Abstract HDL functions are impaired by myeloperoxidase (MPO), which selectively targets and oxidizes human apoA1. We previously found that the 4WF isoform of human apoA1, in which the four tryptophan residues are substituted with phenylalanine, is resistant to MPO-mediated loss of function. The purpose of this study was to generate 4WF apoA1 transgenic mice and compare functional properties of the 4WF and wild-type human apoA1 isoforms in vivo. Male mice had significantly higher plasma apoA1 levels than females for both isoforms of human apoA1, attributed to different production rates. With matched plasma apoA1 levels, 4WF transgensics had a trend for slightly less HDL-cholesterol versus human apoA1 transgensics. While 4WF transgensics had 31% less reverse cholesterol transport (RCT) to the plasma compartment, equivalent RCT to the liver and feces was observed. Plasma from both strains had similar ability to accept cholesterol and facilitate ex vivo cholesterol efflux from macrophages. Furthermore, we observed that 4WF transgenic HDL was partially (~50%) protected from MPO-mediated loss of function while human apoA1 transgenic HDL lost all ABCA1-dependent cholesterol acceptor activity. In conclusion, the structure and function of HDL from 4WF transgenic mice was not different than HDL derived from human apoA1 transgenic mice.—Berisha, S. Z., G. Brubaker, T. Kasumov, K. T. Hung, P. M. DiBello, Y. Huang, L. Li, B. Willard, K. A. Pollard, L. E. Nagy, S. L. Hazen, and J. D. Smith. HDL from apoA1 transgenic mice expressing the 4WF isoform is resistant to oxidative loss of function. J. Lipid Res. 2015. 56: 653–664.

High levels of high density lipoprotein-cholesterol (HDL-C) are associated with lower risk for cardiovascular disease in epidemiological studies (1). Although several mechanisms may play a role in HDL’s protective effect, HDL and its major protein constituent, apoA1, are critical components of the reverse cholesterol transport (RCT) pathway, in which cholesterol is removed from peripheral tissues and transferred to the liver for excretion. In the first step of the RCT pathway, lipid-poor apoA1 acts as an acceptor for cell cholesterol and phospholipids via the cell membrane protein ABCA1, generating nascent HDL.

Recently, “HDL is the good cholesterol” hypothesis has suffered several setbacks. For example, several trials of HDL-Craising drugs, including cholesteryl ester transfer protein inhibitors and niacin, failed to demonstrate clinical benefit (2–5). Furthermore, a Mendelian randomization study did not associate gene variants that solely alter HDL-C with coronary artery disease (CAD) incidence (6). That said, human and mouse models with defective RCT, via mutations in ABCA1, apoA1, or scavenger receptor class B type I (SR-BI), are more susceptible to atherosclerosis independent

Abbreviations: AcLDL, acetylated LDL; BMDM, bone marrow-derived macrophage; CAD, coronary artery disease; FCR, fractional catabolic rate; FPLC, fast-protein liquid chromatography; HDL-C, high density lipoprotein-cholesterol; MPO, myeloperoxidase; PR, production rate; RCT, reverse cholesterol transport; SR-BI, scavenger receptor class B type I; Trp, tryptophan.

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of their effects on HDL-C, which goes down in ABCA1 and apoA1 mutations, and up in SR-BI deficiency (7–10). Conversely, mouse models with over expression of apoA1 are resistant to atherosclerosis (11, 12). Thus, it appears that it is the functions of apoA1 and/or the HDL particle, rather than the levels of HDL-C, that are protective.

It is now apparent that not all HDL particles are equivalent. Several studies have reported that individuals with CAD have HDL that is “dysfunctional” and has lost its anti-inflammatory activity, has gained a proinflammatory activity, and has diminished cholesterol acceptor activity (13–15). Oxidation of HDL lipids and proteins has been reported to be associated with the transformation of normal HDL into a proinflammatory and dysfunctional HDL (16). The Hazen and Smith labs (17, 18) reported that myeloperoxidase (MPO), an enzyme found mainly in neutrophils and monocytes, generates chlorinating and nitrating oxidants that selectively target apoA1 in human atheroma for covalent modification. We found that these oxidative posttranslational modifications of apoA1 were higher in plasma of CAD subjects compared with controls, and that these modifications were even higher in apoA1 isolated from human atheroma (17), where MPO is abundant (19, 20). Our in vitro studies demonstrated that MPO modifications of human apoA1 led to diminished ABCA1-dependent cholesterol acceptor activity (21), a finding that was independently replicated in the Heinecke lab (22, 23). Further proteomic studies in apoA1 isolated from human atheroma revealed MPO-mediated modifications at all four of its tryptophan (Trp) residues, as well as tyrosine, lysine, and methionine modifications (21, 24). By site-directed mutagenesis, we determined that the four apoA1 Trp residues, and not the tyrosine or methionine residues, are crucial for the observed MPO-mediated loss of cholesterol acceptor activity (21, 24). We created a novel recombinant apoA1 protein isoform in which all four Trp residues are replaced by phenylalanine, called the 4WF isoform, and demonstrated in vitro that it is resistant to MPO-mediated loss of function (21). The purpose of this study was to generate 4WF apoA1-expressing transgenic mice and compare them with transgenic mice expressing matched levels of wild-type human apoA1. We found that the 4WF transgenics, compared with the human apoA1 transgensics, had less HDL-C and RCT to the plasma compartment, but equivalent overall RCT to the liver and feces. Although plasma from both strains of mice had similar cholesterol acceptor activity, HDL isolated from the 4WF transgenics was partially protected from MPO-mediated loss of ABCA1-dependent cholesterol acceptor activity, while all of this activity was lost upon MPO treatment of HDL from human apoA1 transgenics.

MATERIALS AND METHODS

Mice

We subcloned a 12.3 kb EcoRI fragment into the EcoRI site of pUC19, derived from a human BAC clone (CTD-2149o15, Invitrogen) that contained the entire APOA1 gene, including 6.3 kb and 3.7 kb of 5′- and 3′-flanking regions, respectively. For comparison, the Rubin apoA1 BamH1 fragment transgene construction contained 5.4 kb and 3.7 kb of 5′- and 3′-flanking regions, respectively. In order to convert all four Trp residues in human apoA1 to phenylalanine residues, generating the 4WF apoA1 isoform, we performed site-directed mutagenesis. The final construct was confirmed by DNA sequencing. The modified EcoRI insert was microinjected into C57BL/6 fertilized oocytes (Case Transgenic Core lab). 4WF human apoA1 transgenic founders were obtained and screened for the human plasma apoA1 levels by dot blot, and the highest expressing line was selected for further breeding and study.

Wild-type human apoA1 transgenic mice [C57BL/6-Tg(APOA1) 1Rub/J] were purchased from the Jackson Laboratory (Bar Harbor, ME) and further bred and maintained in-house. These mice were obtained as homozygous for the transgene and bred to C57BL/6J mice, and the progeny that were heterozygous for the transgene were used in this study. Mice were maintained on a chow diet and a 12 h light/dark cycle. The research was conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All experiments were performed in accordance with protocols approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

Plasma apoA1, HDL-C, and total cholesterol quantification

Blood samples were collected and spun in a microcentrifuge for 10 min at 13,200 rpm at 4°C. Plasma was transferred to new 1.5 ml tubes and used for human apoA1 and HDL-C quantification. Ten microliters of plasma was diluted in 70 μl (8×) of PBS for human apoA1 quantification, which was done by a US Food and Drug Administration-approved apoA1 immunoturbidimetric assay on the Abbott ARCHITECT ci8200 Integrated Analyzer System (Abbott Labs) using a goat polyclonal antibody specific for human apoA1 in a kit from Kamiya Biomedical (catalog #KAI-002). The instrument was calibrated by running six apoA1 standards of known concentration in every run.

Plasma was always used undiluted for HDL-C quantification. To prepare samples for HDL-C quantification, 10% (v/v) of precipitating reagent (catalog #0599, STANBIO, Boerne, TX) was added to plasma from each sample; the samples were mixed well by vortexing, allowed to sit at room temperature for 5 min, and spun down for 5 min at 13,200 rpm (room temperature). The supernatant was used to quantify HDL levels (10 μl per each well). HDL-C samples as well as standards were loaded in duplicates in a 96-well plate, and a 250 μl aliquot of enzymatic cholesterol reagent (catalog #1010-430, STANBIO) was added to each well. The plate was incubated for 20 min in a 37°C incubator, and the optical density for the samples and standards was read at a 505 nm wavelength in a plate spectrophotometer. The mean value for each sample’s HDL-C was determined after adjustment for the appropriate dilution.

HDL and apoA1 turnover studies in apoA1 and 4WF transgenic mice

Plasma human and mouse apoA1 levels in wild-type mice and human apoA1 and 4WF transgenic mice were determined as previously described (25). In addition, a turnover study for HDL-C and human apoA1 was performed, as previously described (25), and fractional catabolic rates (FCRs) and production rates (PRs) were determined for each analyte. The method takes advantage of the integrated peak ratio(s) of the endogenous apoA1 peptides generated upon protein digestion to known quantities of a stable heavy-isotope-labeled internal standard. Briefly, 3 days after taking baseline tail vein blood, the mice received a loading
dose of $^2$H$_2$O saline solution (22 µl/g body weight by intraperitoneal injection) and were given drinking water enriched with $^2$H$_2$O (6%). Tail vein blood samples (~70 µl) were collected at 4, 8, 24, and 72 h. Mice were maintained on 6% $^2$H$_2$O for 7 days and were euthanized after obtaining a terminal retro-orbital blood sample. Blood samples were centrifuged (2,000 g for 10 min) upon collection. Plasma was used for the analysis of $^2$H labeling of the total body water (5 µl) and for isolation of the HDL-C fraction (30 µl). To isolate apoB-depleted plasma, 30 µl of plasma from each sample and time point was overlaid with 30 µl sterile PBS and spun on a mini-ultracentrifuge for 3 h at 213,000 g (4°C) to float up VLDL. Then the lower phase of the solution was mixed with HDL-precipitating reagent as described above, and the supernatant containing apoB-depleted plasma was used for further analysis. To separate the lipids from proteins, 600 µl ice-cold acetone was added to each sample, mixed well, and stored at −20°C overnight. The next day, the samples were spun down at 8,000 g for 10 min at 4°C, and the supernatant was separated from the pellet. The pellets were saved for the analysis of apoA1 by LC/MS/MS analysis. The supernatant was used for the analysis of HDL-C. After the evaporation of acetone, total HDL-C was analyzed by GC/MS as previously described (25). HDL-C turnover data were normalized for water body labeling prior to performing statistical analysis.

**Lipid analysis and HDL profile measurement**

HDL (1.063 < d < 1.21) was isolated by sequential ultracentrifugation from pooled plasma (n = 3–5 mice per group) collected from human apoA1 and 4WF transgenic mice (both genders) as described previously (20). Total and free cholesterol in HDL were enzymatically measured using the colorimetric Amplex Red cholesterol assay kit (Molecular Probes) in the presence or absence of cholesterol esterase, respectively. Cholesterol esters were reported as the difference between total and free cholesterol from each sample. HDL total protein content was quantified using the alkaline Lowry assay as previously described (27). Cholesterol mass values were normalized to cellular protein. Phospholipids, including sphingomyelin, in HDL were quantified using an enzymatic colorimetric kit from Wako Chemicals USA (Catalog #433-36201). HDL particle size and profile were characterized by native and SDS-PAGE gel electrophoresis using Novex 4-20% Tris-Glycine Mini Protein gradient gels (Catalog #EG6025BOX; Life Technologies). Samples and native gel standards (Amersham #179-445-01) were run at 125 V for 6 h at room temperature. Gels were stained with 1 h with 0.04% Coomassie blue and destained overnight with destaining solution (10% acetic acid, 40% methanol, 50% water). Gels were scanned and analyzed with TotalLab 1D Gel Image Analysis Software (Nonlinear Dynamics Ltd., Newcastle, UK).

**Culturing bone marrow-derived macrophages for RCT and cholesterol efflux assays**

Bone marrow-derived macrophages (BMDMs) from wild-type C57BL/6 mice were used for RCT and cholesterol efflux assays. Mice were euthanized by CO2 inhalation, femoral and humeral bones were removed from each mouse, and marrow was flushed out of the bones into a 50 ml sterile tube using a 10 ml syringe with a 26 gauge needle filled with sterile DMEM. Upon collection, cells were centrifuged for 5 min at 1,800 rpm at 4°C. Media were aspirated, and the cells were washed twice with sterile PBS. The cells were resuspended in sterile-filtered BMDM growth media [DMEM with 7.6% fetal bovine serum, 15% L-cell conditioned media (which serves as a source of macrophage colony-stimulating factor), and 0.76% penicillin/streptomycin mixture], plated in 10 cm bacterial-grade plastic dishes and incubated at 37°C for 14 days. Cell media were replaced every 2–3 days, and after 2 weeks, the cells had proliferated and differentiated into confluent BMDMs.

**RCT assay**

These studies were performed using methods similar to those described previously (28) with some modifications. BMDMs were cholesterol loaded and labeled by incubation with DMEM containing 20% L-cell conditioned medium, acetylated LDL (AcLDL, 50 µg/ml), and [3H]cholesterol (2 µCi/ml; Perkin Elmer, preincubated with the AcLDL for 30 min) for 24 h. Foam cells were washed twice with DMEM before harvesting for in vivo injection, and ~2–4 million cells containing ~3–5 million [3H]cholesterol dpm in a volume of 0.25 ml were injected subcutaneously in the midback of the recipient mice (human apoA1 and 4WF transgenic mice). Mice were housed separately in cages with metal mesh bottom inserts (without bedding) postinjection. The injected amount of radioactivity was quantified from three aliquots of the foam cell suspension intended for in vivo injection by lipid extraction using hexane-isopropanol (5:2) and measuring radioactivity by liquid scintillation counting in cell pellets and media. Retro-orbital blood was collected 3 days after cell injections and centrifuged to isolate plasma. The plasma radioactivity was determined, and total plasma dpm was calculated by estimating blood volume to be equal to 7% of the body weight and plasma to be 55% of the blood volume. RCT to the plasma was calculated as the percentage of dpm appearing in plasma/total dpm injected. In addition, feces were collected daily, dried overnight at 55°C, weighed, and allowed to soften in a 50% ethanol solution. After hydration, the feces were homogenized, and an internal recovery standard of 10,000 dpm of [14C]cholesterol (Perkin Elmer) was added to each sample. The radioactivity in a 0.3 ml aliquot of the fecal homogenate was measured by liquid scintillation counting after keeping samples in the dark for 24 h to reduce chemiluminescence. The amount of [14C]cholesterol radioactivity was used to back calculate the [3H]cholesterol recovery for the entire fecal homogenate. RCT to the feces was calculated as the percentage of dpm in feces/injected dpm and adjusted for fecal weight when significant differences of fecal weight were observed among study groups. At the time of euthanization (3 days postinjection), the chest cavity was opened, and each mouse was perfused with 10 ml of PBS injected into the left ventricle after making an incision in the right atrium. The liver was harvested and, weighed, and a ~0.2 g fraction was suspended in PBS and homogenized. RCT to the liver compartment was determined similarly as described above for the feces. Any sample with % RCT >2 SDs away from the mean of the remaining data within each group was excluded from statistical analysis.

**MPO modification of HDL from human apoA1 and 4WF transgenic mice**

HDL (1.063 < d < 1.21) was isolated by sequential ultracentrifugation from plasma collected from human apoA1 and 4WF transgenic mice as described previously (26). MPO-mediated modification of HDL was carried out at a molar ratio of 10 mol of H2O2/mol of apoA1 as described previously (18). HDL modification by MPO/H2O2/Cl− system was ascertained by visualizing apoA1 cross-linking on a 4-12% Tris-glycine gel stained with Coomassic Brilliant Blue (10 µg of apoA1 protein per lane).

**MPO modification of human apoA1 in vivo**

C57BL/6 wild-type and mouse MPO-deficient mice (29) were injected subcutaneously with human apoA1 (0.35 mg/g of body weight) at 0 and 48 h (n = 3 each strain). At the 0 h time point, the mice received an intraperitoneal injection of sterile zymosan suspension (0.07 mg/g of body weight). Blood was collected from mice upon euthanization at 72 h postzymosan injection.
and plasma was separated by centrifugation. Levels of Trp72 modification were quantified by a newly developed ELISA assay (30). The levels of human oxidized Trp72-apoA1 were normalized to the levels of total plasma human apoA1.

**Ex vivo macrophage cholesterol efflux assay**

Cholesterol efflux experiments were performed according to established procedures (31, 32) with minor modifications. Murine RAW264.7 macrophages were incubated in 24-well plates and maintained in DMEM containing 10% fetal bovine serum. Cells were cholesterol labeled and loaded by incubation with serum-free DMEM in the presence of either 1 µCi/ml [3H]cholesterol or 0.5 µCi/ml [14C]cholesterol in 50 µg/ml AcLDL for 24 h. In some wells, ABCA1 expression was induced by a 24 h preincubation with 0.34 mM 8Br-cAMP (Sigma, St. Louis, MO) as previously described (31, 32). The cells were washed and then chased for 4 h with the same media containing either no cholesterol acceptors or 0.4% (v/v) plasma from human apoA1 or 4WF transgenic mice in the absence or presence of 8Br-cAMP (for the pretreated wells). Radioactivity in the media, after spinning out cell debris, was counted directly, whereas radioactivity in the cells was determined after extraction with hexane-isopropanol (3:2). The cholesterol efflux from cells was calculated as 100 × (medium dpm) / (medium dpm + cell dpm). The ABCA1-dependent efflux was calculated as the difference between efflux in the presence of 8Br-cAMP (total efflux) and the absence of 8Br-cAMP (ABCA1-independent efflux). Total cholesterol efflux from BMDMs was determined similarly (without 8Br-cAMP pretreatment). In a separate study, RAW264.7 cells were chased with unmodified or MPO-oxidized HDL (50 µg apoA1 protein/ml) from human apoA1 and 4WF transgenic mice in presence or absence of 8Br-cAMP pretreatment.

**HDL binding and uptake assays by primary mouse hepatocytes**

Hepatocytes were isolated from male C57BL/6 mice, as previously described (33), and suspended in William’s E Medium with 10% FBS and primary hepatocyte supplement pack (Gibco, Grand Island, NY). Hepatocytes were then plated and cultured at a density of ~95,000 cells/ml in William’s E (incubated overnight at 56°C), and total protein concentration was measured by Micro BCA Protein assay (Thermo Scientific, Rockford, IL). The fluorescence intensity was normalized by the total protein concentration in each well.

**Statistical analysis**

Data are reported as mean ± SD. Kolmogorov-Smirnov tests were performed to assess whether the data were normally distributed. For normally distributed data, a parametric unpaired t-test was performed to compare the values between two groups, and one-way ANOVA with Newman-Keuls multiple comparison post-test was performed when comparing three or more groups. Statistical analyses were done using Prism software, version 5.0 for Windows (GraphPad Software, San Diego, CA).

**RESULTS**

**Plasma apoA1 and HDL-C levels in homozygous 4WF versus heterozygous apoA1 transgenic mice**

We generated one line of transgenic mice with high expression of 4WF human apoA1 isoform in which each of the four Trp residues was replaced by a phenylalanine residue. We quantified plasma apoA1 and HDL-C levels in 4WF heterozygous mice at 8, 18, and 28 weeks of age and compared them with commercially available wild-type human apoA1 heterozygous transgenic mice and found that the 4WF apoA1 levels were lower than those in the human apoA1 transgenics, and that the apoA1 and HDL-C levels did not change during this time course. In order to obtain 4WF and wild-type human apoA1 transgenic mice with matched plasma apoA1 levels, we bred the 4WF strain to homozygosity. Despite both transgenic strains being genetically inbred, we observed a range of human apoA1 and HDL-C levels in each strain. This allowed us to select mice with plasma 4WF apoA1 levels that matched the human apoA1 levels in human apoA1 transgenics for use in different experiments.

Fig. 1 shows a cohort (n = 5 for each genotype and gender) matched for plasma apoA1 levels in human apoA1 and 4WF transgenic mice (397.4 ± 14.4 mg/dl vs. 390.0 ± 9.0 mg/dl in male mice, and 271.2 ± 9.4 mg/dl vs. 270.2 ± 26.7 mg/dl in female mice; Fig. 1A). We observed a striking gender difference in plasma human apoA1 levels with male mice having higher plasma apoA1 than the female mice (P < 0.001 for both strains; Fig. 1A).

Among the males in this cohort, plasma HDL-C levels were ~23% lower in the 4WF versus the human apoA1 transgenic mice. In the females of this cohort, plasma HDL was ~7% higher in the 4WF strain; however, in both genders the difference was not statistically significant (Fig. 1B). Female mice from each strain had plasma HDL-C levels roughly comparable or slightly higher to the levels found in male human apoA1 transgenic mice, despite having significantly lower plasma apoA1 levels (Fig. 1A, B). Overall, the transgenic mice in this cohort had higher levels of HDL-C (~113 to 145 mg/dl) compared with those we commonly observe in wild-type C57BL/6 mice (~90 to 95 mg/dl).

Fast-protein liquid chromatography (FPLC) was used to separate plasma lipoproteins by size in pooled plasma samples. FPLC profiles mirrored the HDL-C levels in the male and female 4WF and human apoA1 transgenic mice (compare Fig. 1B with Fig. 1C, D). Furthermore, the FPLC profiles demonstrated that most of the cholesterol in both transgenic lines and genders was found in the HDL size range (HDL fractions numbers 22–28; Fig. 1C, D). Human apoA1 protein was quantified for the male mice in the
Characterization of 4WF-human apoA1 transgenic mice

HDL profile and composition

To examine HDL size heterogeneity, we isolated HDL from both transgenic strains and analyzed it by nondenaturating gradient gel electrophoresis. We identified five different peaks of particle diameter based on protein staining (Fig. 3A): peak 1 at ~12.2 nm roughly corresponding to even-numbered fractions, and it was found in a peak corresponding to the HDL fractions 22–28 (Fig. 1E), suggesting that most of the plasma apoA1 protein in both transgenic lines was associated with HDL-C.

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**Plasma levels of human and mouse apoA1**

In a separate group of female human apoA1 and 4WF transgenic mice with matched human apoA1 levels (294 ± 2.6 mg/dl and 288 ± 56.0 mg/dl, respectively), we quantified the relative abundance of human and mouse plasma apoA1 levels. Mouse apoA1 levels were determined by mass spectrometry after protease digestion and the addition of a heavy-isotope-labeled mouse apoA1 peptide as internal standard (25). The plasma mouse apoA1 levels in C57BL/6 mice were ~90.2 ± 16 mg/dl (Fig. 2). In the two transgenic lines, we observed striking 65% to 69% reductions in the levels of mouse apoA1 (28 ± 4 mg/dl and 31 ± 10 mg/dl mouse apoA1 in the human apoA1 and 4WF transgenic mice, respectively) compared with mouse apoA1 protein levels in C57BL/6 wild-type mice (Fig. 2). The level of human apoA1 in plasma of the 4WF and human apoA1 transgenic mice represented ~90% of the total plasma apoA1 protein. Our data are consistent with findings from previous studies that have reported that mouse apoA1 levels represent only a small fraction of total apoA1 in human apoA1 transgenic mice (25, 37, 38).

**Fig. 1.** Characterization of plasma apoA1 and HDL-C in human apoA1 heterozygous and 4WF homozygous transgenic mice. Plasma apoA1 (A) and HDL-C (B) levels were quantified in apoA1 heterozygous and 4WF homozygous female and male transgenic mice (n = 5 per group). Data are presented as mean ± SD. Different numbers above bars show P value <0.0001 (A) by ANOVA with Bonferroni posttest. Cholesterol profiles of human apoA1 and 4WF male (C) and female (D) transgenic mice were generated by FPLC analysis of pooled plasma samples. Human apoA1 protein levels in males (E) were quantified in even-numbered FPLC fractions by an immunoturbidimetric assay.

**Fig. 2.** Quantification of mouse and human apoA1 in plasma from C57BL/6, human apoA1, and 4WF transgenic mice. Mouse apoA1 (gray bars) and human apoA1 (open bars) levels were quantified in plasma samples isolated from apoA1 and 4WF female transgenic mice with matched human apoA1 levels. Data are presented as mean ± SD (n = 3 per group). For mouse apoA1 levels, different numbers above bars show P value <0.01 by ANOVA with Newman-Keuls posttest.
and 4WF transgenic mice (supplementary Table 1). In HDL from both transgenic lines and genders the total cholesterol represented 31–35% of the total lipids present on HDL particles, and phospholipids represented 59–65% of the total lipids levels. We did not have sufficient sample to quantify HDL TGs in male samples; however, we found similar TG levels in human apoA1 and 4WF HDL isolated from female mice (representing 1.6% and 1.3% of the total lipids, respectively).

Human apoA1 and HDL-C turnover

We performed a human apoA1 turnover study in cohorts of human apoA1 and 4WF transgenic mice using \(^{2}\)H\(_{2}\)O labeling as previously described (25). As seen above in Fig. 2, males in this study had consistently higher human apoA1 levels compared with females, which we replicated in our turnover cohort (\(P < 0.01\) and \(P < 0.001\) for human apoA1 and 4WF transgenic mice, respectively, after Bonferroni correction; Table 1). Among the human apoA1 transgenics, this was associated with a 60% increase in PR (\(P < 0.01\)), and a 27% increase in FCR (\(P < 0.05\)).

Table 1. Plasma levels and kinetics of human apoA1 in human apoA1 and 4WF male and female transgenic mice (n = 4)

|          | ApoA1 Male (1) | ApoA1 Female (2) | 4WF Male (3) | 4WF Female (4) | P, 1 versus 2 | P, 3 versus 4 | P, 1 versus 3 | P, 2 versus 4 |
|----------|----------------|------------------|--------------|----------------|---------------|---------------|---------------|---------------|
| ApoA1 levels (mg/dl) | 438.9 ± 15.4 | 351.4 ± 16.6 | 456.5 ± 23.3 | 329.5 ± 23.6 | <0.01 | <0.001 | NS | NS |
| ApoA1 PR (mg/kg/h) | 12.13 ± 0.03 | 7.62 ± 0.2 | 10.29 ± 1.65 | 6.28 ± 0.87 | <0.01 | <0.01 | NS | NS |
| ApoA1 FCR (%)h) | 6.41 ± 0.03 | 4.83 ± 0.32 | 5.24 ± 0.8 | 4.22 ± 0.30 | <0.05 | NS | NS | NS |

Fig. 3. HDL profile and protein composition. Non-denaturing gradient gel electrophoresis (A; male samples) and SDS-PAGE (B; female samples) were performed on human apoA1 and 4WF transgenic HDL isolated by sequential gradient ultracentrifugation. HDL samples were run at 10 µg of total HDL protein per lane, stained with Coomassie blue, and scanned by densitometry.
Similarly, among the 4WF transgenics, this was associated with a 64% increase in PR ($P < 0.01$) and a nonsignificant 24% increase in FCR. Thus, the major reason for higher human apoA1 levels in male versus female transgenic mice is the increase in apoA1 PR. Within each gender, we compared human apoA1 turnover in the wild-type and 4WF transgenics. In males, the wild-type human apoA1 versus the 4WF transgenics had nonsignificant 18% and 17% increases in apoA1 PR and FCR, respectively. In females, the human apoA1 versus the 4WF transgenics had marginal 21% and 14% increases in apoA1 PR and FCR, respectively. Thus, there were only small effects of the 4WF apoA1 isofrom on apoA1 turnover.

RCT in human apoA1 and 4WF transgenic mice

RCT was assessed in ~6-month male human apoA1 and 4WF transgenic mice matched for human apoA1 levels ($n = 5$ per group, the same mice shown in Fig. 1) by subcutaneous injection of [3H]cholesterol-labeled foam cells and following the cholesterol efflux and radioactivity into the plasma, liver, and feces at 72 h postinjection. The 4WF transgenic hosts had ~31% less [3H]cholesterol transfer to the plasma when compared with the human apoA1 transgenic hosts (Fig. 4A, $P = 0.006$), which is larger than the 23% difference in HDL-C levels between the two transgenic lines. Despite the large difference in RCT to the plasma compartment, RCT of [3H]cholesterol to the liver and feces was not different between the human apoA1 and 4WF transgenic lines (liver RCT = 6.97 ± 0.6% and 7.10 ± 0.62%, and fecal RCT = 21.7 ± 2.1% and 20.7 ± 1.9% in human apoA1 and 4WF transgenics, respectively; Fig. 4B, C).

Ex vivo evaluation of plasma efflux capacity from apoA1 and 4WF transgenics

To further examine the functional properties of the human apoA1 and 4WF apoA1 isoforms produced in vivo, we tested the ability of plasma from apoA1 and 4WF transgenic mice with matched human apoA1 levels to promote cholesterol efflux from either [14C]cholesterol-loaded wild-type BMDMs or RAW264.7 macrophages. Our prior study demonstrated that cholesterol efflux increased linearly with mouse plasma concentrations up to 0.5% (30); thus, we used 0.4% (v/v) in our efflux studies (40). The plasma from human apoA1 and 4WF transgenic mice similarly increased cholesterol efflux from BMDMs by 2.9- and 3.2-fold, respectively, compared with no plasma control (Fig. 5A, $P < 0.001$). In cholesterol-loaded RAW264.7 macrophages, transgenic plasma significantly increased [14C] cholesterol ABCA1-independent and total efflux when compared with no plasma control (Fig. 5B). Total cholesterol efflux from these cells was similar in presence of plasma from human apoA1 versus 4WF transgenic mice (17.2 ± 1.7% vs. 17.7 ± 1.2%, respectively; Fig. 5B). However, we found that ABCA1-independent efflux was slightly higher in presence of plasma from human apoA1 transgenics versus 4WF transgenic mice (7.7 ± 0.6% and 7.0 ± 0.2%, respectively, $P < 0.05$; Fig. 5B). Plasma from the human apoA1 and 4WF strains had efficient and similar ABCA1-dependent acceptor activity (9.2 ± 2.0% vs. 10.7 ± 1.2%, respectively; Fig. 5B). Overall, our data indicate that plasma from 4WF transgenic mice had similar total cholesterol efflux capacity compared with plasma from apoA1 transgenic mice.

Ex vivo evaluation of native and oxidized apoA1 and 4WF HDL ability to efflux cholesterol from RAW 264.7 macrophages

Previous in vitro studies have shown that purified recombinant 4WF apoA1 maintains its activity as an ABCA1-dependent cholesterol acceptor upon oxidation by the MPO/H$_2$O$_2$/Cl$^-$ system, while the wild-type isoform becomes dysfunctional under the same conditions (21). We hypothesized that oxidized HDL from 4WF transgenic mice would maintain its cholesterol acceptor activity better than oxidized human apoA1 HDL. To test our hypothesis, we isolated HDL from male human apoA1 and 4WF transgenic mice with matched plasma apoA1 levels via ultracentrifugation. A portion of the HDL isolated from both strains was oxidized by MPO/H$_2$O$_2$/Cl$^-$ system (10 mol of H$_2$O$_2$/mol of apoA1), and modified and unmodified HDL at 50 µg human apoA1 per ml was used as a cholesterol acceptor incubated with [3H]cholesterol labeled RAW264.7 cells. The ABCA1-independent cholesterol acceptor activity of HDL obtained from human apoA1 transgenic mice was significantly lower (reduced by 27%) after oxidation (Fig. 6A). The HDL obtained from 4WF transgenic mice had slightly less ABCA1-independent cholesterol acceptor activity (17% lower) compared with HDL from apoA1 transgenics, but its ABCA1-independent cholesterol acceptor activity was not impaired upon oxidation (Fig. 6A). ABCA1-dependent cholesterol efflux is higher in magnitude and, as expected, appeared to be more sensitive to MPO oxidation than the ABCA1-independent cholesterol efflux. Unmodified 4WF HDL had 12% lower cholesterol ABCA1-dependent acceptor activity than unmodified apoA1 HDL (9.2 ± 0.2% vs. 10.5 ± 0.6%, respectively,
In vivo MPO-dependent modification of wild-type human apoA1

We tested whether human apoA1 is modified in vivo by MPO by injecting human apoA1 protein subcutaneously and zymosan intraperitoneally in C57BL/6 wild-type and mouse MPO-deficient mice. We previously demonstrated that zymosan induces an acute inflammatory response in mice, which reduces RCT (40). Three days later, we assayed the mouse plasma for human apoA1 containing the MPO-specific oxTrp72 modification using a newly developed sensitive and specific ELISA assay (30). There was no detectable oxTrp72-apoA1 in MPO-deficient mice lacking the MPO gene, whereas in MPO-expressing mice there was 4.24 ± 0.07% oxTrp72 per mg of total plasma human apoA1. The findings provided further evidence that plasma wild-type human apoA1 can be oxidized by MPO in vivo.

Native and oxidized apoA1 and 4WF HDL binding and uptake by primary mouse hepatocytes

To assess the ability of unmodified and MPO-modified HDL from human apoA1 and 4WF transgenic mice to interact with SR-BI, we performed Dil-labeled HDL uptake and binding assays using freshly isolated primary hepatocytes from wild-type C57BL/6 mice, which take up HDL primarily via SR-BI (36). We isolated HDL from female human apoA1 and 4WF transgenic mice via ultracentrifugation and a portion of each was oxidized by the MPO/H2O2/Cl-/H11002 system (10 mol of H2O2/mol of apoA1). We observed that MPO oxidation of HDL from human apoA1 transgenic mice significantly impaired its binding by ~47% (P < 0.01 by ANOVA; Fig. 7A). The unmodified HDL from 4WF transgenic mice had equivalent binding to hepatocytes compared with unmodified HDL from human apoA1 transgenic mice. However, oxidized 4WF HDL still maintained all of its hepatocyte binding activity compared with its unmodified form (Fig. 7A). The uptake of HDL from apoA1 transgenic mice was 23% higher than the uptake of HDL from 4WF transgenic mice (P < 0.01 by ANOVA; Fig. 7B). However, upon MPO oxidation, the uptake of apoA1 transgenic HDL was markedly impaired (reduced by ~60%; P < 0.001 by ANOVA), while the uptake of oxidized HDL from 4WF transgenic was only reduced by ~26% (P < 0.05 by ANOVA). Thus, the 4WF HDL was more resistant to MPO oxidation in regard to its ability to be bound and taken up by hepatocytes, presumably via SR-BI.

P < 0.05; Fig. 6B); however, MPO oxidized apoA1 HDL lost all of its ABCA1-dependent cholesterol acceptor activity (not significant vs. no plasma control; P < 0.001 vs. unmodified apoA1 HDL), while MPO oxidized 4WF HDL still maintained half of its cholesterol acceptor activity (P < 0.001 vs. oxidized human apoA1 HDL; Fig. 6B). Similar results were observed for total cholesterol acceptor activity (Fig. 6C), with oxidized HDL from 4WF mice having 1.8-fold higher total acceptor activity than oxidized HDL from human apoA1 mice (P < 0.001 by ANOVA posttest).

In vivo MPO-dependent modification of wild-type human apoA1

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Fig. 4. RCT in human apoA1 and 4WF transgenic mice with matched levels of human plasma apoA1. RCT to plasma (A), liver (B), and cumulative feces (C) 72 h post [3H]labeled foam cell injection into human apoA1 and 4WF male transgenic hosts. Data are presented as mean ± SD (n = 5 per group). Values represent % of the injected [3H]cholesterol counts. P value shown for significant differences by unpaired t-test.

Fig. 5. Ex vivo evaluation ofapoA1 and 4WF plasma cholesterol acceptor activity. A: Plasma cholesterol acceptor activity from wildtype mouse BMDMs. B: Plasma cholesterol acceptor activity from RAW264.7 cells in the absence or presence of 8Br-cAMP treatment to distinguish ABCA1-independent, total, and ABCA1-dependent (by subtraction of independent from total) cholesterol efflux. For both panels, no plasma control is shown in black bars; 0.4% (v/v) plasma from human apoA1 (blue bars) or 4WF (green bars) male transgenic mice with matched human apoA1 levels was used as cholesterol acceptor. Data are presented as mean ± SD (n = 5 per group; each sample run in triplicate). Different numbers above bars show P values <0.001 (A) or P values of <0.05 (B) by ANOVA with Newman-Keuls posttest.
does not play a major role in regulating HDL-C, as HDL stays relatively constant in human females during puberty and menopause (45). Instead, testosterone appears to be the major sex hormone in humans that physiologically regulates HDL-C levels. Boys and girls before puberty have similar HDL-C levels, which drops in males during puberty (45, 46). In addition, blockage of endogenous testosterone in males increases HDL-C levels (46, 47). From these human HDL-C data, we infer that testosterone lowers apoA1 PR in humans. Thus, the effect of sex on human apoA1 levels appears to be in the opposite directions in humans versus transgenic mice. In our study, we observed that the human apoA1 PR was significantly higher in male apoA1 transgenic mice compared with females. By combining our finding with the prior studies, we can only speculate that the sex effect on human apoA1 levels in transgenic mice is probably due to a sex hormone effect on human apoA1 PR.

DISCUSSION

Functional properties of HDL are considered to play a major role in determining its protective effects against cardiovascular disease. Several studies have demonstrated that HDL isolated from subjects with CAD is dysfunctional, becomes proinflammatory, and loses its cholesterol acceptor activity (13–17). MPO-dependent apoA1 oxidation has been identified as one mechanism for transforming normal HDL into dysfunctional HDL (17, 41). Different studies have identified specific residues in human apoA1 that are targeted and modified by MPO under oxidative conditions. Peng et al. (21) found that replacing all four Trp residues with phenylalanine, creating the 4WF variant, led to a fully functional apoA1 that is highly resistant to MPO-mediated oxidation as determined by its cholesterol acceptor activity. Thus, the 4WF isoform of apoA1 may have a therapeutic potential to promote RCT in individuals who are exposed to oxidative conditions and have high levels of dysfunctional HDL. Human atheroma is highly oxidative and contains abundant MPO; therefore, the 4WF oxidant-resistant variant of human apoA1 may be superior in preventing plaque formation or promoting RCT and atherosclerosis regression. In this study, we expanded on the previous work by comparing the oxidant-resistant 4WF variant with its human apoA1 wild-type isoform made in vivo in mouse models. We generated a new line of transgenic mice carrying the 4WF variant of human apoA1 protein on C57BL/6 background. By breeding the 4WF transgenic mice to homozygosity, we obtained mice with plasma human apoA1 levels that matched those found in mice heterozygous for the wild-type isoform of human apoA1.

We found that gender had a significant effect on the plasma levels of human apoA1 in both lines of transgenic mice, with male mice having significantly higher levels than female mice (≈1.6-fold). It is well known that there is a significant gender effect on apoA1 and HDL-C levels in humans with females having higher plasma apoA1 and HDL-C than males (42, 43). Schaefer et al. (42) reported that this gender effect was due to a higher apoA1 PR in females versus males. Although postmenopausal estrogen therapy in women is associated with increased HDL-C levels due to increased apoA1 PR (44), physiological estrogen

Fig. 6. Plasma HDL-C acceptor activity in the absence or presence of ex vivo MPO-mediated oxidation. ABCA1-independent (A), ABCA1-dependent (B), and total (C) [3H]cholesterol effluxed from RAW 264.7 cells in the presence of either MPO buffer control, native (filled bars) or MPO-oxidized (open bars) HDL isolated from apoA1 (blue) or 4WF (green) transgenic mice. HDL containing human apoA1 or the 4WF isoform, isolated form male mice, was added at a final concentration of 50 µg of apoA1/ml of media. Data are presented as mean ± SD (each condition run in triplicate). Different numbers above bars show P values <0.001 (A) or <0.05 (B, C), by ANOVA with Newman-Keuls posttest.

Fig. 7. HDL uptake and binding by primary mouse hepatocytes. Binding at 4°C (A) and uptake at 37°C (B) of DiI-labeled HDL by primary mouse hepatocytes normalized to cellular protein. Native (filled bars) and oxidized (open bars) DiI-labeled HDL from apoA1 (blue) or 4WF (green) transgenic mice was added at a final concentration of 25 µg of apoA1/ml of media. Data are presented as mean ± SD (each condition run in quadruplicates). Different numbers above bars show P values <0.05 by ANOVA with Newman-Keuls posttest.
We observed that, despite being matched for plasma human apoA1 levels, 4WF transgenic mice usually had lower plasma HDL-C levels than the human apoA1 transgenic mice in two different cohorts (a trend for males in the first cohort; significant for females in the second cohort, males not studied). HDL from both human apoA1 and 4WF transgenic mice was polydisperse on native gel electrophoresis, similar to human HDL, and in contrast to mouse HDL, which has one major species (37). We did not observe much difference in the HDL subspecies between the two transgenic lines. In addition, we did not observe meaningful differences in HDL protein or lipid composition between these two strains. Circulating levels of HDL-C are determined by its degradation and PRs (39). Based on our HDL turnover study performed only in females, the difference in HDL-C levels between the 4WF and human apoA1 transgenic mice can be attributed to a significantly lower PR in the 4WF strain, with no significant effect on FCR. Thus, 4WF apoA1 appears to be less efficient in generating new HDL-C than human apoA1 for reasons that we do not understand. Thus, we cannot easily explain why at matched human apoA1 levels there is a trend for decreased HDL-C in the 4WF transgenic line, which reaches significance in some, but not all of our paired cohorts. It is possible that our apoA1 immunoassay slightly overestimates the level of 4WF apoA1 versus human apoA1, although we used recombinant 4WF and human apoA1 spiked into mouse lipoprotein-deficient plasma to calibrate our assay. Our RCT data in these mice are consistent with the difference in plasma HDL-C, in that RCT to the plasma was significantly lower in 4WF transgenic mice. However, the overall or global extent of RCT to the liver and fecal compartments was very similar in both strains.

Thus, RCT to the plasma compartment does not appear to be rate limiting for the eventual excretion of macrophage foam cell-derived cholesterol. Although different mouse genetic background may alter the response to apoA1 transgenes (48), both our 4WF and the human apoA1 transgenic model previously made by Rubin’s laboratory (11) are on the C57BL/6J background. It is possible that subtle genetic variation between these C57BL/6J strains could account for the observed differences in RCT between these transgenic strains, but we think it is much more likely that the RCT effect is inherent to these two human apoA1 isoforms.

Despite strong epidemiological data showing an inverse correlation between HDL-C levels and CAD risk, it is now apparent that increasing HDL-C pharmacologically or genetically may not be associated with less CAD (39). Thus, it is the functions of HDL, rather than HDL-C levels, that may protect against atherosclerosis. These functions include RCT, anti-inflammatory, antiapoptotic, antithrombotic, and endothelial repair activities. Recently, apoB-depleted serum cholesterol acceptor activity was found to be an independent predictor of CAD status in a case-control study (14). Although our group confirmed this result for prevalent CAD, we found a paradoxical result in that higher apoB-depleted serum cholesterol acceptor activity was associated with higher incident CAD in a longitudinal study (15). To test the function of the 4WF isoform to facilitate cholesterol efflux from macrophages and act as a cholesterol acceptor, we performed ex vivo cholesterol efflux assays in the presence of plasma from the 4WF and human apoA1 transgenic mice with matched human apoA1 levels. Plasma from 4WF transgenic mice had similar activity as the plasma from human apoA1 transgenic mice in facilitating cholesterol efflux from bone marrow-derived and RAW264.7 macrophages. This supports the previous study by Peng et al. (21), which reported that the 4WF and wild-type recombinant human apoA1 isoforms have equivalent cellular cholesterol acceptor activity.

Taken together, the data from our study demonstrated that despite differences in plasma HDL-C levels, 4WF transgenic mice, compared with human apoA1 transgenic mice, retain similar levels of RCT to the liver and feces, as well as similar plasma activity to accept cholesterol and facilitate ex vivo cholesterol efflux from macrophages under normal conditions. In vitro studies using lipid-free apoA1 found that the 4WF isoform versus wild-type human apoA1, while still susceptible to MPO mediated cross-linking, was highly resistant to MPO-mediated loss of its cholesterol acceptor activity (21). We tested the susceptibility of HDL from 4WF and human apoA1 transgenic mice to MPO oxidation and found that ABCA1-dependent cholesterol acceptor activity was almost completely lost in apoA1 transgenic HDL, while oxidized 4WF HDL retained ~50% of its ABCA1-dependent cholesterol acceptor activity. 4WF HDL had a modest 17% reduction in ABCA1-independent cholesterol acceptor activity, which we attribute to a small reduction in HDL-C in samples with matched apoA1 levels. We did not directly test the effect of 4WF transgenic HDL on ABCG1-mediated cholesterol efflux. However, because ABCG1-mediated efflux is one of the pathways involved in ABCA1-independent efflux, we can assume that 4WF HDL has at most a small effect on ABCG1-mediated cholesterol efflux. Why was the 4WF HDL only 50% protected from the MPO-mediated loss of its ABCA1-dependent cholesterol acceptor activity, while 4WF recombinant apoA1 protein was almost completely protected from this MPO-mediated loss of activity? First, 4WF transgenic HDL contains ~10% mouse apoA1, which due to its conserved Trp residues is expected to be susceptible to MPO-mediated loss of function. Second, 4WF HDL contains lipids that may also be oxidized by MPO leading to the formation of lipid peroxides that may create other types of apoA1 modification associated with loss of cholesterol acceptor activity.

Recently, Huang et al. (30) described that the MPO-mediated oxidation of human apoA1 Trp72 is the single most important modification resulting in the loss of its cholesterol acceptor activity. Furthermore, it was found that ~20% of human apoA1 recovered from aortic plaques is modified by oxidation at Trp72. In addition, it was demonstrated that oxidized Trp72-apoA1 recovered from human atheroma has impaired ABCA1-dependent HDL biogenesis activity with cultured cells and in vivo after injection into apoA1-deficient mice (30).
Previously, Wu et al. (49) demonstrated that MPO oxidation of reconstituted HDL (rHDL) impaired its ability to activate LCAT. Recently, DiDonato et al. (50, 51), made recombinant human apoA1 with the specific substitution of Tyr166 by nitro-tyrosine, incorporated by the use of an orthogonal nitro-tyrosine tRNA synthase. rHDL made with nitro-Tyr166 apoA1 was markedly impaired in its ability to activate LCAT compared with wild-type apoA1 (50, 51). Our HDL compositional analysis demonstrated that human apoA1 and 4WF transgenic HDL have similar cholesterol esters to total cholesterol ratios; thus, we do not think that 4WF apoA1 has impaired ability to activate LCAT. However, we suspect that MPO will equally impair LCAT activation of these two HDL isoforms, as both species retain the MPO-sensitive Tyr166 residue.

In conclusion, we have 1) generated and characterized a new line of transgenic mice carrying the 4WF isoform of human apoA1 protein; 2) demonstrated that the 4WF transgenic mice retain similar levels of RCT to the liver and feces as human apoA1 transgenics; 3) established that 4WF plasma has a similar ability to accept cholesterol and facilitate ex vivo cholesterol efflux from macrophages as human apoA1 transgenic plasma; and 4) found that the 4WF transgenic HDL is superior to human apoA1 transgenic HDL in retaining its cholesterol acceptor activity after ex vivo MPO-mediated oxidation, providing further proof that apoA1 Trp residues play a major role in apoA1 dysfunction. Further studies are needed to test the superiority of 4WF isoform compared with wild-type human apoA1 in delaying atherosclerosis progression and promoting lesion regression.

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