Methods

Human subjects

Patients undergoing cardiac surgery (coronary artery bypass, valve replacement) using a membrane oxygenator, donated blood prior to (pre-acute phase, pre-AP), 24 hr post-operatively (acute phase, AP- 1d), and at discharge, 5 days after surgery (AP- 5d). This study was approved by the University of Kentucky Medical Institutional Review Board (IRB) and blood was only collected from patients who underwent successful uncomplicated surgery and who gave informed consent.

Blood sampling

Blood was collected into EDTA-tubes. Plasma was isolated after a 10 min centrifugation at 530 x *g* and stored at minus 80ºC after addition of protease inhibitor (aprotinin (2 µg/ml)). Measurements of SAA, $sPLA_2$, CETP, HDL-C and apoA-I were performed on individual patient plasma samples. Due to IRB restrictions, only small volumes of blood could be obtained from each patient. This necessitated the pooling of plasma from 4 – 7 patients for HDL isolation. The HDL remodeling studies are representative of two such HDL preparations. For efflux studies, plasma was clotted in a glass tube using 25 mM $CaCl_2$[1].

Measurement of plasma proteins and lipids

Plasma SAA levels were determined by ELISA (Biosource International, Camarillo, CA). CETP activity was determined using the Roar Biomedical kit (NY, New York). $sPLA_2$ activity was measured according to the protocol of Wooton-Kee using 1-palmitoyl-2-oleoyl-

phosphatidylglycerol (POPG) as a substrate[2]. Plasma CETP and apoA-I was determined by densitometric scanning of Western blots (Kodak 1D, New Haven, CT). Briefly, 1 µl of plasma from patients was separated by 4 – 20% SDS-PAGE, transferred onto a PVDF membrane and immunoblotted using either anti-human CETP TP2 (University of Ottawa, Canada) or anti-human apoA-I (Calbiochem, San Diego, CA). A standard curve was obtained from the same gel by quantifying the densitometric signal produced by four different amounts of recombinant human CETP (Cardiovascular Targets, Inc, NY) or apoA-I (Biodesign International, Saco, Maine). CETP or apoA-I mass in plasma samples was determined by densitometric analysis against this standard. HDL-cholesterol levels were determined using a commercial kit (Wako Diagnostics, Richmond, VA). For the efflux experiments, apoA-I was measured using an automated turbidimetric immunoassay (Mayo Medical Laboratories, Rochester, MN). Total protein was measured by the Lowry method[3].

HDL isolation

Pre-AP and AP- 1d HDL ($1.063 < \rho < 1.21$ g/ml) were isolated from pooled patient plasma by sequential ultracentrifugation as described[4], dialyzed against 150 mM NaCl, 0.01% (w/v) EDTA (pH 7.4), sterile filtered and stored at 4ºC under argon gas. VLDL was isolated from normal volunteers by ultracentrifugation ($\rho < 1.019$ g/ml), dialyzed and stored as above. SAA-HDL was prepared by incubating 1.4 mg HDL_2 with 1 mg recombinant human SAA (Biovision, Mountain View, CA) for 4 hr at room temperature. The SAA-HDL was recovered by ultracentrifugation ($\rho < 1.25$ g/ml), dialyzed and stored as above.

In vitro *HDL remodeling*

Pre-AP and AP- 1d HDL (0.7-0.9 mg/ml total HDL protein, 30 µg apoA-I) were incubated in a final reaction volume of 50 µl in Tris-buffered saline, pH 7.4 at 37ºC for 24 hr with human recombinant CETP (0, 1, 2 or 4 µg per mg HDL protein, Cardiovascular Targets, Inc, NY), human VLDL (4 mM final triglyceride concentration), fatty-acid free bovine serum albumin (BSA) (10 mg/ml) and $CaCl_2$ (2 mM). We attempted to make our incubations as close as possible to physiological conditions, by using physiological concentrations of HDL, CETP, VLDL, calcium and albumin. In order to allow the HDL remodeling to proceed to completion, reactions were incubated for 24 hr. These methods are comparable to those employed in the literature [5],[6],[7]. Reactions were terminated by the addition of EDTA (final concentration 20 mM). Human VLDL was used as a cholesteryl ester acceptor and triglyceride donor for CETP action. SAA-HDL (1 mg/ml) was incubated with CETP (2 µg/mg HDL) and human recombinant $sPLA_2$-IIA (2 µg/mg HDL) under the same conditions described above. The purity of the recombinant CETP was assessed by SDS-gel electrophoresis which showed only one band corresponding to the size of CETP at ~ 70kDa. The specific activity was reported by the manufacturer at 25 fold over normal human plasma. In the *in vitro* studies, we used CETP at physiological concentrations (approx 1 in 25 dilution of the stock CETP) which is in the activity range of CETP *in vivo*.

Gradient gel electrophoresis and Western blots

Aliquots of incubation mixtures (1 µg apoA-I) were electrophoresed on 4-20% non-denaturing polyacrylamide gels for 3.5 hr at 200 V, 4ºC. They were then transferred to PVDF membranes (100 min at 100 V at 4ºC) for subsequent Western blotting with anti-human apoA-I (Calbiochem) or anti-human SAA (Behring, Germany) antibodies. Bound antibodies were detected by enhanced chemiluminescence (GE Healthcare, NJ).

Immunoaffinity chromatography

HDL was radiolabeled by the iodine monochloride method[8] and characterized using the Seize® Primary Mammalian Immunoprecipitation kit (Pierce, Rockford, IL). Briefly, immunoaffinity spin columns were made containing anti-SAA or anti-apoA-II antibodies (200 µg antibody/ 200 µl coupling gel) according to the manufacturer's instructions. ^{125}I-labeled pre-AP and AP HDL (10 µg total protein) was allowed to bind to the column (with rotation) overnight at 4ºC. Unbound HDL particles (flow through, FT) were spun down (1 min at 1180 x *g*). After 5 washes with 400 µl Tris-buffered saline (pH 7.4), the bound HDL was eluted using 200 µl elution buffer containing a primary amide (pH 2.8) into 10 µl of 1M Tris (pH 9.5) to give a final pH of 7.4. Equal counts (2000 dpm) of the FT and eluted fractions (E1-E5) were subjected to SDS-PAGE using 4 – 20% acrylamide gels and analyzed by subsequent autoradiography (ARG).

Cholesterol Efflux

ABCA1-dependent cholesterol efflux was determined in skin fibroblasts isolated from normal and Tangier disease patients as described previously[9]. Cells were labeled with 0.2 µCi/ml [^{3}H]cholesterol in medium for 48 h. Cellular ABCA1 expression was stimulated with free cholesterol loading by incubating fibroblasts with 30 µg/ml cholesterol in medium containing 0.2% fatty acid-free BSA for 48 h. Total cellular efflux was calculated following overnight incubation in 300 µl serum-free DMEM containing 0.2% fatty acid-free BSA and 2.5% pre-AP, AP- 1d or AP- 5d AP- 5d serum. ApoA-I (10 µg/ml) was used as a positive control. ABCA1-specific efflux was calculated by subtracting the value obtained in Tangier disease fibroblasts from the value in normal fibroblasts. ABCG1-dependent cholesterol efflux was determined in mock- and ABCG1 transfected cells as described previously[10]. Cells were labeled as described above and treated with 10 nM mifepristone for 18 hr prior to efflux experiments which were

carried out as outlined above. ABCG1-dependent cholesterol was calculated as the difference in cholesterol efflux between mock- and ABCG1 transfected cells. HDL (25 µg/ml) was used as a positive control). All the cell lines were kindly provided by Dr. John Oram (University of Washington).

References

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