Triacylglycerol-rich lipoproteins alter the secretion, and the cholesterol-effluxing function, of apolipoprotein E-containing lipoprotein particles from human (THP-1) macrophages

E. Marie LINDHOLM, Anna M. PALMER and Annette GRAHAM[1]

Department of Biochemistry and Molecular Biology, Royal Free and University College Medical School of University College London, Royal Free Campus, Rowland Hill Street, London NW3 2PF, U.K.

Elevated plasma levels of triacylglycerol-rich lipoproteins (TGRLP) are associated with increased risk of atherogenesis and abnormal reverse cholesterol transport, as illustrated in Type II diabetes. Here we examine the effect of plasma triacylglycerol-rich or cholesteryl ester-rich lipoproteins on the secretion of nascent apolipoprotein E (apoE)-containing lipoprotein E (LpE) particles by human (THP-1) macrophages. As expected, preincubation with low-density lipoprotein (LDL) yielded small but significant increases in total cellular cholesterol content and also the secretion of apoE by macrophages. By contrast, preincubation with TGRLP resulted in higher, dose-dependent, increases in apoE secretion that reflected, but were not dependent on, cellular triacylglycerol accumulation. Secreted apoE was incorporated into a pre-β migrating LpE fraction that differed in lipid composition and flotation density depending on preincubation conditions. Specifically, the LpE-containing lipoprotein fraction produced by macrophages preincubated with TGRLP was cholesterol-poor, markedly heterogeneous and of higher peak flotation density (d 1.14–1.18) when compared with particles produced after preincubation with LDL. Both the conditioned medium and the isolated ($d < 1.21$) LpE-containing fraction, yielded by macrophages preincubated with TGRLP, seemed poorer at inducing cholesterol efflux than the equivalent fractions from cells preincubated with LDL, as judged by [^{3}H]cholesterol efflux from untreated 'naïve' macrophages. Thus, although the interaction of TGRLP with macrophages can enhance apoE output from these cells, the LpE particles produced seem to be relatively inefficient mediators of cholesterol efflux. These factors might contribute to the increased risk of atherosclerosis in individuals with Type II diabetes.

Key words: atherosclerosis, dyslipidaemia, high-density lipoprotein, lipoprotein E, Type II diabetes.

INTRODUCTION

Elevated levels of triacylglycerol-rich lipoproteins (TGRLP), including chylomicrons, chylomicron remnants, very-low-density lipoproteins and intermediate-density lipoproteins, are an independent risk factor for premature coronary artery disease [1–3]. The association between plasma triacylglycerol levels and atherosclerosis is particularly apparent in conditions associated with elevated levels of TGRLP, such as Type II (non-insulin dependent) diabetes ('NIDDM') [4], Type III hyperlipoproteinaemia [5] and familial combined hyperlipidaemia [6]. Current research suggests that TGRLP might contribute directly to the atherosclerotic process [7] by triggering endothelial dysfunction [8], enhanced monocyte adhesion [9] and lipid accumulation within the artery wall [10]. Undegraded TGRLP and their remnants are present within human and experimental atherosclerotic lesions [11–13]; Proctor and Mamo [13] provided visual evidence that chylomicron remnants directly transgress the endothelial cell layer and become trapped within the subendothelial space, leading to focal accumulation of these pro-atherogenic lipoproteins.

Exposure to TGRLP, particularly those isolated from patients with Type II diabetes [14], induces the intracellular accumulation of triacylglycerol and/or cholesteryl ester in human monocyte-derived [14,15] and murine [10,16] macrophages. Indeed, TGRLP are the only native, non-modified lipoproteins to cause macrophage 'foam cell' formation *in vitro* [10,14–17]. Elevated levels of plasma TGRLP also seem to cause foam cell formation *in vivo*. Macrophage foam cells are found in bone marrow, spleen and skin in hypertriglyceridaemic subjects, with persistent chylomicrons/remnants in the postprandial state [18]; similarly, patients with Type II diabetes can also develop eruptive xanthomas consisting of foam cells laden with triacylglycerol and cholesteryl ester [19]. The process of lipid deposition within arterial macrophages might be exacerbated by the abnormal reverse cholesterol transport associated with hypertriglyceridaemia [20–23]. In general, high-density lipoprotein (HDL) isolated from individuals with Type II diabetes and/or coronary heart disease are characterized by their small size, enrichment in triacylglycerol and depletion of free cholesterol, and show a decreased ability to induce reverse cholesterol transport [20–23].

Intriguingly, evidence exists indicating that TGRLP might directly affect at least one key component of the reverse cholesterol transport pathway by enhancing the secretion of apolipoprotein E (apoE) by human monocyte-derived macrophages [24]. The secretion of apoE by macrophages is anti-atherogenic in the absence of changes in serum lipoproteins [25,26] and can enhance the removal of cholesterol from the vessel wall [26]. In particular, the endogenous production of lipoprotein particles containing apoE as their sole apolipoprotein (lipoprotein E, LpE) mediates the removal of cholesterol from human monocyte-derived macrophages in the absence of serum or exogenous (apo)lipoprotein 'acceptors' [27,28]. Similarly, low-dose

Abbreviations used: apoE, apolipoprotein E; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LpE, lipoprotein E; TGRLP, triacylglycerol-rich lipoproteins.

[1] To whom correspondence should be addressed (e-mail agraham@rfhsm.ac.uk).

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expression of the human apoE3 transgene by murine apoE$^{-/-}$ macrophages restores the diminished cholesterol effluxing capacity of plasma isolated from these animals [29] and represses the rampant atherogenesis seen in apoE-deficient mice [25,26,29]. Nascent LpE can also interact with exogenous 'acceptors' such as lipid-poor apoAI and might 'shuttle' cellular cholesterol to large acceptor particles. Indeed, the presence of exogenous apoAI enhances both apoE secretion and cholesterol efflux from macrophages [30] and further decreases lesion size in apoE-deficient mice transplanted with apoE-expressing macrophages [31]. Finally, macrophage-derived apoE might exert indirect effects on the reverse cholesterol transport pathway by altering the plasma lipoprotein distribution of apoAI [32] or by activating lecithin:cholesterol acyltransferase ('LCAT') [33] and hepatic lipase [34].

Here we examine the hypothesis that increases in the secretion of apoE/LpE by human macrophages, after exposure to TGRLP [24], might enhance cholesterol efflux from macrophages and provide some protection against the accelerated atherogenesis seen in Type II diabetes. We determine the effects of preincubation with TGRLP, or native low-density lipoprotein (LDL), on the secretion, physical properties and cholesterol effluxing function of the apoE-containing lipoprotein fraction produced by differentiated human (THP-1) macrophages. The results show that although the interaction of macrophages with TGRLP enhances apoE output from these cells, the LpE-containing fraction produced seems to be a relatively inefficient mediator of cholesterol efflux compared with that produced by macrophages preincubated with LDL.

MATERIALS AND METHODS

Materials

Monoclonal (1-H4) mouse anti-(human apoE), polyclonal goat anti-(human apoE) and apoE-Tek ELISA kits were purchased from PerImmune (Rockville, MD, U.S.A.). [^{3}H]Cholesterol was purchased from Amersham Life Science Ltd (Little Chalfont, Bucks., U.K.); tissue culture media and consumables were purchased from Life Technologies Ltd (Paisley, Renfrewshire, U.K.). All other chemicals were from Sigma (Poole, Dorset, U.K.) except Orlistat (tetrahydrolipstatin), which was generously given by Dr G. Gibbons (Radcliffe Infirmary, Oxford, Oxon., U.K.).

Lipoprotein isolation

Human lipoproteins were isolated from freshly collected blood [0.27 % (w/v) EDTA] from healthy volunteers. The total plasma TGRLP fraction, including very-low-density lipoproteins, intermediate-density lipoproteins and any residual chylomicrons/remnants [peak flotation relative density (d) < 1.019], and the LDL fraction ($d = 1.019$–1.065), were isolated by differential ultracentrifugation flotation [35]. Analyses of cholesterol, phospholipid and triacylglycerol mass were performed with Infinity™ cholesterol (Sigma), Phospholipids B (Alpha labs, Southampton, U.K.) and GPO-Trinder (Sigma) kits.

Macrophage culture

Human THP-1 monocytes (genotype E3/E3; [35a]) were maintained exactly as described [36]. For use in experiments, cells were seeded at either 10^6 per well in six-well plates or at 3.0×10^7 in 125 cm^2 flasks. Cells were incubated in RPMI 1640 medium, supplemented with glutamine (4 mM), penicillin (20 i.u./ml), streptomycin (20 μg/ml) and bovine fetal calf serum (10 %, v/v) and differentiated into macrophages by the addition of PMA (100 ng/ml). Macrophages were maintained for 7 days; media containing PMA were replaced at 2-day intervals [36].

Before each experiment, macrophages were washed three times with PBS and left overnight (12 h) in RPMI medium containing BSA (1.5 %, w/v) (RPMI-BSA medium). The cells were then preincubated in RPMI-BSA medium (12 h) in the presence or absence of TGRLP (0–200 μg/ml), LDL (0–200 μg/ml) or Orlistat (5 μM), as indicated in figure legends. Macrophages were then washed thoroughly (three times) with RPMI-BSA medium (with or without heparin or heparinase) and incubated for a further 12 h in the same medium but without any lipoproteins. At the end of this incubation, the medium was harvested, cellular debris was removed by centrifugation at 10000 $\boldsymbol{g}$ for 5 min and immunoreactive apoE was quantified by ELISA (PerImmune); the cells were also retained for determination of their lipid and protein contents.

Lipid analyses

Macrophages were washed (three times) with PBS; their lipid content was extracted by the addition of 1 ml of hexane/propan-2-ol (3:2, v/v) for 1 h at room temperature. Lipid extracts were dried under N_2 and resuspended in propan-2-ol. Total cholesterol and triacylglycerol analyses were performed as above. Residual cell protein was dissolved in 0.3 M NaOH and determined with the bicinchoninic acid assay (Pierce, Rockville, IL, U.S.A.), with BSA as standard.

Isolation of LpE particles

Conditioned media from flasks of THP-1 macrophages (125 cm^2) were harvested from the final incubation without any exogenous lipoproteins. Flotation densities of LpE particles were determined by discontinuous density-gradient ultracentrifugation [37]. Fractions (1 ml) were collected from the bottom of each tube; the apoE content (ELISA) and density (refractive index) of each were determined.

In other experiments the total LpE fraction ($d < 1.21$) was isolated by ultracentrifugation [35], concentrated and desalted; total cholesterol, phospholipid and apoE mass were determined as described above and the cholesterol effluxing potential of the fraction was assessed (see below). The electrophoretic mobility of LpE particles was characterized by electrophoresis with Hydragel Lipo + Lp(a) agarose gels (Sebia, Issy-les-Moulineaux, France), followed by staining with Sudan Black. The apoE content of pre-β migrating particles was confirmed by transfer to nitrocellulose followed by incubation with goat anti-(human apoE) polyclonal antibody (PerImmune) or an irrelevant immunoaffinity-purified IgG fraction (Dako). Primary antibody binding was detected with anti-goat IgG, streptavidin ABC:alkaline phosphatase (DAKO), and 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium (Sigma) as substrate. The lipoprotein particle size ($d < 1.21$ fraction) was determined by electron microscopy, essentially by standard methods described in [27].

Cholesterol efflux assay

Cholesterol efflux from THP-1 macrophages was measured exactly as described [38]. In brief, macrophages were radiolabelled with [^{3}H]cholesterol (1 μCi/ml) during incubation (24 h) in RPMI medium containing foetal calf serum (5 %, v/v). Cells were then incubated in serum-free medium (RPMI-BSA) for a further 18 h before measurement of cholesterol efflux. The efflux potentials of LpE ($d < 1.21$) lipoprotein fractions, isolated from flasks of

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THP-1 macrophages (see above), were determined by adding the total LpE fraction (100 μl) to 3.5 ml of RPMI-BSA medium. Samples (1 ml) of this medium were then added to triplicate wells of labelled macrophages and incubated at 37 °C for 12 h. Media were removed from the cells and centrifuged at 10000 g to remove any floating cells. Released and intracellular [^{3}H]-cholesterol were assessed by determining the radioactivity in an aliquot of the medium and of the cell extract (see above) by scintillation counting. Fractional cholesterol efflux was calculated as the amount of radiolabel released to the medium divided by the total label in each well [38].

Statistics

Results are expressed as means ± S.E.M.; statistical differences ($P < 0.05$) were determined by Student's two-tailed t test.

RESULTS

Effects of lipoproteins on apoE secretion by human (THP-1) macrophages

Macrophages were preincubated (12 h) in the absence (basal) or presence of TGRLP (100 μg/ml protein; 100 μg/ml total cholesterol) or LDL (100 μg/ml protein; 270 μg/ml total cholesterol). Analysis of the lipid composition of these isolated lipoprotein fractions yielded molar ratios of total cholesterol, triacylglycerol and phospholipid of 1:1.72(±0.14):0.101(±0.06) for TGRLP and 1:0.092 (±0.01):0.065(±0.01) for LDL (means ± S.E.M., $n = 4$). Macrophages preincubated under these conditions exhibited predictably different lipid contents and secreted markedly different amounts of apoE into the culture medium in the subsequent incubation period (12 h) (Figure 1).

The results confirm the finding that cholesterol accumulation in THP-1 cells is associated with the synthesis and secretion of apoE by the cells [39,40]. Macrophages preincubated with native LDL (12 h) exhibited a small but significant ($P < 0.05$) increase in total cholesterol content (14 %) (Figure 1B) and secreted more apoE into the culture medium over a subsequent 12 h incubation period (a 36 % increase; $P < 0.05$) than did control macrophages (Figure 1A). In contrast, macrophages preincubated with TGRLP accumulated large deposits of triacylglycerol (Figure 1C) without any apparent increase in total cholesterol content.

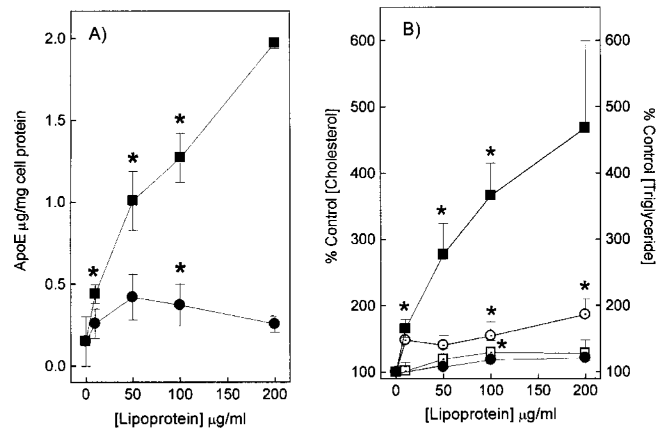

Figure 2 Secretion of apoE into serum-free culture medium (12 h) (A), compared with (B) macrophage total cholesterol (○,□) or triacylglycerol ('Triglyceride') (●,■) after preincubation for 12 h with LDL (○,●) or TGRLP (□,●) at the concentrations indicated

Values are means ± ranges of at least two independent experiments; where significant (*$P < 0.05$) differences from the basal condition are shown, values are means ± S.E.M. for at least three experiments.

Despite this, much higher levels of apoE were measurable (a 214 % increase) in the medium after preincubation with TGRLP (12 h), compared with those in macrophages preincubated under basal conditions (Figure 1A).

The effects of preincubation with TGRLP were apparent at concentrations as low as 10 μg/ml protein (Figure 2). Again, TGRLP caused dose-dependent increases in macrophage triacylglycerol but not total cholesterol content (Figure 2B), which were accompanied by increased apoE secretion in the subsequent incubation (12 h) (Figure 2A). Small but significant ($P < 0.05$) increases in apoE production (Figure 2A) and cholesterol accumulations (Figure 2B) were again evident in macrophages preincubated with LDL (100 μg/ml).

In some experiments, the macrophages were washed with heparin (20 i.u./ml) or with heparinase (type I, 5 units/ml) after

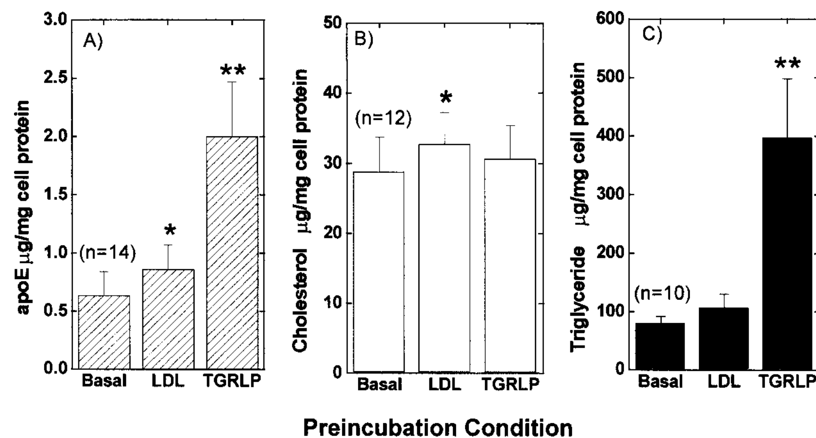

Figure 1 Secretion of apoE into serum-free cell culture medium (12 h) (A) compared with macrophage total cholesterol (B) and triacylglycerol ('Triglyceride') (C) content, after preincubation for 12 h in the presence or absence of LDL (100 μg/ml protein) or TGRLP (100 μg/ml protein)

For details see the Materials and methods section. Results are expressed as means ± S.E.M. for the numbers of experiments indicated in parentheses. *$P < 0.05$ compared with the basal condition; **$P < 0.05$ compared with the basal and LDL preincubation conditions.

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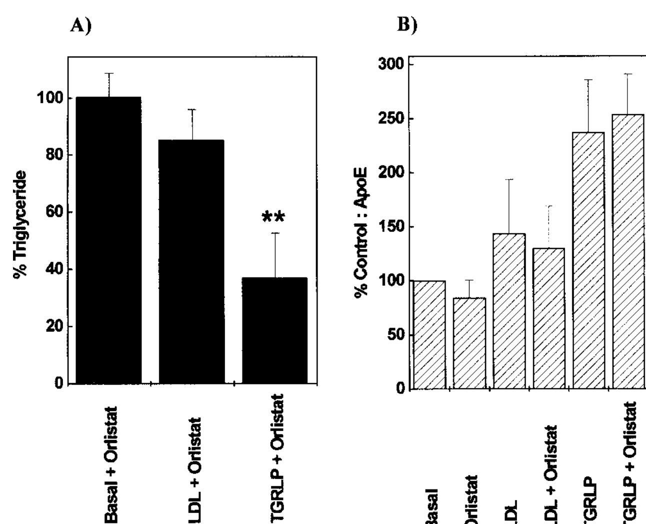

Figure 3 Effect of decreases in macrophage triacylglycerol ('Triglyceride') accumulation (A) on the secretion of apoE (B) (12 h) by THP-1 macrophages

Cells were preincubated for 12 h under basal (serum-free) conditions, or with LDL (100 μg/ml) or TGRLP (100 μg/ml), in the presence or absence of Orlistat (5 μM). Values shown in (**A**) are expressed as percentages of cellular triacylglycerol content in the absence of Orlistat, calculated from three independent experiments (means $\pm$ S.E.M.); values in (**B**) are means $\pm$ ranges for two independent experiments, where the absolute value for apoE secretion was 1.55 $\pm$ 0.98 μg/mg of cell protein.

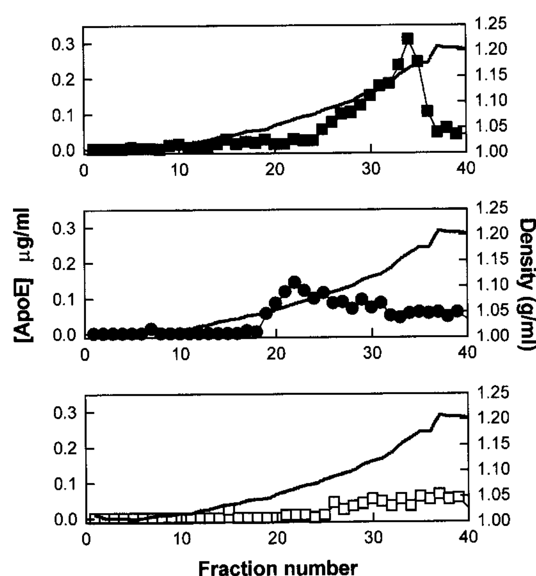

Figure 4 Density-gradient analysis of apoE from medium conditioned by macrophages (12 h) previously incubated for 12 h with TGRLP (100 μg/ml) (top panel), LDL (100 μg/ml) (middle panel) or under basal conditions (bottom panel)

The distribution of apoE was determined by ELISA after rapid density-gradient ultracentrifugation for 2.5 h.

the preincubation phase and before the incubation in which apoE accumulation was assessed, to remove any apoE bound to proteoglycans on the cell surface. Neither treatment substantially altered the subsequent accumulation of apoE in the medium. Washing with heparin released approx. 10–20 % of the total apoE measurable after 12 h, but this effect was observed uniformly for all preincubation conditions (results not shown). ApoE secretion from macrophages preincubated under basal conditions or in the presence of TGRLP or LDL (100 μg/ml) was subsequently inhibited to similar extents (89.8 $\pm$ 4.8 %, 83.3 $\pm$ 5.7 % and 82.2 $\pm$ 7.6 %, respectively; means $\pm$ ranges for two independent experiments) by using cycloheximide (5 μg/ml) to block protein synthesis. Equally, the addition of an inhibitor of transcription, actinomycin D (5 μg/ml), during the preincubation phase caused substantial decreases in apoE secretion in a subsequent incubation (12 h); again, secretion was inhibited by 90.5 $\pm$ 2.1 %, 84.4 $\pm$ 1.7 % and 88.9 $\pm$ 0.1 % respectively in two separate determinations. Thus syntheses of both mRNA and protein seem to be necessary to maintain apoE secretion under each of the preincubation conditions tested.

Triacylglycerol content of macrophages and apoE secretion by THP-1 macrophages

Results indicated an apparent association between macrophage triacylglycerol content and apoE secretion (Figures 1 and 2). We investigated this putative link by modulating the triacylglycerol content of THP-1 macrophages with the lipoprotein lipase inhibitor Orlistat (tetrahydrolipstatin) (5 μM) [41]. Orlistat was present during preincubation with or without TGRLP (100 μg/ml) or LDL (100 μg/ml). As expected, Orlistat did not affect the triacylglycerol content of cells preincubated under basal conditions or with LDL (Figure 3); total macrophage cholesterol content was similarly unaffected (results not shown). However, Orlistat did significantly ($P < 0.05$) decrease macrophage triacylglycerol accumulation due to TGRLP (by 63.0 $\pm$ 9.1 %; mean $\pm$ S.E.M., $n = 3$) (Figure 3A) but did not affect the secretion of apoE (Figure 3B) under the same conditions.

In a further series of experiments we incubated macrophages with non-esterified fatty acids before assessing the secretion of apoE. Incubation with 100 μM $C_{16:0}$, $C_{18:1}(n-9)$ or $C_{18:2}(n-6)$ increased the basal macrophage triacylglycerol content (77.3 $\pm$ 3.8 μg/mg of cell protein) by 132 $\pm$ 23.7 %, 159.9 $\pm$ 45.9 % and 154.3 $\pm$ 30.8 % respectively (means $\pm$ ranges, $n = 2$). However, no substantial changes in apoE secretion were detected in the same experiments after incubation with $C_{16:0}$ (95.5 $\pm$ 3 %), $C_{18:1}(n-9)$ (106.4 $\pm$ 16.6 %) or $C_{18:2}(n-6)$ (84.4 $\pm$ 8.1 %). Thus alterations in macrophage triacylglycerol accumulation do not seem to alter apoE secretion.

Effects of TGRLP on lipid association of apoE

Previous studies have shown that human monocyte-derived macrophages produce LpE particles only after cholesterol enrichment [27,28] and that, under basal conditions, apoE remains lipid-poor ($d > 1.21$) [28]. However, the effect of preincubation with TGRLP on macrophage LpE production was not established. We therefore examined the flotation density of apoE produced by macrophages that had previously been preincubated (12 h) under basal conditions or in the presence of TGRLP (100 μg/ml) or LDL (100 μg/ml) (Figure 4), with the use of rapid discontinuous density-gradient ultracentrifugation [37]. This technique permitted a direct comparison of the flotation densities of LpE particles produced under differing incubation conditions with values published in the literature [27,28]. However, it should be noted that the use of salt gradients can result in the dissociation of a proportion of lipoprotein-bound apolipoproteins.

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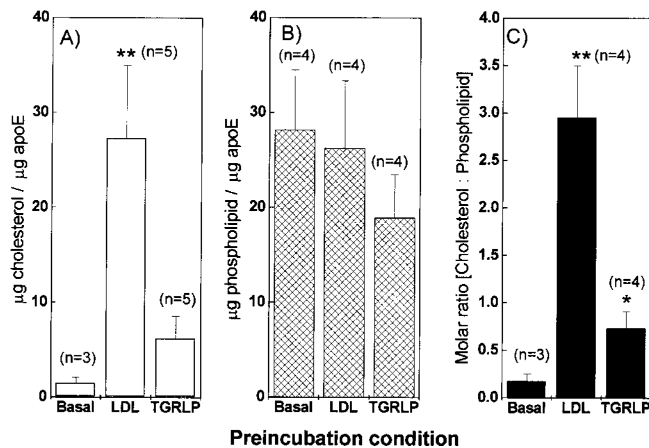
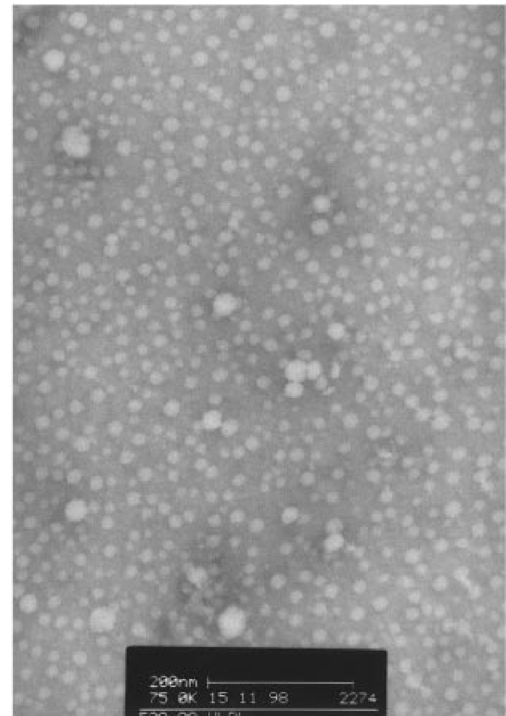


Figure 5 Analysis of the lipid composition of the apoE-containing lipoprotein fraction, isolated by ultracentrifugation ($d < 1.21$) of conditioned medium from macrophages previously incubated for 12 h under basal conditions or in the presence of LDL (100 μg/ml) or TGRLP (100 μg/ml)

(**A**) The total cholesterol and (**B**) phospholipid contents of the concentrated lipoprotein fraction were determined enzymically (see the Materials and methods section); the apoE content of each fraction was determined by ELISA. (**C**) Molar ratios of cholesterol/phospholipid for each lipoprotein fraction, calculated using an estimated molecular mass of 830 for phospholipid. Values shown are means ± S.E.M. for the numbers of experiments shown in parentheses. $^{*}P < 0.05$ compared with the basal condition; $^{**}P < 0.05$ compared with both the basal condition and cells preincubated with TGRLP.

Macrophages preincubated under basal conditions secreted apoE which remained relatively lipid-poor (Figure 4, bottom panel), whereas macrophages preincubated with LDL secreted apoE particles that floated at $d = 1.08$–1.10 [28] (Figure 4, middle panel). By contrast, macrophages preincubated with TGRLP secreted a markedly heterogeneous population of apoE-containing particles, with most apoE floating at a d value of 1.14–1.18 (Figure 4, top panel). These results suggest that apoE-containing particles produced from THP-1 macrophages preincubated with TGRLPs differed markedly in lipid composition from those produced from macrophages preincubated under basal conditions or in the presence of LDL.

We therefore analysed the lipid composition of the total $d < 1.21$ fraction isolated by ultracentrifugation of tissue culture medium yielded by flasks (125 cm^2) of THP-1 macrophages (Figure 5). This fraction contains LpE but can also contain other lipid vesicles or lipoproteins produced by the THP-1 macrophages [27,28]. Phospholipid and cholesterol, but no triacylglycerol, were detected in this fraction. As predicted (see above), macrophages previously incubated with LDL subsequently produced a lipoprotein fraction with a higher cholesterol-to-phospholipid ratio than seen under basal conditions or after preincubation with TGRLP (Figure 5C). It should be noted that the cellular cholesterol-to-phospholipid molar ratio did not vary to a corresponding degree: preincubation with LDL increased the macrophage cholesterol-to-phospholipid ratio by 1.4 ± 0.05-fold ($n = 2$), whereas the cholesterol-to-phospholipid ratio in the $d < 1.21$ fraction increased 17-fold. Similar changes in the cholesterol-to-apoE ratio (Figure 5A) but not the phospholipid-to-apoE ratio (Figure 5B) were also observed for this lipoprotein fraction. Indeed, the phospholipid-to-apoE ratio remained remarkably constant under all the conditions tested, varying (non-significantly) by only approx. 30 % (Figure 5B). By contrast, preincubation with TGRLP yielded a $d < 1.21$ fraction that contained much less cholesterol/μg of apoE (Figure 5A) and a much lower cholesterol-to-phospholipid molar ratio (Figure 5C). Thus, although macrophages preincubated with TGRLP exhibit increased apoE secretion, the $d < 1.21$ fraction containing LpE produced by these cells seems to be relatively lipid-poor compared with that generated by macrophages preincubated with LDL.



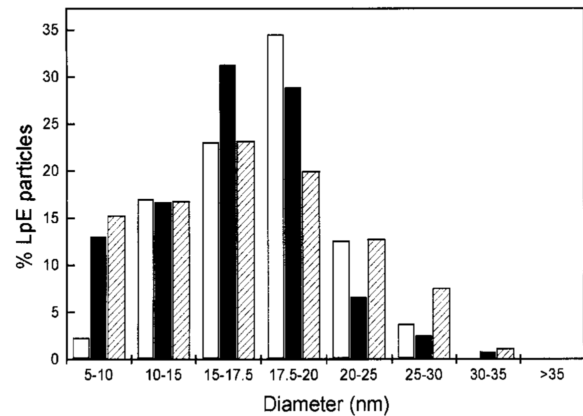


Figure 6 Size distribution of lipoproteins within the $d < 1.21$ fraction

Upper panel: electron microscopic analysis of the $d < 1.21$ lipoprotein fraction isolated from medium conditioned by macrophages previously exposed to TGRLP (100 μg/ml). Lower panel: size distribution of lipid particles isolated by ultracentrifugation ($d < 1.21$) of culture medium from macrophages previously incubated for 12 h under basal conditions (open bars) or in the presence of LDL (hatched bars) (100 μg/ml) or TGRLP (filled bars) (100 μg/ml). Samples of each concentrated lipoprotein fraction were negatively stained with 2% (w/v) sodium phosphotungstic acid and examined with an electron microscope.

The observed differences in lipid composition of lipoprotein particles within the $d < 1.21$ fraction were also reflected in the size distribution of these particles as assessed by electron microscopy (Figure 6). The particles produced were within the HDL size range: most particles had diameters of 10–20 nm and

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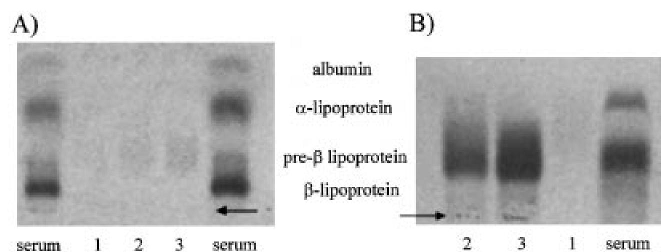

Figure 7 Agarose-gel electrophoretic analyses of apoE-containing lipoprotein fractions ($d < 1.21$) isolated by ultracentrifugation of culture medium from macrophages previously incubated under basal conditions (lane 1), 100 μg/ml TGRLP (lane 2) or 100 μg/ml LDL (lane 3); human serum (5 μl) was included as a positive control

(**A**) Samples (5 μl) of each lipoprotein fraction separated by agarose-gel electrophoresis, then stained for lipid with Sudan Black. The arrow indicates the position of the origin where the lipoproteins were loaded on the gel. (**B**) Equivalent lipoprotein fractions transferred to nitrocellulose membranes and probed for the presence of immunoreactive apoE (see the Materials and methods section).

seemed spherical (Figure 6, upper panel). Under basal conditions, approx. 16.4% of the particles produced had a diameter of more than 20 nm; this proportion increased slightly after preincubation with LDL (21.5%) but decreased substantially after preincubation with TGRLP (9.9%) (Figure 6, lower panel). However, the average particle size differed significantly ($P < 0.005$) only for macrophages preincubated under basal (15.33 ± 0.24 nm; mean of 345 particles, from two fields) or TGRLP (14.38 ± 0.20 nm; mean of 485 particles, from two fields) loading conditions.

However, despite their differences in lipid content, apoE-containing lipoproteins produced by macrophages after preincubation with LDL or TGRLP yielded identical pre-β migrations on agarose-gel electrophoresis (Figure 7). Equal volumes (5 μl) of each concentrated lipoprotein fraction were loaded on an agarose gel. Direct visualization, with Sudan Black, clearly indicated the presence of β/pre-β migrating lipoproteins; however, more intense staining was noted after preincubation with LDL, reflecting the higher lipid content of these particles (Figure 7A).

Lipoproteins ($d < 1.21$) were then transferred to nitrocellulose, probed with anti-(human apoE) polyclonal antibody (PerImmune) and shown to contain immunoreactive apoE (Figure 7B). No immunoreactivity was noted with a control immunoaffinity-purified IgG fraction. This process was also repeated for concentrated (10-fold) culture medium that had not undergone ultracentrifugation (results not shown). Identical results were obtained, indicating that the migration of these lipoprotein particles was not altered by ultracentrifugation.

Cholesterol effluxing capacity of macrophage LpE-containing media or lipoprotein fractions: effects of preincubation with TGRLP

The size and lipid composition of the ($d < 1.21$) LpE-containing lipoprotein fractions suggested that particles produced from macrophages preincubated with TGRLP or LDL would exhibit differing cholesterol effluxing abilities. To investigate this we compared the ability of (1) conditioned media and (2) isolated ($d < 1.21$) LpE-containing fractions, derived from macrophages incubated under these conditions, to efflux [^{3}H]cholesterol from naïve macrophages, not previously exposed to these lipoprotein fractions [38].

As expected, the ($d < 1.21$) fraction isolated from macrophages preincubated with TGRLP contained more apoE (0.22 ± 0.11 μg/ml, $n = 6$) than was produced under basal conditions (0.043 ± 0.01 μg/ml, $n = 5$) or after exposure to LDL (0.12 ± 0.08 μg/ml, $n = 6$). However, despite its enhanced apoE content, the $d < 1.21$ fraction derived from TGRLP did not increase cholesterol efflux to a greater extent than did the fraction derived from macrophages preincubated with LDL (Figure 8A). Similar findings were observed for conditioned media. Fractions derived from macrophages preincubated under basal conditions did not increase the cholesterol efflux from macrophages above

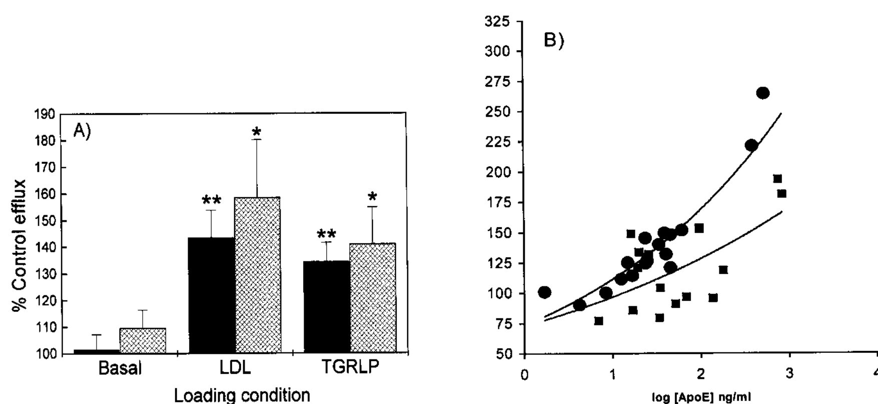

Figure 8 Cholesterol-effluxing capacity of macrophage LpE-containing media or lipoprotein fractions

(**A**) Efflux of [^{3}H]cholesterol (12 h) from radiolabelled 'donor' macrophages to conditioned medium (filled bars) or LpE fractions ($d < 1.21$) (cross-hatched bars), isolated by ultracentrifugation of culture medium, from macrophages previously incubated for 12 h under basal conditions, or in the presence of TGRLP (100 μg/ml) or LDL (100 μg/ml). Results are expressed as percentages of cholesterol efflux from macrophage to non-conditioned RPMI-BSA medium. Values are means $\pm$ S.E.M. for at least eight independent experiments for the conditioned medium, and for at least six independent experiments for LpE fractions ($d < 1.21$) derived from macrophages incubated in the presence of lipoproteins. *$P < 0.05$ compared with the control incubation; **$P < 0.05$ compared with both the control incubation and medium conditioned by macrophages incubated under basal conditions. (**B**) Percentage increases in cholesterol efflux due to conditioned medium ($n = 6$)/LpE ($n = 6$), derived from macrophages preincubated in the presence of TGRLP (100 μg/ml) (■) or LDL (100 μg/ml) (●). Values shown are the pooled results from 12 independent experiments, expressed as a function of the final (logarithmic) concentration of apoE supplied to naïve radiolabelled macrophages.

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that seen with non-conditioned medium (Figure 8A). Under identical conditions, plasma HDL (10 μg/ml), apoAI (10 μg/ml) and exogenous apoE (10 μg/ml) induced $168 \pm 36\%$, $17.1 \pm 2.5\%$ and $10.6 \pm 2.9\%$ increases in cholesterol efflux respectively (means $\pm$ S.D., $n = 3$).

Cholesterol efflux to fractions containing conditioned medium or $d < 1.21$ LpE were then plotted as a function of the final concentration of apoE supplied to naïve radiolabelled macrophages (Figure 8B) ($n = 12$). Samples derived from macrophages previously incubated with LDL seem to efflux more cholesterol than those derived from TGRLP-preincubated macrophages. Indeed, some of the $d < 1.21$ fractions isolated from TGRLP-preincubated macrophages decreased rather than enhanced the cholesterol-accepting capacity of the RPMI-BSA medium, possibly by diluting the medium and thereby decreasing non-specific desorption of cellular cholesterol to albumin. The cholesterol-effluxing abilities of the conditioned-medium/$d < 1.21$ fractions produced by TGRLP-preincubated macrophages were also more variable, possibly reflecting the heterogeneous nature of LpE particles produced from these cells (Figure 4). These results suggest that the apoE-containing lipoproteins derived from LDL-preincubated macrophages are more efficient acceptors of cholesterol than those derived from TGRLP-loaded macrophages. However, it should be noted that because the entire $d < 1.21$ lipoprotein fraction, or conditioned medium, was used in these experiments, it is possible that other particles released by macrophages might also influence the cholesterol efflux seen here.

DISCUSSION

The secretion of apoE by macrophages might be important in initiating cholesterol efflux within the arterial wall, thereby accelerating the reverse cholesterol transport process [24–33]. In the present study we have shown that the interactions of lipoproteins rich in triacylglycerols and cholesteryl esters with human (THP-1) macrophages alters the secretion of apoE from these cells. Pretreatment with TGRLP yielded dose-dependent increases in apoE secretion that were dependent on the continuing synthesis of mRNA and protein but independent of the accumulation of intracellular triacylglycerol. Secreted apoE was incorporated into LpE particles; however, the endogenous lipoproteins produced by macrophages incubated with TGRLP, LDL or under basal conditions differed in lipid composition, flotation density and size. In particular, the LpE-containing fraction ($d < 1.21$) produced by macrophages previously exposed to TGRLP was relatively cholesterol-poor, and of higher density, compared with those produced by macrophages preincubated with LDL. The LpE-containing fraction ($d < 1.21$) isolated from macrophages preincubated with TGRLP also seemed correspondingly poorer at effluxing cholesterol from naïve 'donor' macrophages than the equivalent fraction isolated from macrophages preincubated with LDL. These results suggest that, although the interaction of TGRLP with macrophages enhances apoE output, the LpE particles produced are relatively inefficient mediators of cholesterol efflux compared with those produced by macrophages preincubated with LDL.

The production of apoE-containing lipoprotein particles by human monocyte/macrophages now seems to be an established mechanism contributing to cholesterol efflux from macrophages in the absence of exogenously added serum or lipoprotein acceptors [27,28]. Previous studies showed that macrophages incubated with acetylated LDL secreted apoE-containing lipoprotein particles containing cholesterol and phospholipid (molar ratio 2.7:1) that floated at densities of 1.08–1.10 [27]. Although the present study used native instead of acetylated LDL, similar results were observed: the LpE-containing lipoprotein fraction produced by cells preincubated with LDL exhibited a similar cholesterol-to-phospholipid molar ratio (2.95:1) and floated at an equivalent density. By contrast, the apoE-containing lipoprotein fraction produced by macrophages preincubated with TGRLP seems to be relatively poorly lipidated (cholesterol-to-phospholipid molar ratio 0.73:1) and much more heterogeneous; it floats at a much higher density (d 1.14–1.18). ApoE-transfected cells are reported to secrete similarly minimally lipidated forms of LpE (d 1.19–1.26) that can mediate neurite outgrowth from neuronal cells [42]. However, the studies described here show that the lipid composition of macrophage-derived ($d < 1.21$) LpE-containing lipoprotein fractions can be determined by prior exposure to differing plasma lipoproteins.

Importantly, this result implied that the apoE-containing lipoprotein fraction produced by TGRLP-loaded macrophages was a less efficient 'acceptor' of cholesterol than that produced after preincubation with LDL, a finding that was subsequently supported by incubation with naïve 'donor' macrophages (Figure 8). This was surprising, as cholesterol-poor LpE particles would intuitively be expected to have a greater potential capacity for uptake of cellular cholesterol than particles already more saturated with cholesterol (Figure 5). We speculate that apoE derived from TGRLP-loaded macrophages might be modified in some way, or might adopt a differing conformation, from that in more cholesterol-enriched LpE particles and that this property is retained even in the presence of naïve cholesterol-'donating' macrophages. Lipid-dependent changes in apoE conformation are known to influence subsequent interactions with cellular receptors [43]; it is intriguing to speculate whether changes in apoE structure, or lipid composition, might affect the ability of LpE particles to interact with the cell surface and/or induce the efflux of cholesterol mediated via the newly characterized cholesterol efflux regulatory protein ('CERP') [44–46].

Previous work investigating the association of apoE with macrophage-derived lipoproteins identified the LpE as either discoidal [27] or spherical [47] particles. Despite their differing lipid contents, however, all the lipoprotein particles isolated by ultracentrifugation in the current experiments seemed spherical by electron microscopic analysis. Lipoproteins ($d < 1.21$) were of the HDL size range (10–20 nm in diameter) and exhibited pre-β mobility on agarose electrophoresis. The latter was somewhat surprising; γ-migrating LpE has been isolated from human plasma [47] and from cell culture medium conditioned by macrophages [47] or Chinese hamster ovary cells overexpressing apoE [48]. These particles were cholesterol-poor and enriched in sphingomyelin; they promoted the efflux of cholesterol from fibroblasts [47]. The reasons for these differences are not clear but we hypothesize that, by analogy with lipid-poor ApoAI and pre-β_1 HDL [49–51], pre-β migrating LpE particles might reflect the progressive maturation of γ-LpE by the subsequent accumulation of cholesterol and phospholipid. Radiolabelled cellular cholesterol has been shown to accumulate in γ-, pre-β- and α-migrating LpE particles in plasma [29] and we have also noted the co-localization of cellular effluxed cholesterol with pre-β-migrating LpE particles without serum or exogenous lipoprotein acceptors (results not shown).

In summary, the interaction of TGRLP with human (THP-1) macrophages results in enhanced apoE secretion, but into a lipoprotein fraction that seems relatively inefficient in mediating cholesterol efflux in comparison with that produced by macrophages previously exposed to LDL. This is the first demonstration, to our knowledge, that the physical and cholesterol-

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effluxing properties of macrophage-derived apoE-containing lipoprotein particles can be modulated by prior exposure to differing plasma lipoproteins. The presence of such particles might not only affect the removal of cholesterol from the vessel wall but might also subsequently influence other components of the reverse cholesterol transport pathway. These factors might contribute to the increased risk of atherosclerosis seen in individuals with Type II diabetes.

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