Apolipoprotein J (clusterin) induces cholesterol export from macrophage-foam cells: a potential anti-atherogenic function?

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Apolipoprotein J (apo J) is a secreted glycoprotein of which the exact function remains a matter for speculation. Apo J has been implicated in such diverse processes as sperm maturation, regulation of complement activation, programmed cell death, tissue remodelling and lipid transport. In this study a possible role for apo J in lipid transport was explored. Mouse peritoneal macrophages were incubated with acetylated low-density lipoprotein (AcLDL) to produce foam cells containing cholesterol and cholesteryl esters. Incubation of the foam cells with physiological concentrations of purified apo J led to a dose-dependent export of cholesterol. The appearance of cholesterol in the medium was associated predominantly with a decline in intracellular chol-

esteryl esters rather than intracellular free cholesterol. The kinetics of cholesterol release to apo J were similar to apo A-I, an established promoter of cholesterol efflux. Apo J was also shown to induce phospholipid efflux from cells, whereas the cholesterol exported to the medium was associated with the apo J. Studies using foam cells from apo E-null mice showed that the cholesterol exported to the medium was independent of apo E production by the cells. These results present the first evidence that apo J can promote cholesterol efflux from foam cells and indicates that it might have a function in cellular cholesterol homeostasis in both normal and pathological situations.

INTRODUCTION

The function of apolipoprotein J (apo J; also known as clusterin, SP-40,40, complement lysis inhibitor, SGP2, TRPM-2, gp80, gpIII) has been the subject of much speculation. This secretory glycoprotein, found in most tissues and body fluids, has been implicated in such diverse processes as sperm maturation, complement inhibition, apoptosis and tissue remodelling (reviewed in [1–4]).

Apo J is a 70–80 kDa disulphide-linked heterodimer with carbohydrate contributing approx. 30 % of its molecular mass. In plasma, apo J is associated only with a distinct subclass of high-density lipoprotein (HDL) [5] at a circulating concentration of approx. 100 mg/l [6,7]. Apo J-containing HDLs are small and lipid-poor, and have a distribution of molecular mass of 70–90 kDa (approx. 50 %) and at least 200 kDa (approx. 50 %). A fraction of apo J-HDL also contain apo A-I at a constant ratio of five apo J molecules per apo A-I [5]. No other apolipoproteins are associated with apo J-HDL. That HDL is a negative risk factor for vascular disease is well established, and might be due at least in part to the role of HDL in reverse cholesterol transport, a process in which excess cholesterol is removed from peripheral cells and returned to the liver for eventual excretion as bile acids [8,9]. In the initial stages of this process, a lipid-poor apo A-I-containing subfraction of HDL (also referred to as pre- β -HDL) is believed to act as an acceptor for cholesterol donated by extrahepatic cells [10]. The fact that apo J-HDLs are also small and lipid-poor and that the protein itself has three predicted amphipathic helices that could serve as lipid-binding domains has led to speculation that apo J might also be involved in reverse

cholesterol transport [5,6]. However, no evidence for this has so far been presented.

Apo J has also been implicated in a number of degenerative diseases including atherosclerosis. It has been detected in human and mouse atherosclerotic lesions but was absent from normal arteries [11,12]. In lesions of mice fed with atherosclerosis-inducing diets, apo J was co-localized with lipid-containing cells [12]. These observations led us to study the potential of apo J to act as a promoter for cholesterol efflux from macrophage foam cells. Our studies show that apo J at physiologically relevant concentrations can promote net cholesterol efflux and induce cellular cholesteryl ester depletion in mouse macrophage foam cells.

These results present the first evidence that apo J can promote cholesterol efflux from foam cells and indicate that it might have a function *in vivo* in cellular cholesterol homeostasis in both normal and pathological situations.

EXPERIMENTAL

Reagents

All reagents used were of analytical grade. BSA (essentially fatty acid-free, fraction V) was purchased from Sigma-Aldrich (Castle Hill, N.S.W., Australia). All solvents were of HPLC grade (Mallinckrodt, Biolab Scientific, Clayton, South Australia).

Lipoprotein isolation and modification

Low-density lipoprotein (LDL) was isolated and acetylated as described previously [13]. Briefly, LDL (relative density 1.021–

Abbreviations used: AcLDL, acetylated low-density lipoprotein; apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; LDL, low-density lipoprotein.

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1.063) was prepared from fasting plasma by density gradient ultracentrifugation and acetylated with acetic anhydride. Oxidation of LDL was performed by incubating LDL (1 mg/ml) with CuCl_2 (20 μM) at 37 °C for 24 h [14]. The degree of modification of the LDL (acetylation and oxidation) was confirmed by non-denaturing gel electrophoresis [13,14].

Apolipoprotein and vitronectin isolation

Apo J was purified from human serum by monoclonal antibody affinity chromatography as described in [15]. SDS/PAGE analyses followed by density scanning showed that the apo J was more than 95% pure. SDS/PAGE and Western blot analyses with anti-apo A-I mAb HA60 showed that the purified apo J contained no detectable apo A-I. Dot-blot analyses with vitronectin-specific mAb HV-8 [16] and purified vitronectin (see below) as standard showed apo J preparations contained approximately 1–2% vitronectin. Similar purity of apo J preparations has been reported previously [17]. The final apo J preparation contained trace amounts of IgM and IgA [15] and no detectable cholesterol or phospholipid.

Vitronectin was purified from human plasma using heparin affinity chromatography as described in [18]. SDS/PAGE analyses followed by density scanning showed that vitronectin purified by this method was more than 95% pure.

Apo A-I was purified from human HDL by FPLC with a Q Sepharose Fast Flow column (Pharmacia Biotechnology, Uppsala, Sweden) and freeze-dried as described in [19]. The purity of the apo A-I was confirmed by SDS/PAGE [20% (w/v) gel] by detection of a single band of molecular mass 28 kDa.

Apo J, apo A-I and vitronectin were reconstituted from freeze-dried stocks, which were stored at –20 °C until use, in 3 M guanidinium chloride solution at approx. 8–10 mg/ml and dialysed for 5 days against five 1-litre changes of 10 mM Tris/HCl (pH 7.4). The proteins were filter-sterilized (0.45 μm pore size) and stored in the dark at 4 °C. Apo A-I was used within 3 months of reconstitution, whereas apo J and vitronectin were used within 2 weeks because there is little information available regarding the stability of these two proteins in solution.

Mouse macrophage isolation, cholesterol loading and efflux

Mouse peritoneal macrophages were isolated and loaded with cholesterol as previously described in detail [13]. Briefly, resident macrophages were collected by peritoneal lavage and plated at approx. 5×10^6 cells per 35 mm diameter culture dish. After they had adhered and been washed, the cells were incubated for 24 h in Dulbecco's modified Eagle's medium (DMEM) containing human lipoprotein-deficient serum (1.5 mg/ml protein, prepared by density gradient ultracentrifugation; $d > 1.25$ [20]) and 25 $\mu\text{g/ml}$ acetylated LDL (AcLDL). After loading with lipid, the cells were washed and incubated overnight (14–16 h) in DMEM containing BSA (1 mg/ml) to allow sufficient time for lysosomal degradation and processing of the AcLDL. These macrophage foam cells were then incubated in efflux media containing either BSA only (1 mg/ml) or BSA plus apo A-I (25 $\mu\text{g/ml}$), apo J (25–200 $\mu\text{g/ml}$) or vitronectin (10–20 $\mu\text{g/ml}$) for various periods. After the efflux period, media and cells were analysed separately for sterols and protein.

For all cell culture experiments except those involving cells derived from apo E knockout mice, macrophages from 6-week-old Quackenbush-Swiss mice were used. Apo E knockout mice, created by gene targeting of C57BL/6 mice [21], as well as C57BL/6 control mice, were obtained from Murdoch University (Perth, Western Australia).

We have previously shown [13,22] that apo A-I-mediated efflux of 7-oxocholesterol is defective from model foam cells. To assess the efficacy of apo J to mediate 7-oxocholesterol efflux, cells were incubated with either copper-oxidized LDL (25 $\mu\text{g/ml}$) or 7-oxocholesterol-enriched AcLDL (25 $\mu\text{g/ml}$) [13]. The cells were then incubated overnight and then for 24 h with efflux media containing BSA alone (1 mg/ml), BSA plus apo A-I (25 $\mu\text{g/ml}$) or apo J (150 $\mu\text{g/ml}$).

All cell loading and efflux experiments were carried out in triplicate cultures.

Sterol analysis

Lipids were extracted and prepared for HPLC analysis as previously described [13,14,22]. Briefly, lipids were extracted from AcLDL, cell lysates or efflux media by using methanol followed by hexane. For cells loaded with copper-oxidized LDL, a portion of the cell extracts was saponified [14] to give total cellular cholesterol and 7-oxocholesterol. The organic extracts were evaporated and redissolved in the mobile phase (see below).

Sterol and sterol esters were analysed by HPLC as previously described [14,23] using a Supelco reverse-phase C_{18} column (5 μm particle size). Cholesterol and cholesteryl esters were detected at 210 nm after elution with propan-2-ol/acetonitrile (70:30, v/v). Free and esterified 7-oxocholesterol were detected at 234 nm after elution with propan-2-ol/acetonitrile/water (54:44:2, by vol.).

Apo J-mediated efflux of phospholipid from cells

Mouse macrophages were loaded with AcLDL (25 $\mu\text{g/ml}$), incubated overnight (14–16 h) in media containing BSA (1 mg/ml), and then incubated for 24 h in the presence of efflux media containing BSA alone or BSA plus apo J (150 $\mu\text{g/ml}$). For each condition (in duplicate), media from six 35 mm diameter culture dishes were pooled, centrifuged (16000 g for 10 min) to remove detached cells and extracted for lipid by using methanol/chloroform (1:1, v/v) [24]. [^{14}C]cholesterol was added as an internal standard. Lipid extracts were evaporated under reduced pressure and phospholipid content was determined by assaying for phosphorus [25]. Equal volumes of cell-free media (with and without added apo J) were also extracted and assayed for phospholipid content.

Binding of cholesterol to apo J in efflux media

Mouse macrophages were loaded for 24 h in DMEM containing AcLDL (25 $\mu\text{g/ml}$), [^{14}C]cholesterol [in ethanol; final ethanol concentration 0.1% (v/v); Amersham International, Little Chalfont, Bucks., U.K.] and human lipoprotein-deficient serum (1.5 mg/ml protein). The cells were then washed and incubated overnight in DMEM containing BSA (1 mg/ml) and then for 24 h in DMEM containing BSA (1 mg/ml) with and without apo J (100 $\mu\text{g/ml}$). The medium was collected and centrifuged to remove any detached cells. Association between apo J and cell-derived [^{14}C]cholesterol in the medium was measured by two methods. Samples of medium (20 μl in 2 μl aliquots) were applied to an eight-lane 1.0% (w/v) Universal Agarose gel (Ciba-Corning, Palo Alto, CA, U.S.A.) and subjected to electrophoresis in Tris/barbitone buffer, pH 8.6, at 125 V for 1 h. Duplicate samples were applied to both halves of the same gel. Freshly isolated human plasma was included as a reference, and [^{14}C]cholesterol in DMEM containing BSA only (1 mg/ml) was included as a control on both. After electrophoresis the gel was cut in half. Proteins were transferred from one half to a

Immobilon-P transfer membrane (Millipore) by press-blotting for 1 h. Apo J was detected with a murine monoclonal anti-human apo J antibody (G7mAb; Quidel, San Diego, CA, U.S.A.) and detected by enhanced chemiluminescence (Amersham) in accordance with the manufacturer's directions. The other half of the gel was stained with Fat Red 7B (Sigma-Aldrich) to determine the migration of the lipoprotein classes in the plasma sample and [14 C]cholesterol was detected by phosphorimaging (Fujix BAS 1000).

Secondly, samples of efflux medium were subjected to sucrose density gradient ultracentrifugation as previously described [26]. Sucrose solutions were prepared at 35% (w/v; $\rho = 1.15$) and 65% (w/v; $\rho = 1.31$) in 10 mM Tris/0.15 M NaCl/1 mM EDTA (pH 7.4). Medium (0.5 ml) was underlaid with 2.0 ml of 35% (w/v) and 1.5 ml of 65% (w/v) sucrose and centrifuged at 417 *g* at 16 °C for 20 h in a TL 100.4 rotor with a Beckman TL-100 ultracentrifuge. Samples (250 μ l) were withdrawn by syringe from the bottom of the tube and aliquots were assayed separately for [14 C]cholesterol, apo J and density. Densities were measured by refractometry. Apo J was measured by slot-blotting with a murine monoclonal anti-(human apo J) antibody (G7mAb) and detected by enhanced chemiluminescence followed by density scanning. [14 C]cholesterol was measured by scintillation counting.

Protein determination

The protein contents of apo A-I, apo J, vitronectin, cell lysates and AcLDL were measured by the bicinchoninic acid method (Sigma-Aldrich) with BSA as standard. Standards were prepared in PBS for apo A-I, apo J, vitronectin, LDL and AcLDL, or 0.2 M NaOH for cell lysates. Absorbance at 562 nm was measured after incubation at 60 °C for 1 h.

Cell viability

Cell viability was measured by both Trypan Blue exclusion staining and retention of lactate dehydrogenase (LDH), a cytoplasmic enzyme. Trypan Blue exclusion staining was assessed in duplicate cultures by two independent observers, whose results were averaged.

LDH was measured in cell lysates [prepared with 0.1% (v/v) Triton X-100 in water] and media by conversion of pyruvic acid into lactic acid, with dinitrophenylhydrazine (Sigma-Aldrich) as the colour reagent. Absorbance was measured at 450 nm and LDH retention was expressed as the proportion of total LDH (cells plus media) present in the cells.

RESULTS

Mouse peritoneal macrophages were loaded with AcLDL (25 μ g/ml) for 24 h to produce foam cells containing free cholesterol as well as a significant proportion of cholesteryl esters. Typically, this loading regime results in a 4–5-fold increase in total cellular cholesterol content relative to non-loaded cells (the approximate total cholesterol contents of non-loaded and loaded cells were 50–80 and 200–400 nmol/mg of cell protein respectively) [13,22]. Approximately 50–80% of this cholesterol is esterified and stored as small droplets in the cytosol. When incubated with lipid-free apo A-I for 24 h, these macrophage foam cells routinely export 45–60% of their total cellular cholesterol to the apo A-I. This percentage is constant over a wide range of cellular cholesterol loadings [13,22]. During efflux, only free cholesterol is exported into the medium containing apo A-I. The net release of free cholesterol mass from cells

Figure 1 Cholesterol efflux from AcLDL-loaded macrophages in the presence of various concentrations of apo J

Mouse macrophages were loaded for 24 h with 25 μ g/ml AcLDL, equilibrated overnight (14–16 h) in medium containing BSA (1 mg/ml), and then incubated for 24 h in the presence of BSA (1 mg/ml) only, or with added vitronectin (VN; 10 or 20 μ g/ml) or apo J (25–200 μ g/ml). Cholesterol efflux is expressed as the amount of cholesterol in the medium relative to the total cholesterol pool (including cholesteryl esters) in cells and media. Results are means $\pm$ S.D. for three independent experiments for BSA and for apo J (100, 150 and 200 μ g/ml) and means $\pm$ range for two independent experiments for VN (10 and 20 μ g/ml) and apo J (25 and 50 μ g/ml).

Figure 2 Kinetics of cholesterol efflux from AcLDL-loaded macrophages to apo A-I and apo J

Mouse macrophages were loaded with 25 μ g/ml AcLDL and equilibrated overnight (14–16 h) in medium containing BSA (1 mg/ml); efflux was then measured over time in the presence of apo A-I (25 μ g/ml) or apo J (150 μ g/ml). Cholesterol efflux is expressed as the amount of cholesterol in the medium relative to the total cholesterol pool (including cholesteryl esters) in cells and media. Results are means $\pm$ S.D. for triplicate cultures, and are pooled from two independent experiments covering different time points (time points 3, 6, 9, 18 and 24 h for experiment 1; time points 2, 4, 6, 8, 20 and 28 h for experiment 2). Symbols: $\circ$, apo A-I; $\bullet$, apo J. Lines of best fit (linear): apo A-I, $y = 2.21x + 4.8$ ($r = 0.991$); apo J, $y = 1.70x + 1.1$ ($r = 0.998$).

is associated with cholesteryl ester hydrolysis and depletion [13,22,27].

To test the potential of apo J to act as stimulator of cholesterol efflux, macrophage foam cells were incubated for 24 h with various concentrations of apo J. Cholesterol and cholesteryl esters were measured in cells and media after efflux. The data are expressed as proportional efflux (Figure 1), calculated as $100 \times (\text{Cholesterol in medium}) / \{[(\text{Cholesterol plus cholesteryl esters in cells}) + (\text{Cholesterol in medium})]\}$.

Figure 3 Distribution of cholesterol and cholesteryl esters from AcLDL-loaded macrophages after efflux with apo A-I or apo J

Mouse macrophages were loaded with 25 $\mu\text{g/ml}$ AcLDL and equilibrated overnight (14–16 h) in medium containing BSA (1 mg/ml); efflux was then measured for up to 24 h in the presence of apo A-I (25 $\mu\text{g/ml}$) or apo J (150 $\mu\text{g/ml}$). Results are expressed as nmol per culture and are means $\pm$ S.D. for triplicate cultures. **(A)** Cholesterol exported to the medium; **(B)** intracellular free cholesterol; **(C)** intracellular cholesteryl esters. Rates of cholesterol release (cholesterol in medium expressed as nmol/h per mg of cell protein) were 50.2 ± 7.5 for apo A-I and 24.2 ± 4.7 for apo J. Data are from expt. 1 presented in Figure 2. Symbols: ○, apo A-I; ●, apo J.

The proportional cholesterol efflux from AcLDL-loaded cells to apo J (Figure 1) increased with increasing concentrations of apo J in the medium. Maximum efflux was achieved at apo J concentrations of 100–150 $\mu\text{g/ml}$, which are similar to total plasma apo J levels in humans [6,7]. At these concentrations approx. 30–40 % of the total cholesterol pool was released into the medium during 24 h. In contrast, efflux to either BSA only (1 mg/ml) or vitronectin (10 and 20 $\mu\text{g/ml}$) was minimal (less than 5%). This confirmed that the small amount of vitronectin

Figure 4 Apo J induces efflux of phospholipid from AcLDL-loaded macrophages

Mouse macrophages were loaded for 24 h with 25 $\mu\text{g/ml}$ AcLDL and equilibrated overnight (14–16 h) in medium containing BSA (1 mg/ml); efflux was then measured at 24 h in the presence of medium containing BSA (1 mg/ml) with or without apo J (150 $\mu\text{g/ml}$). Results are given as nmol of phospholipid per ml of medium (mean $\pm$ half range for duplicates, each pooled from six independent cultures). Open bars, cell-free control medium; tinted bars, medium after incubation with cells.

in the apo J preparations was not responsible for the cholesterol efflux.

Cell viability after incubation with BSA only, apo A-I, vitronectin (10 and 20 $\mu\text{g/ml}$) and apo J (up to 200 $\mu\text{g/ml}$) was tested by Trypan Blue exclusion and LDH retention. Incubation with apo J did not affect cell viability (results not shown), confirming that its effects on cholesterol export were not due to toxicity. Similarly, the recovery of cell protein was not affected by incubation of macrophage foam cells with apo J or vitronectin (results not shown).

The proportional efflux of cholesterol to apo J over time was compared with that of apo A-I. AcLDL-loaded cells were incubated with either apo A-I at 25 $\mu\text{g/ml}$, a concentration previously shown to stimulate cholesterol efflux maximally [22], or apo J at 150 $\mu\text{g/ml}$ for various times (Figure 2). The proportional cholesterol efflux increased steadily over time for both acceptors, with apo A-I being a more efficient stimulator of efflux at all time points. Efflux to both apolipoproteins approximated linear kinetics and at the latest time point measured (28 h) did not seem to have reached completion.

Figure 3 demonstrates the kinetics of the sterol redistribution between cells and media during exposure to apo A-I or apo J. Cholesterol levels in the media (Figure 3A) increased for both acceptors, again with apo A-I being slightly more efficient than apo J over the duration of the experiment. The export of free cholesterol in the presence of apo A-I was associated with only a modest decrease in intracellular free cholesterol (Figure 3B), with most loss occurring from the intracellular cholesteryl ester pool (Figure 3C), confirming previous findings [13,22,27]. Similarly the increases in extracellular free cholesterol in the presence of apo J arose mainly from depletion of the intracellular cholesteryl ester pool (Figure 3C). These results indicate that exposure of foam cells to apo J can lead to cholesterol export and cholesteryl ester depletion.

Others have shown that apo A-I-mediated sterol efflux involves the removal of cellular phospholipid [26,28]. We investigated whether apo J also induces phospholipid efflux by comparing phospholipid content in pooled efflux media and in media that had not been incubated with cells. Figure 4 shows that the addition of apo J to cell-free DMEM containing BSA did not influence measured phospholipid levels, demonstrating that the

Figure 5 Cholesterol is associated with apo J after efflux from AcLDL-loaded macrophages

Mouse macrophages were loaded for 24 h with 25 $\mu\text{g/ml}$ AcLDL and [^{14}C]cholesterol, equilibrated overnight (14–16 h) in medium containing BSA (1 mg/ml) and then incubated for 24 h in the presence of efflux medium containing BSA (1 mg/ml) and apo J (100 $\mu\text{g/ml}$). Samples were subjected to electrophoresis in 1% (w/v) agarose as described in the Experimental section. (A) Apo J was detected by reaction with murine monoclonal anti-human apo J followed by anti-mouse IgG conjugated with horseradish peroxidase. (B) [^{14}C]cholesterol was revealed by phosphorimaging. Lane 1, fresh plasma; lane 2, efflux medium not exposed to cells; lane 3, efflux medium after exposure to cells; lane 4, [^{14}C]cholesterol in efflux medium containing BSA (1 mg/ml) only.

apo J preparation was free of phospholipid. Apo J very clearly caused an efflux of phospholipids from AcLDL-loaded mouse macrophages. The molar ratio of phospholipid to apo J was 2.35 ± 0.18 (mean $\pm$ range; $n = 2$), whereas that of phospholipid to cholesterol was 1.98 ± 0.18 .

To establish that the cholesterol released was physically associated with apo J in the efflux media, radiolabelled cholesterol was incorporated into macrophage foam cells. Non-denaturing agarose gel electrophoresis of the medium allowed the separation of free [^{14}C]cholesterol from that associated with apo J. Western blotting of the gels (Figure 5A) indicated one broad apo J band

Figure 6 Cholesterol and apo J co-localize in density gradient ultracentrifugation after efflux from AcLDL-loaded macrophages

Mouse macrophages were loaded for 24 h with 25 $\mu\text{g/ml}$ AcLDL and [^{14}C]cholesterol, equilibrated overnight (14–16 h) in medium containing BSA (1 mg/ml) and then incubated for 24 h in the presence of efflux medium containing BSA (1 mg/ml) and apo J (100 $\mu\text{g/ml}$). Medium samples were subjected to sucrose density gradient ultracentrifugation as described in the Experimental section. Samples were withdrawn by syringe from the bottom of the tube and aliquots were assayed separately for apo J, [^{14}C]cholesterol and density. Symbols: ■, apo J; □, [^{14}C]cholesterol; ○, density.

Figure 7 Cholesterol efflux from apo E-knockout and control foam cells in the presence of BSA only, apo A-I or apo J

Mouse macrophages from apo E-knockout and control mice were loaded with 25 $\mu\text{g/ml}$ AcLDL, equilibrated overnight (14–16 h) in medium containing BSA (1 mg/ml) and then incubated for 24 h in the presence of BSA only (1 mg/ml) or with added apo A-I (25 $\mu\text{g/ml}$) or apo J (150 $\mu\text{g/ml}$). Cholesterol efflux is expressed as the amount of cholesterol in the medium relative to the total cholesterol pool (including cholesteryl esters) in cells and media. Results are means $\pm$ S.D. for triplicate cultures. Filled bars, apo E-knockout; hatched bars, controls.

in freshly isolated plasma (lane 1). In efflux media, with and without exposure to cells (Figure 5A, lanes 3 and 2 respectively), a band of the same size and mobility was detected. In Figure 5(B) the co-migration of [^{14}C]cholesterol with the apo J band is shown. Free [^{14}C]cholesterol in medium, not exposed to cells (Figure 5B, lane 4), did not migrate from the origin. No [^{14}C]cholesterol could be detected in association with albumin, which was present at a 10-fold greater concentration than apo J in the efflux medium. In contrast, [^{14}C]cholesterol present in the medium of foam cells incubated with apo J (Figure 5B, lane 3) co-migrated with the leading edge of the apo J band. This discrete band represented more than two-thirds of the radioactivity (determined by density scanning) in this lane.

Medium from the same experiment was also subjected to sucrose density ultracentrifugation. Figure 6 indicates that apo J and most of the [^{14}C]cholesterol co-localized at $d = 1.1$, further indicating a physical association.

Apo E, like apo A-I, is known to stimulate cholesterol efflux from mouse peritoneal macrophages [26]. Macrophages are known to synthesize apo E themselves and secrete it as a complex with phospholipid and cholesterol, especially when the cells are loaded with cholesterol [29]. To exclude the possibility that apo J induced cholesterol efflux from mouse foam cells indirectly through stimulation of apo E secretion, cholesterol efflux from apo E-knockout mice was compared with corresponding apo E-competent controls (Figure 7). Apo J stimulated cholesterol export from apo E-knockout cells to an extent that was very similar to that from control cells, demonstrating that apo E secretion is not a component of apo J-mediated cellular cholesterol export. Basal, unstimulated cholesterol export was slightly lower from apo E-knockout cells than from controls; this minor difference might reflect the contribution of endogenously secreted apo E to efflux. However, it is clearly only a minor contributor to cholesterol export in the presence of an excess of extracellular acceptors such as apo J.

We have previously shown [22] that apo A-I-mediated efflux of cholesterol from oxidized LDL-loaded cells is impaired and that efflux of 7-oxocholesterol, the major oxysterol present in copper-oxidized LDL [14], is even more severely limited. In the present study we found that both cholesterol and 7-oxocholesterol were also poorly exported from oxidized LDL-loaded cells to apo J

Figure 8 Efflux of cholesterol and 7-oxocholesterol from oxidized LDL-loaded macrophages

Mouse macrophages were loaded with 25 $\mu\text{g/ml}$ copper-oxidized LDL, equilibrated overnight (14–16 h) in medium containing BSA (1 mg/ml) and then incubated for 24 h in the presence of BSA only (1 mg/ml), or with added apo A-I (25 $\mu\text{g/ml}$) or apo J (150 $\mu\text{g/ml}$). Sterol efflux is expressed as the amount of cholesterol or 7-oxocholesterol in the medium relative to the total pool of cholesterol or 7-oxocholesterol (including esters) in cells and media. Results are means $\pm$ S.D. for triplicate cultures. Filled bars, cholesterol efflux; hatched bars, 7-oxocholesterol efflux.

(Figure 8). In addition, defective apo J-mediated efflux of 7-oxocholesterol was evident when cells were loaded with AcLDL selectively enriched with this oxysterol (results not shown), as we have previously reported for apo A-I [13].

DISCUSSION

The results above show that apo J, at concentrations similar to those found in plasma, induced concentration-dependent cholesterol ester depletion in murine foam cells. We have similarly found apo J to promote cholesterol efflux from the human macrophage cell lines THP-1 and U937 (T. Hochgrebe, unpublished work), suggesting that the results reported here might represent a function of apo J in human cells also. This raises the possibility that apo J might have a role in the initial stages of reverse cholesterol transport, and specifically that it might contribute to the removal of excess cholesterol from foam cells in atherosclerotic lesions. Consistent with this suggestion are reports that apo J occurs at sites of atherosclerosis. Apo J has been found in human atherosclerotic lesions [11,12] and in lesions of mice with diet-induced atherosclerosis [12]. At present it is not fully understood whether apo J present in human lesions is produced by cells in the lesion or is derived from apo J transported in blood. In a small number of human lesions ($n = 3$ [11]), no apo J mRNA was detected. However, platelets were shown to store apo J in their granules and release the protein on activation, suggesting that the apo J in human lesions could be deposited from activated platelets [11].

Moreover, it is known that the biosynthesis of apo J is up-regulated at sites of tissue damage or injury (reviewed in [3]) and in a wide variety of instances of cellular 'stress'. Examples of this up-regulation include regression of the rat prostate after androgen ablation; the kidney after nephrotoxic injury, or damage caused by hydrostatic pressure after obstruction of the ureter; a number of degenerative conditions including Alzheimer's and Pick's diseases, myocardial infarction, and a variety of cancers (reviewed in [30]). Thus it is clear that there is an increased local concentration of apo J in the interstitial fluid surrounding such tissues. The quantitative importance of apo J depends on its local concentration relative to other acceptors such as apo A-I and

apo E. However, our demonstration that apo J can promote net cellular cholesterol efflux suggests that apo J present at high concentrations around stressed cells has the potential to remove cholesterol from damaged cell membranes and then deliver it to HDL particles for subsequent disposal through reverse cholesterol transport.

To achieve maximum cholesterol efflux to apo J, a 100–150 $\mu\text{g/ml}$ concentration of the protein was required. Previous work from our group has shown that the maximum rate of efflux to apo A-I from AcLDL-loaded cells can be achieved with as little as 20 $\mu\text{g/ml}$ apo A-I [22]. However, considering that apo J has a molecular mass three times that of apo A-I (70–80 kDa with 30% carbohydrate, compared with 28 kDa and no carbohydrate), in molar terms approximately twice as much apo J was needed compared with apo A-I to stimulate maximum cholesterol efflux. The maximum rate of efflux induced by apo J was close to the maximum rate obtained with apo A-I. Plasma levels of apo J are approximately one-tenth of those of apo A-I [6,7].

However, because the majority of apo J is in small lipid-poor particles (apo J-HDL) [5], whereas lipid-poor apo A-I comprises less than 10% of total circulating apo A-I [31,32], the concentration of apo J-HDL can effectively exceed that of lipid-poor apo A-I in plasma and interstitial fluid. Taken together, these facts suggest that in tissues that are directly accessible to plasma, apo J might be quantitatively as important as apo A-I in initiating reverse cholesterol transport. Furthermore, as discussed above, at sites of tissue damage or cellular stress, locally produced apo J might be even more significant in primary removal of sterols from these sites.

Apo A-I-mediated sterol efflux involves the removal of cellular phospholipid, which can then serve to solubilize cholesterol desorbed from the cell, resulting in the formation of apo A-I/lipid complexes (reviewed in [32,33]). We have shown in the present paper that apo J also induces efflux of phospholipid from model foam cells. Furthermore we found, with two independent methods, that radiolabelled cholesterol and apo J were co-localized after exposure to cells. The density of the cholesterol/apo J complex was estimated to be 1.1, which is the same as that found previously for the apo A-I/lipid complexes generated under similar conditions [26]. Therefore our studies suggest that apo J might form similar HDL-like acceptor particles as apo A-I.

Incubation of copper-oxidized LDL-loaded macrophages with apo J showed that efflux of cholesterol and 7-oxocholesterol from these cells was defective, and comparable to apo A-I-mediated efflux from the same cells [22]. The mechanisms underlying this inhibition of efflux are at present poorly understood. One possibility is that the presence of oxysterols such as 7-oxocholesterol in the plasma membrane affects the desorption of cholesterol and/or phospholipid to acceptor particles.

Even though many functional roles have been suggested for apo J [1–4], promotion of cell aggregation and regulation of complement are the only putative physiological functions for which direct experimental evidence has been produced [34–38]. The work reported here is novel, as it provides the first evidence that apo J might have an important role in lipid transport *in vivo*. We have shown that apo J stimulated cholesterol and phospholipid efflux from macrophage foam cells. This observation, together with the knowledge that apo J is present in human atherosclerotic plaque, raises the possibility that apo J has a protective and potentially anti-atherogenic function in facilitating cholesterol removal from foam cells. Whether this is a major or minor function of apo J remains to be elucidated.

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