Materials and Methods

Paradoxical association of enhanced cholesterol efflux with increased incident cardiovascular risks

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General Procedures

ApoB-depleted serum was prepared by addition of PEG 6000 using methods similar to that previously described Rothblat and colleagues[1, 2]. Briefly, 40 parts 20% PEG 6000 (QIAGEN Science, Germantown, MD) was added to 100 parts serum with gentle mixing, and then incubated at room temperature for 20 minutes. ApoB-depleted serum was then obtained by recovery of supernatant following centrifugation (10,000 rpm, 30 minutes, 4°C). Apolipoproteins, lipoprotein a (Lp(a)), albumin, complete lipid profiles (including direct LDL cholesterol determinations) and cardiac troponin I levels were determined using the Abbott Architect platform (Abbott Diagnostics, Chicago, Illinois). All lipid and lipoprotein measurements employed FDA-cleared in vitro diagnostic assays, and were performed in a CAP accredited and CLIA licensed laboratory that also annually participates in the CDC lipid standardization program. VLDL ($1.006<d<1.019$ g/ml fraction), LDL ($1.019<d<1.063$ g/ml fraction) and HDL ($1.063<d<1.21$ g/ml fraction) were isolated from plasma, serum or tissue culture media by sequential buoyant density ultracentrifugation using sucrose and D_2O to avoid high ionic strength associated alterations to protein compositions of lipoprotein particles observed with KBr use[3]. The fraction remaining following removal (floatation) of lipoproteins was called "lipoprotein-depleted" (LPD) ($d>1.21$ g/ml). Fast protein liquid chromatography (FPLC)-based isolation of human albumin was purified from fresh lipoprotein-depleted plasma by ion exchange chromatography using Q-SepharoseTM XL resin (GE Healthcare, Uppsala, Sweden) with discontinuous salt gradient, and delipidation performed using methanol:ether:chloroform as described[4]. The purity of FPLC isolated albumin was characterized by SDS-PAGE and scanning densitometry of Coomassie Blue stained gel, which showed >98% purity. Protein content was determined by the Markwell-modified Lowry protein assay[5]. Paraoxonase activity measures were performed as recently described[6].

Cellular Cholesterol Efflux Activity Assays

Cholesterol efflux activity assays were performed essentially as recently described by Khera et al[7] except for a key change that radiolabeling counts were monitored within both cell compartment and media for every sample (i.e. not just within media and a handful of control samples for cell pellet). All assays employed radiolabeled free cholesterol enriched cultured macrophages under conditions that have nominal cholesterol ester (CE) labeling (similar to that in[7]), and the procedures employed were similar to those previously described[8], but adapted for high-throughput screening. Briefly, macrophages (typically RAW264.7; ATCC,Rockville, MD; though other macrophage cell lines as outlined below were also employed) in 48- or 96-well plates were first plated, cultured and loaded with 0.2 µCi/ml radiolabeled cholesterol (either [^{3}H]-cholesterol (47.9 Ci/mmol, Perkin Elmer, Boston, MA) or [^{14}C]-cholesterol (50 mCi/mmol, American Radiolabeled Chemicals, St Louis, MO) in Dulbecco's modified Eagle's medium (DMEM) (tissue culture core facility, Lerner Research Institute, Cleveland, OH) for 24 hours at 37°C. The day after labeling, the cells were washed with phosphate buffered saline, and then incubated in DGGB (DMEM supplemented

with 50 mM glucose, 2 mM glutamine, 0.2% BSA and 1% penicillin-streptomycin) with (to induce ABCA1) or without 0.3 mM (final) 8-Br-cAMP(Sigma-Aldrich, St Louis, MO) for 24 hours. Efflux to cholesterol acceptor (2%, v/v, ApoB-depleted serum unless otherwise indicated) was measured after an additional 4 hours of incubation in the presence of 8-Br-cAMP, with recovered media either briefly centrifuged or passed through a glass filter to ensure removal of all potential cellular debris. All efflux assays were performed with at least duplicate determinations. Where indicated, instead of murine macrophage RAW 264.7 cells, J774A.1 (ATCC, Rockville MD) cells were used in RPMI media (tissue culture core facility, Lerner Research Institute, Cleveland, OH). Separate one time use aliquots from three different pools of sera (healthy controls with low normal, mid and high HDLc levels) were included on each plate to allow for monitoring of plate to plate and day to day variations. Overall intra- and inter-plate coefficients of variation were excellent, with 3.1-4.5% intra-batch, and 7.6-11.4% (depending on low, mid versus high HDLc control) inter-batch CVs throughout the duration of analyses of all study samples. The relative cholesterol efflux activity was calculated for each sample as the radioactivity in the medium divided by total radioactivity (medium plus cell extracted from hexane/ isopropanol) in sample.

Immuno-precipitation of ApoA1 and albumin in lipoprotein-depleted media.

Lipoprotein-depleted media (d>1.21 g/ml) recovered from cholesterol efflux activity measures was first mixed with a cocktail of mouse anti-human ApoA1 monoclonal antibodies (homemade monoclonal antibodies 2D10.5, 4G11.2, 2H7, 6C8.1, 5H8.1, and 10G1.5, and a commercial mouse anti-human ApoA1 mAb from BIODESIGN International) and then total IgG and IgG:ApoA1 complexes were quantitatively removed using immobilized protein A (Pierce, Rockford, IL). Quantitative removal of ApoA1 was independently confirmed by SDS-PAGE followed by Western blot analyses and scanning densitometry with ApoA1 levels titrated in separate lanes for generating standard curve. Under the conditions employed, 97% removal of ApoA1 from starting material/media, and 95% recovery in the eluted fraction was documented. Immunoprecipitation of human albumin in lipoprotein-depleted media was similarly performed by first adding purified anti-human albumin polyclonal antibody (Thermo Scientific, Rockford, IL, catalog number PA1-65133), incubation, followed by removal with protein A/G resin (Thermo Scientific, Rockford, IL). Analyses revealed albumin removal (via immuno-precipitation) was near complete (~95% removed) with overall recovery in eluted fractions being ~90%. For both ApoA1 and albumin immuno-precipitation studies, parallel experiments employing non-specific isotype control IgG antibodies were employed and revealed non-specific binding to resins was negligible (<2%) under the conditions employed. Radiolabeled cholesterol before and following ApoA1 and albumin removal were quantified by liquid scintillation counting using a Beckman Scintillation Counter (Fullerton, CA).

Quantification of radiolabel in free vs. esterified cholesterol cellular pools.

The proportion of radiolabeled cholesterol in the cholesteryl ester (CE)

versus free cholesterol pools of cholesterol-enriched macrophages was measured by first recovering radiolabeled sterols from cell pellets with sequential (x 3) hexane/ isopropanol (3/2, v/v) extractions. The combined organic extracts were dried under nitrogen, resuspended in chloroform/methanol (2/1, v/v), and organic extracts spotted onto silica gel plates with preadsorbent strip (GE Life Sciences, Piscataway, NJ). After brief drying of solvents with cool air using a hair dryer, the thin layer chromatography plates were developed using hexane:diethyl ether:acetic acid (70:30:1, v/v/v) in saturated tanks. The bands corresponding to free cholesterol and CE were identified, scraped into vials, and radiolabel sterol in each fraction quantified by liquid scintillation.

Study populations.

Serum samples and associated clinical data were collected from two distinctively different populations, each of which were divided into a case:control cohort based upon clinical evidence of CAD. CAD in all subjects was defined by a clinical history of documented CAD, documented history of prior revascularization, or history of MI confirmed by angiography records, EKG or cardiac enzyme testing. For subjects in the angiographic cohort (see below), CAD was also defined angiographically by ≥50% stenosis in one or more major coronary vessels. All subjects studied gave written informed consent approved by the Cleveland Clinic Institutional Review Board. The first cohort (Cohort A – Angiographic Cohort; N=1150) was comprised of sequential participants enrolled in the study GeneBank[9-13], which consists of sequential stable subjects without evidence of acute coronary syndrome (cardiac troponin I <0.03 ng/mL) who underwent elective diagnostic coronary angiography (cardiac catheterization or coronary computed tomography) for evaluation of CAD. All subjects had extensive clinical and longitudinal outcomes data monitored, including adjudicated outcomes over the ensuing 3 years for all participants after enrollment. Major adverse cardiovascular event (MACE) was defined as death, nonfatal MI, or nonfatal cerebrovascular accident (stroke) following enrollment. The second cohort (Cohort B – Outpatient Cohort; n=577) was comprised of sequential consenting subjects undergoing cardiac risk factor evaluation/modification in an outpatient preventive cardiology clinic at the Cleveland Clinic[11, 14], as well as sequential consenting volunteers undergoing health screenings that included demographics/medical history collection, physical examination and blood laboratory testing. Serum samples were collected in all subjects using serum separator vacutainer tubes. Whole blood was allowed to clot at room temperature for 60 min, and then immediately placed into ice/water bath until processing. For all samples (both cohorts), serum was isolated and aliquots were frozen into cryovials at -80°C within 4 hours of time of blood draw. Healthy volunteers provided serum that was pooled (at least n>5 units per pool) and used as controls during efflux activity assays. Sera pools from healthy volunteers were also used to monitor the lipoprotein compartments into which radiolabeled cholesterol partitions during the cellular cholesterol efflux activity assay. For these latter studies, all sera used showed normal range HDLc (>45 mg/dL) and ApoA1 (>150 mg/dL). For studies assessing the efficiency of PEG

depletion of Lp(a), a dozen samples from subjects in the angiographic cohort with identified Lp(a) levels in excess of 30 mg/dL and as high as 133 mg/dL were used.

Statistical Analyses.

Variables are expressed as mean ± standard deviation or median (interquartile ranges [IQR]). Spearman's correlation was used to determine association between cholesterol efflux activities and fasting lipids/lipoproteins and cardiovascular phenotypes. The main outcome measure is the association of cholesterol efflux activity (using ApoB-depleted serum as cholesterol acceptor) with prevalent CAD and prospective MACE at 3 years follow-up. Odds ratio (OR) for prevalent CAD and corresponding 95% confidence intervals (CI) were calculated using both univariate (unadjusted) and multivariate (adjusted) logistic regression models. Hazard ratio (HR) for adverse incident cardiovascular events (death, MI, stroke) and corresponding 95% CI were estimated using both univariate (unadjusted) and multivariate (adjusted) Cox models. Adjustments were made for individual traditional cardiac risk factors (including age, gender, diabetes mellitus, hypertension, cigarette smoking, HDLc, low-density lipoprotein cholesterol). All analyses were performed using R 2.13.1 (Vienna, Austria) and p-values <0.05 were considered statistically significant.

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