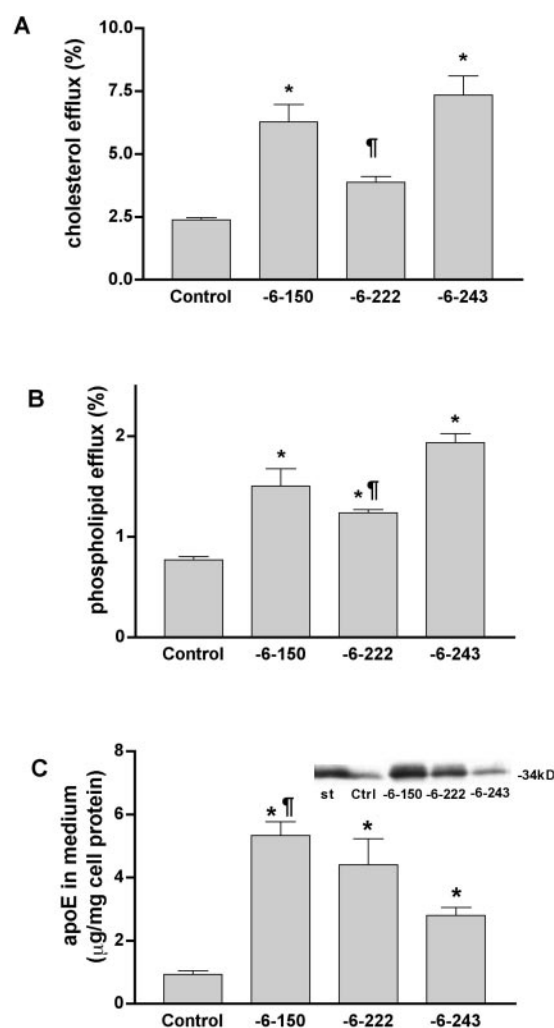


**FIG. 4. ApoA-I stimulates release of cell surface pools of apoE.** HMDM were cholesterol-enriched with AcLDL and radiolabeled with  $^{35}\text{S}$ -methionine/cysteine for 16 h. **A**, [ $^{35}\text{S}$ ]methionine-labeled HMDM were placed on ice ( $4^\circ\text{C}$ ) and cell surface proteins biotinylated as described under "Experimental Procedures." Cells were then incubated in medium without (*Control*) or with  $25\ \mu\text{g}/\text{ml}$  apoA-I (apoA-I) for 30 min at  $37^\circ\text{C}$ . Total  $^{35}\text{S}$ -labeled apoE was immunoprecipitated, the biotinylated pool further immunoprecipitated from this pool, and quantified. Total  $^{35}\text{S}$ -labeled apoE immunoprecipitated from cell cultures at  $T_0$  was  $838,768 \pm 33,266$  dpm/mg cell protein. **B**, [ $^{35}\text{S}$ ]methionine-labeled HMDM were placed on ice and incubated with or without apoA-I for 30 min at  $4^\circ\text{C}$ .  $^{35}\text{S}$ -labeled apoE released to medium was immunoprecipitated and quantified, and represented  $3.2 \pm 0.4\%$  and  $6.1 \pm 1.5\%$  of total cellular apoE at  $T_0$  for *Control* and apoA-I, respectively. \*, significantly different *versus* *Control* ( $p < 0.05$ ).

(-6-243) with truncations of the C terminus (-6-150) and (-6-222) (Fig. 5). Initial studies confirmed that pro-apoA-I and native human apoA-I induced similar cholesterol efflux and apoE secretion (data not shown). Consistent with previous studies in HepG2 cells (27), deletion of the C-terminal 21 amino acids (-6-222) decreased cholesterol efflux relative to whole apoA-I (-6-243), whereas deletion of the C-terminal 93 amino acids (-6-150) had no effect. This correlated with the relative abilities of the proteins to stimulate phospholipid efflux (Fig. 5B). In contrast, both C-terminal deletions increased apoE secretion relative to full-length pro-apoA-I. The discrepant effects of C-terminal deletion on lipid efflux and apoE secretion suggest that induction of apoE secretion is not linked to apoA-I lipid affinity or its stimulation of cholesterol efflux via ABCA1.

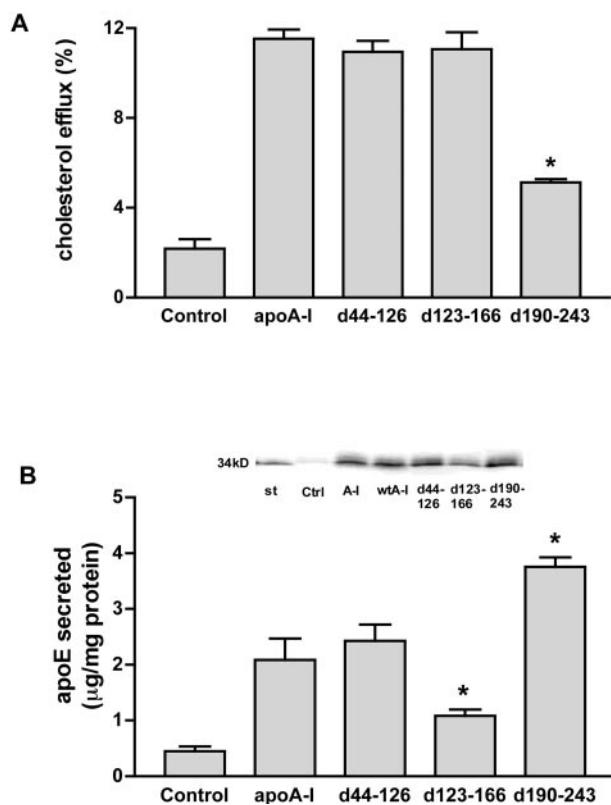
**Selected Deletions and Point Mutations of Recombinant A-I Modulate the Stimulation of ApoE Secretion**—The 6-amino acid residue N-terminal prosegment of pro-apoA-I does not interfere with cholesterol efflux to apoA-I (27). However, as structural requirements for apoE secretion and cholesterol efflux appeared to be dissociated, we studied a second series of recombinant apoA-I molecules lacking the propeptide. The roles of the C terminus, N terminus, and central-protein LCAT activation domains (d123-166) in mediating apoA-I-stimulated apoE secretion and cholesterol efflux were determined (Fig. 6).

Deletion of major domains within the N-terminal segment



**FIG. 5. Truncation of the C-terminal domain of recombinant pro-apoA-I increases apoE secretion from human macrophages.** Monocytes were differentiated, incubated for 4 days with  $50\ \mu\text{g}/\text{ml}$  AcLDL- $[\text{H}]$ cholesterol (**A**), or AcLDL then  $[\text{H}]$ choline (**B**), as described under "Experimental Procedures." Cells were then washed, equilibrated, and incubated with RPMI 1640 (*Control*) or  $25\ \mu\text{g}/\text{ml}$  of indicated recombinant whole pro-apoA-I (-6-243), or pro-apoA-I with varying C-terminal truncations (-6-222 and -6-150). Aliquots of media were removed after 8 h, and cholesterol efflux (**A**), phospholipid efflux (**B**), and apoE secretion (**C**, Western blot *inset*) were measured as described under "Experimental Procedures." Lipid composition after loading (at  $T_0$ ): FC  $38.6 \pm 10.7$  nmol/mg cell protein, CE  $54.9 \pm 5.5$  nmol/mg cell protein, specific activity FC  $17856 \pm 3188$  dpm/nmol, specific activity CE  $22771 \pm 6928$  dpm/nmol. **B**, specific activity PL  $45817 \pm 6110$  dpm/nmol. \*, significantly different *versus* control values ( $p < 0.05$ ); †, significantly different *versus* -6-243 (pro-apoA-I,  $p < 0.05$ ).

(d44-126) or central-protein segment (d123-166) stimulated equivalent cholesterol efflux to that of whole apoA-I, whereas recombinant apoA-I with a C-terminal deletion (d190-243) was significantly less effective than intact apoA-I (Fig. 6A), consistent with previous reports (29). Although d190-243 caused much less cholesterol efflux, it induced significantly more apoE secretion than native, intact apoA-I. Significantly, deletion of the central region of apoA-I (domain d123-166) was important in the stimulation of apoE secretion, as absence of this domain halved apoE secretion relative to whole apoA-I, while having no effect on cholesterol efflux. This is consistent with an important role of apoA-I flexibility, as recently described (29) contributing to apoE secretion. These data also support findings of our experiments using pro-apoA-I indicating that C-terminal mem-



**FIG. 6. Recombinant ApoA-I with selected deletions differentially promote apoE secretion from human macrophages.** Monocytes were differentiated and cholesterol-enriched and incubated with RPMI 1640 (Control), 25 µg/ml whole apoA-I, or 25 µg/ml recombinant apoA-I proteins with deletions of C-terminal (d190–243), N-terminal (d44–126), or central (d123–166) domains. Aliquots of media were removed after 8 h, and cholesterol efflux (A) and apoE secretion (B) were measured as described under “Experimental Procedures.” Lipid composition after loading (at  $t = 0$ ): FC  $79.4 \pm 2.1$  nmol/mg of cell protein, CE  $26.0 \pm 7.3$  nmol/mg of cell protein, specific activity FC  $63622 \pm 3234$  dpm/nmol, specific activity CE  $74321 \pm 15523$  dpm/nmol. Recombinant whole apoA-I and isolated human apoA-I induced identical apoE secretion and cholesterol efflux (data not shown). \*, significantly different versus apoA-I ( $p < 0.05$ ).

brane binding and lipid efflux are not rate limiting in apoA-I-mediated apoE secretion.

**Synthetic Peptides Representing  $\alpha$ -Helical Segments of ApoA-I Induce ApoE Secretion**—ApoA-I contains 10  $\alpha$ -helical segments of 11- and 22-amino acids in length (24). Peptides representing each of the segments induce cholesterol efflux approximately in proportion to the lipid bilayer binding (lipid affinity) of each segment (50). To investigate whether the apparent differences between recombinant apoA-I molecules in their stimulation of apoE secretion were attributable to specific  $\alpha$ -helical domains within apoA-I, particularly in the N-terminal or central domains, we compared apoE secretion to 11- and 22-mer  $\alpha$ -helical peptides representing specific amphipathic  $\alpha$ -helical segments of apoA-I (Table I). Only the C-terminal peptide 220–241 stimulated cholesterol efflux significantly above basal (control medium) level. Although significant, cholesterol efflux to peptide 220–241 was less than that to intact apoA-I (6.2 versus 8.1%  $p < 0.05$ ), consistent with previous literature (50). In contrast to cholesterol efflux, all  $\alpha$ -helical peptides stimulated apoE secretion. This ranged from 2.0- to 6.0-fold that of control medium, and many peptides achieved stimulation of apoE secretion equivalent to that achieved by apoA-I (e.g. peptides 1–33, 44–87, 88–120, 143–186, 220–241). There was no correlation between the known  $\alpha$ -helical content of these peptides (24) and stimulation of apoE secretion ( $R^2 =$

TABLE I

Cholesterol efflux and apoE secretion to  $\alpha$ -helical segments of apoA-I

HMDM were incubated for 8 h with RPMI 1640 medium alone (control), control medium containing 25 µg/ml apoA-I, or control medium containing 25 µg/ml  $\alpha$ -helical peptides representing segments of apoA-I. Values are mean  $\pm$  S.D. from triplicate cultures of one experiment, representative of three experiments. Percent cholesterol efflux was calculated by dividing [ $^3$ H]cholesterol released to the medium by the total [ $^3$ H]cholesterol in the cells after equilibration ( $T_0$ ), and apoE was determined by Western blotting.

ApoA-I peptide	Number of amino acids	Helicity <sup>a</sup>	Cholesterol efflux	ApoE secretion
		%	%	µg/mg cell protein
1–33	33	52	$5.6 \pm 1.1$	$2.3 \pm 0.4^b$
44–87	44	45	$5.9 \pm 1.3$	$2.5 \pm 0.4^b$
66–98	33	30	$4.4 \pm 0.4$	$1.4 \pm 0.6^b$
66–120	55	62	$5.1 \pm 0.6$	$1.9 \pm 0.2^b$
88–120	33	40	$5.0 \pm 0.2$	$2.2 \pm 0.5^b$
99–120	22	48	$4.6 \pm 0.4$	$1.9 \pm 0.4^b$
99–142	44	67	$5.0 \pm 0.1$	$1.5 \pm 0.2^b$
143–164	22	54	$4.4 \pm 0.4$	$2.0 \pm 0.1^b$
143–186	44	40	$4.6 \pm 0.2$	$2.9 \pm 1.1^b$
165–186	22	42	$4.6 \pm 0.3$	ND <sup>c</sup>
165–208	44	55	$5.1 \pm 0.6$	$1.7 \pm 0.3^b$
187–208	22	36	$5.6 \pm 0.5$	$1.7 \pm 0.4^b$
187–219	33	44	$3.8 \pm 0.3$	$1.0 \pm 0.2^b$
220–241	22	54	$6.2 \pm 0.1^b$	$3.4 \pm 0.8^b$
ApoA-I	243	93	$8.1 \pm 0.3^b$	$2.5 \pm 0.3^b$
Control			$4.3 \pm 0.8$	$0.5 \pm 0.2$

<sup>a</sup> Taken from Mishra *et al.* (24).

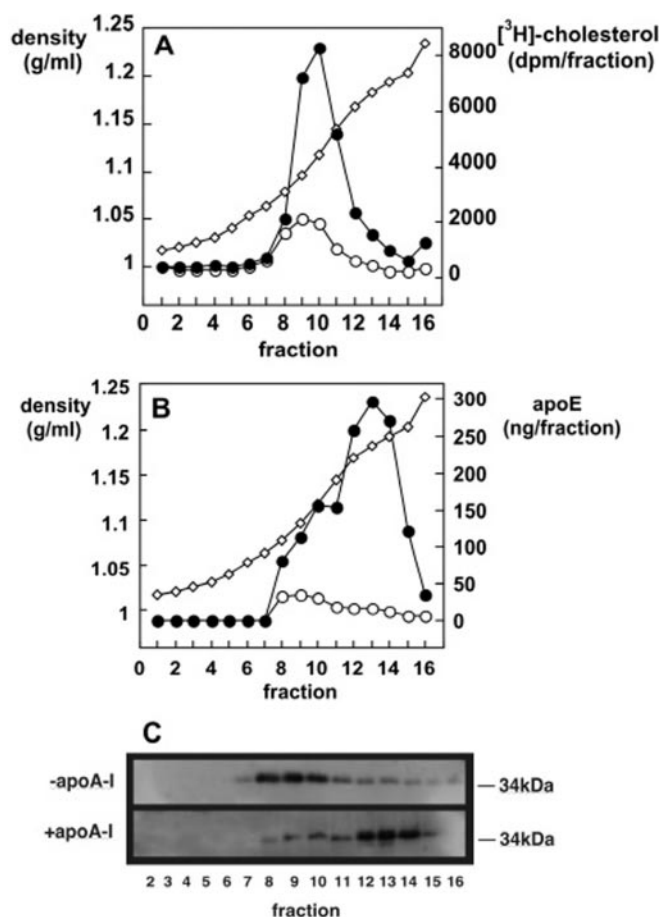
<sup>b</sup> Significantly different from RPMI 1640 by one-way analysis of variance.

<sup>c</sup> Not determined.

0.002,  $p = 0.87$ , data not shown). The peptides studied represent segments of the N terminus, central region, and C terminus of apoA-I, many of which display low lipid affinity (50). This indicates that stimulation of apoE secretion by whole apoA-I or recombinant apoA-I molecules was not attributable to individual  $\alpha$ -helical segments or isolated domains within apoA-I.

**Buoyant Density of ApoE Secreted in Response to ApoA-I**—Previous studies have shown that secreted apoE is predominantly lipidated (density  $< 1.21$  g/ml) (12, 51), and a proportion of secreted apoE and cell surface apoE are lipid-poor (48, 52). As apoA-I-mediated apoE secretion appeared independent of apoA-I lipid affinity and apoA-I-mediated lipid efflux, we investigated whether apoA-I induced secretion of non-lipidated, non-buoyant, apoE (Fig. 7). Under basal conditions (without apoA-I), secreted apoE was found in a buoyant density range of 1.07–1.22 g/ml, with a predominant density of 1.08–1.1 g/ml. Addition of apoA-I, stimulated an increase in apoE across the density range of 1.07–1.22 g/ml, although the proportion present in the less buoyant fractions (density 1.18–1.20 g/ml) was much greater than controls. The density distribution of cell-derived cholesterol in media was the same under basal and stimulated conditions (1.07–1.17 g/ml), corresponding to the more buoyant apoE fractions. Thus, apoE secreted under control and stimulated conditions was buoyant; however, in the presence of apoA-I, a significant proportion was more dense than cholesterol-containing particles in the medium. These observations are consistent with independent regulation of apoE secretion and cholesterol efflux during apoA-I exposure and suggest that apoA-I induces a pathway of apoE secretion distinct from that involved in basal apoE secretion.

**Stimulation of ApoE Secretion Is Induced by Other  $\alpha$ -Helix-containing Apolipoproteins**—The broad specificity for stimulation of apoE secretion by  $\alpha$ -helical peptides of apoA-I suggested that other amphipathic-containing apolipoproteins might induce apoE secretion. Such apolipoproteins (specifically apoA-II and apoA-IV) (53) also stimulate cholesterol and phospholipid

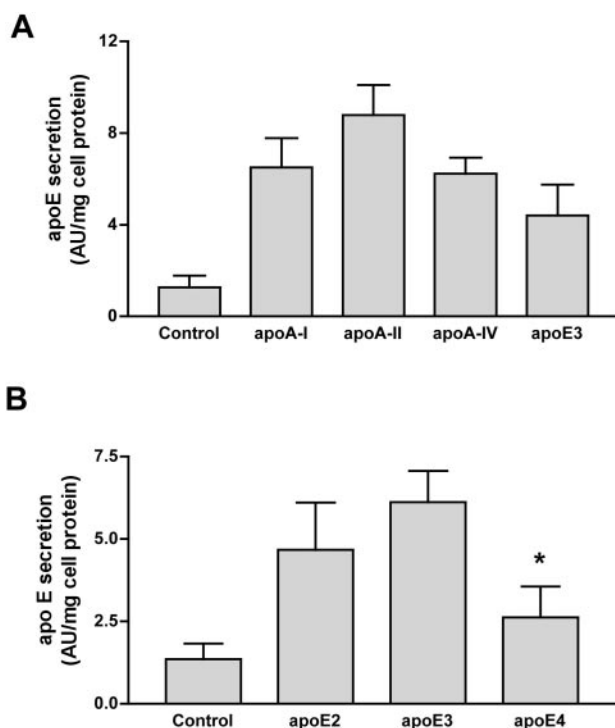


**FIG. 7. Density of secreted apoE with or without stimulation by apoA-I.** Monocytes were differentiated, loaded for 4 days with 50  $\mu\text{g}/\text{ml}$  AcLDL- $^3\text{H}$ cholesterol and then washed, equilibrated, and incubated with control medium  $\pm$  25  $\mu\text{g}/\text{ml}$  apoA-I. Aliquots of media were subjected to ultracentrifugation on 1.33–65% sucrose gradients and analyzed for  $^3\text{H}$ cholesterol, and apoE as described under “Experimental Procedures.” **A**, cholesterol efflux without (open circles) and with apoA-I (closed circles); **B**, apoE secretion without (open circles) and with apoA-I (closed circles). Density of fractions is indicated (open diamonds). Total cholesterol efflux for control and apoA-I were  $3.6 \pm 0.4$ , and  $8.9 \pm 2.0\%$ , respectively, and total apoE secretion for control and apoA-I were  $0.4 \pm 0.1$  and  $3.1 \pm 0.3 \mu\text{g}/\text{ml}$ , respectively. In **C**, fractions analyzed by Western blot, after diluting media from cultures exposed to apoA-I 4.0-fold to facilitate comparison of relative distribution of apoE among fractions. Data are representative of three independent experiments.

efflux from cells (50), but vary markedly in their total  $\alpha$ -helical content and hydrophobicity (54). We incubated these apolipoproteins with HMDM under saturating conditions (all 25  $\mu\text{g}/\text{ml}$ ) and found that human apoA-I, apoA-II, and apoA-IV stimulated apoE secretion equally effectively (Fig. 8). They also induced similar modest cholesterol efflux from primary human macrophages, ranging from 8–15% over 8 h (2–3-fold that of control medium), and there was no significant difference between the apolipoproteins in induction of cholesterol efflux over multiple independent experiments.

ApoE is also HDL-associated, contains  $\alpha$ -helices and, if extracellular apoE were to stimulate secretion of apoE by macrophages, it may mediate a potentially anti-atherogenic auto-crine loop in the artery wall. ApoE did stimulate the secretion of *de novo* synthesized  $^3\text{S}$ -labeled-apoE, and was comparable in its efficiency to apoA-I, apoA-II, and apoA-IV.

The three apoE isoforms (e.g. E2, E3, E4) differ in their effects on the risk of atherosclerosis and differ substantially in  $\alpha$ -helix organization, N- and C-terminal domain organization (55), and in the respective lipid binding properties of the C



**FIG. 8. ApoE secretion is stimulated by apolipoproteins other than apoA-I.** Monocytes were differentiated, cholesterol-enriched with AcLDL, and radiolabeled with  $^3\text{S}$ methionine, before incubation with 25  $\mu\text{g}/\text{ml}$  of each indicated apolipoprotein. **A**, aliquots of media were removed after 8 h, apoE immunoprecipitated, and secretion to media containing apoA-I, apoA-II, apoA-IV, apoE3, determined as AU of  $^3\text{S}$ apoE per mg of cell protein by phosphorimaging of 34-kDa band as described under “Experimental Procedures.” ApoE secretion to apoA-I, A-II, and A-IV was confirmed by Western blot as well as by phosphorimaging. In **B**, all data are expressed as AU of  $^3\text{S}$ methionine-labeled apoE secreted/mg cell protein. Cholesterol efflux to all apolipoproteins was 2–3-fold that of control, and did not differ between apolipoproteins (data not shown). \*, significantly different versus apoE3 ( $p < 0.05$ ).

terminus (56). We therefore investigated whether these structural differences affected their stimulation of apoE secretion. All three isoforms stimulated macrophage apoE secretion above basal levels. However, apoE3 stimulated more apoE secretion than did apoE4 ( $p < 0.01$ ). In contrast, cholesterol efflux to the three apoE isoforms did not differ (data not shown), consistent with a previous study (2). On non-reducing SDS-PAGE, apoE3 and E2, which contain one and two cysteine molecules respectively, self-associated into trimers and tetramers which were reducible with dithiothreitol, whereas apoE4 did not (data not shown). Thus it is possible that either the N- and C-terminal organization of exogenous apoE4, or its lesser self-association, may contribute to lesser apoE secretion to this isoform.

**ApoA-I Maintains the Ability to Stimulate ApoE Secretion in Its Lipid-bound Conformation**—When complexed with phospholipids (e.g. nascent HDL discs) apoA-I undergoes conformational change, which increases the  $\alpha$ -helicity of the molecule (54, 57), and potentially increases the exposure of polar residues to the cell surface. Such changes may affect the efficacy of apoA-I-mediated apoE secretion in lipidated particles. ApoE secretion may thus be diminished if critical domains of apoA-I are obscured by lipidation or may be enhanced if greater exposure of polar domains enhances apoE secretion. There is also potential for synergy between phospholipid and apoA-I, as previous studies have identified a lipid-soluble pool of apoE associated with the extracellular matrix of HepG2 cells and macrophages (13, 41, 48, 58).

ApoA-I-phospholipid discs containing POPC (A-I-POPC),

TABLE II  
Lipidation enhances apoE secretion to apoA-I but not apoA-II

	Diameter	Stoichiometry	Concentration of acceptor	Cholesterol efflux	ApoE secretion
	nm	POPC/SPM/apoA-I mol/mol	μg/ml	relative to control	relative to control
<b>A<sup>a</sup> efflux medium</b>					
RPMI (control)				1.0 ± 0.07	1.0 ± 0.64
ApoA-I			25.0	2.08 ± 0.11 <sup>b</sup>	2.5 ± 0.36
POPC	2 × 10 <sup>2</sup>		78.0	0.99 ± 0.21	1.7 ± 0.11
POPC/apoA-I	9.9	115.7/0/1.0	78.0/0.0/25.0	2.22 ± 0.24 <sup>b</sup>	5.1 ± 0.68 <sup>b,c</sup>
POPC/SPM/apoA-I	9.7	76.4/19.4/1.0	50.9/9.2/25.0	2.95 ± 0.38 <sup>b,c</sup>	6.3 ± 0.51 <sup>b,c</sup>
<b>B<sup>d</sup> efflux medium</b>					
RPMI (control)				1.0 ± 0.31	1.0 ± 0.24
ApoA-II			25.0	1.77 ± 0.35 <sup>e</sup>	2.45 ± 0.17 <sup>e</sup>
POPC	2 × 10 <sup>2</sup>		104.0	1.46 ± 0.15	1.79 ± 0.35
POPC/apoA-II	9.7/10.6	48.2/1.0	104.0/25.0	3.23 ± 0.31 <sup>e,f</sup>	2.85 ± 0.46 <sup>e</sup>

<sup>a</sup> Part A, ApoA-I: HMDM were incubated with or without apoA-I, POPC or apoA-I-phospholipid discs for 8 h. Results of cholesterol efflux and apoE secretion all normalized to control (RPMI 1640) to facilitate comparison of changes to cholesterol efflux and apoE secretion resulting from lipidation.

<sup>b</sup> Significantly different from RPMI 1640 (control) and POPC ( $p < 0.05$ ).

<sup>c</sup> Significantly different from apoA-I ( $p < 0.05$ ).

<sup>d</sup> Part B, ApoA-II: HMDM were incubated with or without apoA-II, POPC or apoA-II discs for 8 h.

<sup>e</sup> Significantly different ( $p < 0.05$ ) from RPMI 1640.

<sup>f</sup> Significantly different from apoA-II ( $p < 0.05$ ).

POPC/SPM (A-I-POPC/SPM) were compared with POPC liposomes and lipid-free apoA-I for their ability to induce cholesterol efflux and apoE secretion (Table II). Acceptors were added at concentrations matched for apoA-I content to allow accurate comparisons of efficiency in stimulating apoE secretion. Lipidation of apoA-I with POPC significantly increased apoE secretion 2–3-fold relative to apoA-I alone, whereas POPC alone did not significantly stimulate apoE secretion. These data suggest that lipidation of apoA-I promotes apoE secretion, and this might be caused by secondary conformational changes, such as increased exposure of polar residues of apoA-I to the cell surface.

Pre-β-HDL are important initial acceptors of cell cholesterol (59) and represent a dynamic pool of apoA-I, which may play a role in stimulating of apoE secretion in the artery wall. As pre-β-HDL contain increased concentrations of SPM relative to more mature HDL (59), we investigated whether SPM modulated the ability of apoA-I to stimulate apoE secretion and whether cholesterol efflux was modulated in parallel (Table II). A-I-POPC/SPM particles containing 25% SPM and 75% POPC achieved 1.4-fold ( $p < 0.05$ ) the cholesterol efflux achieved by A-I-POPC, but did not further increase apoE secretion relative to A-I-POPC. This indicates that the synergistic interaction between apoA-I and phospholipids in inducing apoE secretion is independent of variations of phospholipid subtype.

ApoA-II has higher average residue hydrophobicity than apoA-I (54). Consequently, its conformation in a POPC disc may differ from that of apoA-I. Unlike secretion to apoA-I, apoA-II-stimulated apoE secretion was not increased significantly by its lipidation with POPC, even though cholesterol efflux was enhanced (Table II, Part B). This suggests that a relatively specific conformation change in apoA-I, which is not seen in apoA-II, is important for the enhanced stimulation of apoE secretion by apoA-I following its lipidation.

To investigate whether secreted apoE associated with apoA-I-POPC particles, density gradient analysis, and non-denaturing gel electrophoresis was performed on efflux media. ApoE shared the same buoyant density as effluxed cholesterol and apoA-I in media containing apoA-I-POPC discs (density range 1.042–1.141). This differed from media containing lipid-free apoA-I, where effluxed cholesterol was of a more buoyant density than secreted apoE, and was consistent with the possibility that apoE associated with apoA-I-POPC discs. This was directly investigated by non-denaturing gel electrophoresis of

media containing apoA-I-POPC and apoA-II-POPC discs, followed by electrophoretic transfer to nitrocellulose membranes and immunoblotting for apoA-I, apoA-II, and apoE (33). After efflux, apoA-I and apoA-II migrated as POPC discs (9.5–10.8-nm diameter and 9.5–10.6-nm diameter, respectively), without evidence of displacement of lipid-free apoA-I or apoA-II from these discs (data not shown). Most secreted apoE migrated as large diameter (17.1 nm) particles, and a small proportion migrated as particles of the same diameter as apoA-I- or apoA-II-POPC-containing discs (9.5–10.8-nm diameter). The association of apoE with apoA-I-POPC or apoA-II-POPC discs would be expected to increase the size of these particles. As the size of apoA-I-POPC or apoA-II-POPC particles was unaltered by secretion of apoE, these data suggest that secreted apoE does not significantly associate with apoA-I- or apoA-II-POPC discs, and does not displace apoA-I or apoA-II from these particles. These results also imply that the amount of apoE secreted to apoA-I-POPC or apoA-II-POPC particles is not explained by differential displacement of apoA-I and apoA-II from POPC particles.

**ApoA-I-mediated ApoE Secretion Is Independent of ABCA1 Activity**—ApoA-I-mediated cholesterol efflux is known to be critically dependent upon ABCA1 activity. Our data dissociating the structural requirements of apoA-I-stimulated apoE secretion and cholesterol efflux imply that apoA-I-mediated apoE secretion may be independent of ABCA1. Patients with Tangier disease have impaired ABCA1 activity, and, although a previous study indicated that basal apoE secretion was impaired in TD macrophages (16), apoA-I-stimulated apoE secretion was not investigated. To directly investigate the role of ABCA1 in apoE secretion from human macrophages, we studied macrophages of two unrelated patients with molecular, clinical, and cellular phenotypes of TD.

The first subject with TD (TD1, see “Experimental Procedures”), was a compound heterozygote for the novel ABCA1 mutations C733R in exon 16 and a deletion of three nucleotides (c.5220–5222 delTCT) in exon 38 (TD1-HMDM). ApoA-I-mediated cholesterol efflux from TD1-HMDM was severely impaired relative to an unrelated healthy control (HC1)-HMDM, confirming the cellular TD phenotype (Table III, A). Basal apoE secretion was lower from TD1-HMDM than from HC1-macrophages, consistent with an earlier study (16). However, apoA-I strongly stimulated apoE secretion from both TD1-HMDM (11-fold increase,  $p < 0.01$  relative to control media) and control

TABLE III  
*ApoA-I-induced apoE secretion is normal in HMDM from subjects with Tangier Disease*

Note that apoA-I-stimulated apoE secretion did not significantly differ between the subjects in panels A or B. All cellular data are mean  $\pm$  S.D. of three cell cultures for each subject.

Subject	Mutation	HDL-C	Basal apoE secretion	ApoA-I-specific cholesterol efflux	ApoA-I-stimulated apoE secretion
		mg/dL	$\mu\text{g}/\text{mg cell protein}$		$\mu\text{g}/\text{mg cell protein}$
A <sup>a</sup>					
HC1		44.0 <sup>b</sup>	2.2 $\pm$ 0.49 <sup>b</sup>	3.1 $\pm$ 0.5 <sup>b</sup>	6.3 $\pm$ 0.9
TD1	C733R;C.5220–5222delTCT	3.1	0.5 $\pm$ 0.21	0.5 $\pm$ 0.5	5.6 $\pm$ 1.2
B <sup>c</sup>					
HC2		43.0 <sup>d</sup>	1.1 $\pm$ 1.6	2.2 $\pm$ 0.4 <sup>d</sup>	4.2 $\pm$ 1.0
TD2 <sup>d</sup>	A1046D;4629insA	2.7	ND <sup>e</sup>	0.2 $\pm$ 0.2	3.8 $\pm$ 0.7
HZ2A	4629insA	25.8 <sup>d</sup>	ND	1.9 $\pm$ 0.3 <sup>d</sup>	2.6 $\pm$ 0.6
HZ2B	A1046D	38.6 <sup>d</sup>	1.4 $\pm$ 0.2	2.8 $\pm$ 0.1 <sup>d</sup>	4.5 $\pm$ 0.9

<sup>a</sup> Part A: macrophages from newly characterized TD1 and unrelated control subject HC1 were compared for cholesterol efflux and apoE secretion in the presence and absence of 25  $\mu\text{g}/\text{ml}$  apoA-I. Cholesterol efflux and apoE secretion were determined at 8 h, and apoA-I-specific efflux derived by subtracting efflux to control medium (without A-I) from that to apoA-I.

<sup>b</sup> Conditions are significantly different between HC1 and TD1,  $p < 0.01$ .

<sup>c</sup> Part B: TD2 has previously been described (44). Macrophages from TD2, his normal brother (HC2), and his heterozygous parents (HZ2A, HZ2B), were compared for cholesterol efflux and apoE secretion in the presence and absence of apoA-I as in part A.

<sup>d</sup> Statistically significant ( $p < 0.001$ ) from TD2.

<sup>e</sup> Not detectable via Western blot analysis (based on amount of protein subjected to SDS-PAGE,  $<0.3 \mu\text{g}$  of apoE/mg of cell protein).

HMDM (3.4-fold increase,  $p < 0.01$  relative to control media), achieving the same total apoE secretion from TD1-HMDM and HC1-HMDM. Thus, despite severely impaired ABCA1-mediated cholesterol efflux, apoA-I stimulated apoE secretion normally from TD macrophages.

To exclude a mutation-specific effect, macrophages from a second unrelated Tangier Patient (TD2-HMDM, see "Experimental Procedures") were studied. Cells from TD2, who is compound heterozygote for mutations A1046D and c.4629–4630insA, were compared with those of his healthy brother known to be free of ABCA1 mutations (HC2-HMDM), and his heterozygous parents with mutations 4629insA (HZ2A-HMDM) and A1046D (HZ2B-HMDM) (44, 45) (Table III, B). Basal cholesterol efflux was similar in the four subjects ( $0.9 \pm 0.2\%$  for HC2-HMDM,  $1.3 \pm 0.5\%$  for TD2-HMDM,  $0.9 \pm 0.2\%$  for HZ2A-HMDM and  $0.8 \pm 0.3\%$  for HZ2B-HMDM). ApoA-I-mediated cholesterol efflux from TD2 cells was severely impaired relative to all other family members, confirming dysfunctional ABCA1. Despite this, apoA-I strongly and equally increased apoE secretion from all macrophages, including TD2-HMDM. These findings support our structural studies and clearly dissociate apoA-I-induced cholesterol efflux via ABCA1, from apoA-I-induced apoE secretion.

#### DISCUSSION

The secretion of apoE by macrophages has been shown to be anti-atherogenic (11), and factors that enhance its secretion may be important targets for the treatment or prevention of atherosclerosis. We have demonstrated that apoA-I stimulates the secretion of a mobile pool of apoE which is only in part derived from the cell surface, and which is otherwise destined for intracellular degradation. We have also identified for the first time a biological activity of apoA-I that is independent of its lipid binding properties, by demonstrating that apoA-I-stimulated apoE secretion is enhanced by C-terminal deletions and is independent of ABCA1-mediated cholesterol efflux. These data establish that non-ABCA1-mediated processes contribute to prosecretory roles and potentially anti-atherogenic effects of apoA-I.

In previous studies, including our own, the secretion of apoE after exposure of cells to HDL or apoA-I has been concurrent with the efflux of cholesterol (12–15,60). However, cyclodextrin-stimulated cholesterol efflux in itself does not induce apoE secretion, and acute inhibition of apoE synthesis and secretion with cycloheximide does not inhibit cholesterol efflux to apoA-I (13). The latter observations dissociate stimulation of apoA-I-

mediated cholesterol efflux and apoE secretion. A similar dissociation was earlier reported for HDL-mediated cholesterol efflux and apoE secretion (61). We have investigated in detail the structural and functional requirements for apoA-I-induced apoE secretion and identify these as being quite distinct from those required for apoA-I-induced cholesterol efflux.

ApoA-I does not increase macrophage apoE mRNA levels, and pulse-chase experiments demonstrate that apoA-I stimulates secretion of newly synthesized apoE, confirming that apoA-I-stimulates apoE secretion post-transcriptionally. Modeling of pulse-chase data indicated that human macrophages contain a larger mobile pool of apoE ( $E_m$ ), and a lesser, more stable pool of apoE ( $E_s$ ). Previous studies indicate that such heterogeneous pools of apoE may include apoE on the cell surface (47). Pronase treatment of human macrophages under our experimental conditions indicates that only a small proportion of  $E_s$  is on the cell surface.<sup>2</sup>  $E_m$  is, on the basis of our biotin-labeling and 4 °C displacement studies, in part derived from the cell surface pool of apoE, and apoA-I increases secretion and concurrently diminishes degradation of apoE from this pool. However, as the biotin-labeled pool represents only a minor proportion of total apoE secreted ( $<2\%$ ), its quantitative significance is unclear. These data are consistent with either continual displacement of apoE from the cell surface with its recycling and ongoing replenishment from intracellular pools (41), or is consistent with two sources of secreted apoE, a smaller cell surface pool and a larger intracellular pool, both of which are mobilized by apoA-I.

It is clear that the structural requirements for apoA-I-mediated apoE secretion are distinct from those required for cholesterol efflux. Our studies of recombinant (pro-)apoA-I molecules demonstrate that deletion of the C terminus of apoA-I enhances apoE secretion whereas it impairs cholesterol efflux and phospholipid efflux. This is remarkable as all previous studies of apoA-I function have emphasized the importance of its lipid binding C-terminal domain for functional integrity and for binding to the extracellular matrix of the cell surface (62). That C-terminal deletion enhances apoE secretion suggests that apoA-I does not bind to extracellular matrix to achieve this (62). This is consistent with our previous studies indicating that heparinase pretreatment does not inhibit apoA-I-mediated apoE secretion from primary macrophages (13).

The central domain of apoA-I regulates LCAT activity (63)

<sup>2</sup> M. Kockx, W. Jessup, and L. Kritharides, unpublished observations.

and HDL-mediated inhibition of macrophage homing to atherosclerotic plaque (64). Deletion of this central region of apoA-I (d123–166) was the only modification that decreased the stimulation of apoE secretion by apoA-I. As this domain affects apoA-I flexibility (29), it suggests that for whole apoA-I, flexibility is more important than lipid affinity in inducing apoE secretion. It is unlikely to contain an epitope specific for apoA-I-induced apoE secretion given that  $\alpha$ -helical peptides away from this domain, and other apolipoproteins, also induced apoE secretion. Whether apolipoprotein flexibility is also important for other apolipoproteins, e.g. apoA-II, apoA-IV, and apoEs, is unknown.

Systematic comparison of individual  $\alpha$ -helical peptides of apoA-I identified that only the C-terminal  $\alpha$ -helical peptide was capable of independently inducing cholesterol efflux from primary human macrophages, consistent with earlier studies (50). In contrast, all  $\alpha$ -helical peptides stimulated apoE secretion. As cholesterol efflux to peptides is related to their lipid affinity (50), this property does not appear to determine induction of apoE secretion. Stimulation of apoE secretion appears to be a general structural property of  $\alpha$ -helical proteins and peptides, which, in the case of whole apoA-I, can be enhanced by overall protein flexibility and inhibited by lipid binding to the plasma membrane.

One of our most striking observations was the enhanced secretion of apoE to apoA-I-POPC discs relative to apoA-I. This was not observed with apoA-II, which stimulated apoE secretion to the same extent in free or lipid-bound state. The difference in response to lipidation of apoA-I and apoA-II is interesting given that cholesterol efflux to both apolipoproteins increased with lipidation. Apolipoprotein-mediated apoE secretion may allow subsequent binding of secreted apoE to exogenous phospholipids, and this could explain some apparent synergy between apoA-I and POPC. However, association of apoE with apoA-I- or apoA-II-POPC discs would be expected to require displacement of apoA-I or apoA-II and/or to increase the apparent size of POPC-containing particles, neither of which were found on non-denaturing gel electrophoresis. The absence of synergy between apoA-II and POPC therefore suggests conformational changes unique to apoA-I occur during its lipidation. We suggest that apoA-I exposes more polar, non-lipid bound residues as a POPC disc than does apoA-II, and that this enhances interaction with exposed residues of apoE on the cell surface, or with undefined plasma membrane sites critical for stimulating vesicular trafficking of apoE to the cell surface.

ApoA-I, A-II, A-IV, E3, and apolipoprotein-phospholipid discs all stimulated apoE secretion efficiently, indicating stimulation of apoE secretion is of broad relevance *in vivo*. The ability of apoE to stimulate apoE secretion is highly significant, as local (autocrine) secretion of apoE in the artery wall could mediate further apoE secretion from neighboring cells, independently of the requirement for plasma-derived HDL species. ApoE4 increases the risk of atherosclerosis and Alzheimer's disease relative to apoE3 (65). Reduced local stimulation of apoE secretion by apoE4 in the artery or in the CNS might contribute to disease states associated with the apoE4 phenotype. ApoE4 differs from apoE3 in a single amino acid substitution, arginine instead of cysteine in position 112. This confers a more positive apoE charge, and causes conformational change to the N-terminal helix of apoE4, as a salt bridge forms between Arg<sup>112</sup> and Glu<sup>109</sup>, and this alters the conformation of Arg<sup>61</sup>, which interacts with Glu<sup>255</sup> (55). E4 also differs from E3 and E2 in lacking the potential for disulfide formation and self-association. In a previous study, Smith *et al.* (2) identified that the binding of E2, E3, and E4 to RAW macrophages, and the cholesterol efflux induced by these isoforms, were the same.

Our studies confirmed similar efflux to the various apoE isoforms, supporting that differences in inducible apoE secretion cannot be explained by altered efflux, or binding. A recent study indicates that apoE4 has a higher lipid affinity than other apoE isoforms (56), and in this respect, these data support our observations that C-terminal deletions of apoA-I, which diminish its lipid affinity enhance its ability to induce apoE secretion.

That apoA-I-mediated cholesterol efflux and apoE secretion are independently regulated was directly confirmed by apoA-I-stimulated apoE secretion in two unrelated subjects with clinical, molecular, and cellular phenotype of Tangier disease. Despite impaired ABCA1-mediated cholesterol efflux and severely reduced basal apoE secretion, apoA-I stimulated apoE secretion normally. Recent studies have shown that apoA-I-mediated cholesterol efflux via ABCA1 is linked to accelerated secretory vesicular transport from the Golgi to the plasma membrane, and this is absent in Tangier disease fibroblasts (66). As apoA-I stimulated apoE secretion normally from Tangier disease macrophages, we conclude that apoA-I must stimulate a distinct pathway of apoE secretion unrelated to its interactions with ABCA1 or its stimulation of cholesterol efflux. As apoE secretion induced by apoA-I is severalfold greater than basal apoE secretion, ABCA1-independent secretion is likely to be quantitatively more important than that related to ABCA1 activity. To our knowledge these findings represent the first reported non-ABCA1-mediated cellular process mediated by apoA-I.

It is unlikely that apoA-I stimulates apoE secretion by directly inhibiting lysosomal or proteasomal degradation of apoE. ApoE is degraded in the lysosomal and proteasomal compartments (67) (68), and, although direct inhibition of lysosomal and cysteine proteases does inhibit apoE degradation, this does not increase apoE secretion (58). The possibility that apoA-I exchanges with cellular apoE such that apoA-I is degraded in place of apoE also seems unlikely, as the mass of apoA-I degraded corresponds to <30% of the mass of apoE secreted from human macrophages during time course experiments.<sup>3</sup> It is more likely that apoA-I promotes apoE secretion by inhibiting its trafficking to degradatory compartments such as the lysosome and proteasome, or by stimulating vesicular traffic to the plasma membrane analogous to effects described in fibroblasts (66). Although HDL stimulates a range of protein kinase C-dependent and -independent signaling pathways, these are not necessarily activated by apoA-I (69), and the mechanisms by which apoA-I regulates vesicle traffic in general, and protein secretion in particular, require further investigation.

We conclude that apoA-I-mediated apoE secretion has structural requirements distinct from those regulating the stimulation of cholesterol efflux, facilitated by the central hinge region of apoA-I but inhibited by its C-terminal domain, and is independent of macrophage ABCA1 activity. This represents a novel, ABCA1-independent, positive feedback process for secretion of anti-atherogenic, cholesterol-efflux-inducing apoE by  $\alpha$ -helix-containing molecules.

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<sup>3</sup> K. Gaus, M. Kockx, W. Jessup, and L. Kritharides, unpublished observations.

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