Pegylation of High-Density Lipoprotein Decreases Plasma Clearance and Enhances Antiatherogenic Activity

Andrew J. Murphy, Samuel Funt, Darren Gorman, Alan R. Tall,* Nan Wang*

Rationale: Infusions of apolipoprotein AI (apoAI), mimetic peptides, or high-density lipoprotein (HDL) remain a promising approach for the treatment of atherosclerotic coronary disease. However, rapid clearance leads to a requirement for repeated administration of large amounts of material and limits effective plasma concentrations. Because pegylation of purified proteins is commonly used as a method to increase their half-life in the circulation, we determined whether pegylation of apoAI or HDL would increase its plasma half-life and in turn its antiatherogenic potential.

Methods and Results: Initial pegylation attempts using lipid-poor apoAI showed a marked tendency to form multi-pegylated (PEG) species with reduced ability to promote cholesterol efflux from macrophage foam cells. However, pegylation of human holo-HDL or reconstituted phospholipid/apoAI particles (rHDL) led to selective N-terminal monopegylation of apoAI with full preservation of cholesterol efflux activity. The plasma clearance of PEG-rHDL was estimated after injection into hypercholesterolemic Apoe–/– mice; the half-life of pegylated PEG-apoAI after injection of PEG-rHDL was increased ≈7-fold compared with apoAI in nonpegylated rHDL. In comparison with nonpegylated rHDL, infusion of PEG-rHDL (40 mg/kg) into hypercholesterolemic Apoe–/– mice led to more pronounced suppression of bone marrow myeloid progenitor cell proliferation and monocytes, as well as reduced atherosclerosis and a stable plaque phenotype.

Conclusions: We describe a novel method for effective monopegylation of apoAI in HDL particles, in which lipid binding seems to protect against pegylation of key functional residues. Pegylation of apoAI in rHDL markedly increases its plasma half-life and enhances antiatherogenic properties in vivo. (Circ Res. 2013;113:e1-e9.)

Key Words: apoAI ■ atherosclerosis ■ cholesterol ■ HDL ■ hematopoiesis

Extensive epidemiological data have shown an inverse relationship between high-density lipoprotein (HDL) levels and coronary heart disease.1,2 There is also convincing evidence for a protective role of HDL against atherogenesis in animal models,3,4 notably after infusion of HDL or reconstituted HDL (rHDL) in hypercholesterolemic animal models.5,6 However, the development of new therapies that increase plasma HDL levels has proved challenging. This was highlighted by the failure of torcetrapib and dalcetrapib, 2 different inhibitors of cholesteryl ester transfer protein, and of extended-release niacin in recent clinical trials (Atherothrombosis Intervention in Metabolic syndrome with low HDL/high triglycerides: impact on Global Health outcomes and Heart Protection Study 2—Treatment of HDL to Reduce the Incidence of Vascular Events).5–12 In addition, carriers of the LIPG 396Ser allele in endothelial lipase had higher HDL cholesterol concentrations, but this allele was not associated with an altered risk of myocardial infarction,13 suggesting that raising HDL cholesterol by inhibition of endothelial lipase will not reduce atherosclerosis risk.14 Ongoing clinical studies with more potent, apparently nontoxic cholesteryl ester transfer protein inhibitors6 will provide further evaluation of cholesteryl ester transfer protein inhibition but will not test the HDL hypothesis because these agents substantially lower low-density lipoprotein levels and increase HDL.

A promising approach to reduce coronary atherosclerosis is the infusion of cholesterol-poor rHDL.15–17 In 2 small-scale randomized clinical trials, infusions of wild type or the Milano variant of apolipoprotein AI (apoAI) in complexes with phospholipids in patients who had recently experienced acute coronary syndromes reduced atheroma volume in coronary arteries.15–17 Infusion of rHDL in patients with peripheral vascular disease also resulted in significant remodeling of the atheroma and suppression of inflammation.16 These results most likely relate to the ability of cholesterol-poor rHDL particles to act as highly efficient acceptors of cholesterol from macrophage foam cells and other vascular cells involved in atherogenesis18,19 and to promote reverse cholesterol transport.20 Animal models have revealed that raising HDL...
levels by genetic methods or infusion of rHDL inhibits vascular inflammation,31 significantly remodels atherosclerotic lesions,7,19,22–24 and improves endothelial function.25

Recent studies also indicate that dysregulated cholesterol homeostasis in hematopoietic stem and multipotent progenitor cells (HSPCs) increases the risk of atherosclerosis as cholesterol accumulation in these cells promotes cell proliferation and causes monocytopoiesis and neutrophilia, which contribute to accelerated atherogenesis.26,27 Accordingly, increasing HDL in hypercholesterolemic Apoe−/− mice reduced HSPC proliferation and monocytopoiesis28 and decreased mobilization of HPSCs and extramedullary hematopoiesis,29 suggesting a potential further application of rHDL in the treatment of myeloproliferative neoplasms.

A major drawback of rHDL infusions in clinical practice is the need for repeated administration of a relatively large amount of material because of the rapid clearance of HDL from the plasma.17 The mechanisms responsible for turnover of plasma HDL are still poorly defined, although liver and kidney are the major organs taking up HDL.30 One approach to reduce clearance has been to generate multimers of recombinant apoAI. ApoAI multimers had increased molecular size, decreased clearance relative to apoAI monomers,31 and caused reduced atherogenesis in hypercholesterolemic mice.30 Current methods to produce HDL rely on the use of bile salts.32 Although the amphipathic nature of bile salt used in the preparation of rHDL facilitates rHDL particle formation, the residual bile salt in the rHDL preparation could cause adverse effects and, therefore, limit the quantity of the rHDL that can be administered.17 These considerations suggest the need for novel strategies to improve the therapeutic efficacy of rHDL preparations.

Protein pegylation (PEG), a process of covalent attachment of polyethylene glycol polymer chain to the target protein, has been used extensively to increase the therapeutic efficacy of protein drugs.31 However, protein pegylation has been used primarily for the modification of purified proteins. It is not clear whether pegylation can also increase the plasma half-life of proteins complexed with other biological molecules, such as lipids in lipoprotein particles, and whether this modification affects their biological activities. The goal of this study was to determine whether pegylation of lipid-poor apoAI or rHDL particles could increase the biological half-life of the preparation while preserving antiatherogenic functions.

**Methods**

**Animals**

C57BL6/J or Apoe−/− mice were from Jackson Laboratory. For atherosclerosis studies, Apoe−/− mice were fed a Western-type diet (WTD; TD88137; Harlan Teklad) for the indicated period of time. Where indicated, vehicle (saline), rHDL, or PEG-rHDL was injected at the indicated dose into the mice via the tail vein. Purified human apoAI or rHDL (CSL-111) was provided by CSL Behring AG, Bern, Switzerland; CSL-111 comprises human apoAI and phosphatidylcholine from soybean in a ratio of 1:150. The Columbia University animal ethics committee approved this study.

**Pegylation of ApoAI or HDL**

Holo-HDL was purified from human plasma, as previously described using KBr density gradients.32 Human apoAI, HDL, or rHDL were pegylated with monomethoxy polyethylene glycol-propionaldehyde (M-PEG-ALD) of MW 20,000 or 40,000. M-PEG-ALD was purchased from JenKem Technology, Allen, TX. After fully equilibrated to room temperature from −20°C storage and dissolved in an aliquot of 50 mmol/L sodium acetate, pH 5.5, 10 mmol/L sodium cyanoborohydride solution, M-PEG-ALD was immediately mixed at an indicated molar ratio with apoAI, HDL, or rHDL reconstituted in the same solution with gentle agitation. The final concentration of apoAI, HDL, or rHDL was ranging from 3 to 6 mg/mL. The mixture was incubated at 4°C for 16 to 72 hours. At the end of incubation, the reaction was quenched by addition of an aliquot of 1 mol/L Tris solution to the mixture to make the final concentration 100 mmol/L Tris. The pegylated apoAI, HDL, or rHDL were subjected to SDS-PAGE and Coomassie Brilliant Blue staining for evaluation of the pegylation efficiency. The unmodified control was processed similarly without M-PEG-ALD. For cholesterol efflux assays or infusion of the pegylated or nonpegylated apoAI, HDL, or rHDL preparations into mice, same amounts of quenched, inactivated M-PEG-ALD were added to the nonpegylated apoAI, HDL, or rHDL preparations, and the pegylated or nonpegylated preparations were dialyzed against phosphate-buffered saline for final formulation. To determine the molecular mass of pegylated human apoAI, we isolated pegylated apoAI with a modified protocol of a method previously reported.33 In brief, pegylated apoAI preparation was subjected to SDS-PAGE and the unfixed, unpegated M-PEG-apoAI band was excised from the gel after its location in the gel was estimated using a sample run in the same gel in parallel. After passive elution from the gel strip, the pegylated apoAI sample was repeatedly diluted with phosphate-buffered saline and concentrated with Amicon centrifugal filters. The molecular mass of peg-ApoAI was determined by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

**Plasma Clearance**

An aliquot of unmodified or pegylated apoAI, HDL, or rHDL at the indicated dose was injected into the mice via tail vein. At the indicated time point, an aliquot of blood was collected from the mice. The blood samples were subjected to SDS-PAGE and Western analysis with anti-human apoAI antibodies. Native or PEG-apoAI was quantified by densitometry analysis with ImageJ.

**Cholesterol Efflux**

Cholesterol efflux from mouse peritoneal macrophages or THP-1 cell-derived macrophage-like cells was performed as described previously.32 In brief, the cells were cholesterol loaded by incubation with the indicated amount of acetyl–low-density lipoprotein containing [3H]cholesterol for 16 hours in the presence of 1 μmol/L TO901317. The cells were then washed and cholesterol efflux was initiated by the addition of indicated amount of cholesterol acceptors before the media and cells were collected for analysis. Cholesterol efflux was expressed as the percentage of the radioactivity released from the cells into the medium relative to the total radioactivity in cells plus medium.

**Analysis of Blood Leukocytes**

Total white blood cell count in freshly collected mouse blood was performed using hematology cell counter (Oxford Science Inc). Monocytes and neutrophils were identified from whole blood as previously described.26 Monocytes were identified as CD45+CD11b+ and neutrophils were identified as CD45+CD11b+Ly6-C/G+ (Gr-1).

**Analysis of Hematopoietic Stem Cells**

Hematopoietic stem and progenitor cells from the bone marrow were analyzed by flow cytometry as previously described.26 Isolated bone
morrow was incubated with a cocktail of antibodies against lineage-committed cells (B220, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, Ly6-C/G: all fluorescein isothiocyanate; eBioscience), Scal-Pacific Blue, and ckit-APC Cy7. HSPCs were identified as lin−, Sca1−, and ckit− (LSK) while the hematopoietic progenitor subsets were separated by using antibodies to CD16/CD32 (FcγRII/III) and CD34, Common myeloid progenitors were identified as lin−, Scal−, ckit−, CD34++ and FcγRII/III++ and granulocyte-macrophage progenitors as lin−, Scal−, ckit−, CD34++, and FcγRII/III++. Cell cycle analysis was performed using 4',6-diamidino-2-phenylindole (Sigma) in cells that had been stained with the above markers and then incubated in cytofix/cytoperm buffer (BD Biosciences). Flow cytometry was performed using an LSRII running FACSDiva software. Flow cytometry data were analyzed using FlowJo software (Tree Star Inc).

Quantification of Aortic Atherosclerosis Lesions

The lesions located in the aorta and aortic sinuses were analyzed using Oil Red O staining. Quantification of Oil Red O staining was performed off-line using Adobe Photoshop CS5 and presented as the percentage of the total surface area of the aorta. Lesion area in the aortic root was quantified by morphometric analysis of hematoxylin and eosin–stained sections as described. Collagen staining was performed using picrosirius red as per the manufactures instructions (PolySciences, Inc). Macrophage staining was performed by staining with antibody against F4/80 (Abcam).

Statistical Analysis

One-way ANOVA with post hoc analysis and r tests were performed using GraphPad Prism version 5.0 for Mac OS X (GraphPad Software, San Diego, CA, www.graphpad.com) or STATVIEW 5.0 (Abacus Concepts, Inc). The post-test was Fisher protected least significant difference and the threshold for significance was P<0.05. Data shown are mean±SEM.

Results

Characterization of N-Terminally Pegylated ApoAI

Ideally, pegylation of apoAI would not adversely affect apoAI lipidation or its capacity to promote cholesterol efflux. ApoAI structure and lipidation have been extensively studied. The carboxyl terminal amphipathic α-helix structure of apoAI seems to be critical for lipid binding and its lipidation by ATP-binding cassette transporter A1 activity. In contrast, ATP-binding cassette transporter A1–mediated cholesterol efflux is relatively insensitive to mutagenesis-induced changes in the N-terminal helix-bundle domain. Thus, we reasoned that amino terminal pegylation of apoAI might not disrupt the part of the structure of apoAI necessary for mediating cholesterol efflux. To test this idea, we took advantage of an acidic reaction condition that favors selective pegylation of the amino terminal residue of the target protein. When a re-active linear PEG molecule with average molecular size of 20 kDa was used for pegylation at 4°C, this strategy yielded a predominant single species of pegylated apoAI with retarded migration in SDS-PAGE (Figure 1A). The apparent molecular mass (Mr; ~70 kDa) was higher than expected (ie, human apoAI: 28 kDa+PEG: 20 kDa=48 kDa), likely reflecting aberrant migration, a common observation that has been attributed to PEG–SDS interactions when running pegylated proteins by SDS-PAGE. We also used mass spectrometry to determine the molecular mass of the gel-purified PEG-apoAI band. This showed a molecular mass of ~48 kDa, as expected for monopegylated human apoAI. Importantly, cholesterol efflux from macrophage foam cells was preserved using this method of pegylation (Figure 1B). This indicated that monopegulation of apoAI does not alter its function.

However, the targeted pegylation of lipid-poor apoAI at low temperature was suboptimal as the majority of the apoAI was not pegylated. We attempted to increase the yield of pegylated apoAI by adjusting variables such as the temperature and time of pegylation; however, this resulted in multi-pegylated species, suggesting pegylation of amino acid residues of apoAI in addition to the N terminus (Figure 1C). Furthermore, the multi-pegylated apoAI had reduced capacity to promote cholesterol efflux from macrophage foam cells (Figure 1D), indicating that unmodified amino acids of apoAI other than the N terminus are required to preserve functionality.

Characterization of Pegylated HDL

In an attempt to reduce the pegylation of residues other than the N terminus of apoAI, we assessed the use of human holo-HDL particles as the substrate for protein pegylation. We reasoned that the use of native HDL rather than lipid-poor apoAI might increase the specificity and efficiency of targeted pegylation, as functionally important amino acid residues might be shielded from pegylation by lipid molecules in HDL particles. Under conditions where lipid-poor apoAI gave rise to multi-pegylated species, pegylation of HDL gave rise mainly to monopegylated apoAI species, as shown by SDS-PAGE and Coomassie Blue staining (Figure 2A). After pegylation, >90% of human apoAI in HDL particles migrated at the Mr of the monopegylated apoAI species. The band that appears in the native HDL preparation of a similar size to PEG-HDL in Figure 2A is likely albumin, a common contaminant of HDL purified from plasma. We also performed a Western analysis using an anti-apoAI antibody, and no band of that size was detected in the native HDL preparation confirming that it was not an apoAI product (Figure 2B).

Figure 1. Characterization of pegylated human apolipoprotein AI (apoAI). A, ApoAI pegylated with monomethoxy polyethylene glycol-propionaldehyde (M-PEG-ALD) 20K at 4°C for 16 hours and subjected to SDS-PAGE and Coomassie Blue staining. B, Cholesterol efflux for 3 hours from cholesterol-loaded mouse peritoneal macrophages to apoAI or PEG-apoAI prepared as in A. C, ApoAI pegylated with M-PEG-ALD 20K at 4°C for 40 and 72 hours and subjected to SDS-PAGE and Coomassie Blue staining. D, Cholesterol efflux for 3 hours from cholesterol-loaded mouse peritoneal macrophages to apoAI or PEG-apoAI (10 μg protein/mL) prepared as in C (72 hour incubation). Mr indicates molecular ratio.
We next carried out studies to determine whether PEG-HDL promoted cholesterol efflux from macrophage foam cells. To rule out the potential effect of free PEG molecules in the efflux assay, the same amount of quenched PEG preparation was added to the unmodified HDL preparation during the efflux assay. The targeted pegylation of HDL particles did not affect the ability of HDL to promote cholesterol efflux. Cholesterol efflux from macrophages to the native HDL or PEG-HDL showed a similar dose response (Figure 2C).

To evaluate the pharmacokinetics of PEG-HDL particles, we infused PEG-HDL into Chow-fed wild-type mice. This preparation of PEG-HDL also contains a portion of nonpegylated human apoAI that served as an internal control. The half-life of HDL protein (apoAI) estimated by Western analysis of an aliquot of plasma was ≈9.2 hours (lower Mr band in Figure 2C), comparable with the estimated half-life of human apoAI infused into Chow-fed wild-type mice as reported by others.38 Infusion of native human HDL at similar dose gave rise to a similar half-life of human apoAI as determined from the internal standard (not shown). The turnover time of PEG-HDL, estimated by the clearance of the distinct PEG-apoAI, was increased to ≈48 hours when compared with that of unpegylated human HDL (Figure 2D higher Mr band=PEG-apoAI versus lower Mr band=non–PEG-apoAI), indicating ≈5-fold increase in circulation time. We also noted a nonspecific band that was detected by Western blot with anti-apoAI antibody (Figure 2D). However, we have confirmed this was not a modified form of human apoAI because this band was also detected in plasma from mice that received a saline infusion (Online Figure I, lane 1).

Characterization of Pegylated rHDL

For further characterization of properties of pegylated HDL, we used a preparation of rHDL that has been used in clinical studies.16 Targeted N-terminal pegylation of rHDL also yielded a largely homogeneous species of monopegylated apoAI (Figure 3A). To verify that apoAI was pegylated at the N terminus, the single species of pegylated apoAI was isolated and subjected to Edman N-terminal protein sequencing. Only ≈20% of the pegylated apoAI could be sequenced by Edman degradation whereas, as a control, ≈98% of apoAI that was subjected to similar treatment but without pegylation could be sequenced (not shown). This suggests that the majority (≈80%) of the monopegylated apoAI was N-terminally pegylated. This sequencing result confirmed the method of preferential N-terminal protein pegylation and suggests that it can be applied to proteins complexed with other biological components so that amino acid residues that are essential for function are protected. In line with this idea, cholesterol efflux assays showed that rHDL and PEG-rHDL promoted similar levels of cholesterol efflux from liver X receptor–activated human THP-1 macrophages (Figure 3B). We next carried out studies to examine rHDL or PEG-rHDL clearance from plasma in a hypercholesterolemic mouse model. rHDL or PEG-rHDL at a dose of 60 mg/kg (based on rHDL protein content) was injected intravenously in Apoe−/− mice fed on a WTD. The clearance of human apoAI or PEG-apoAI from plasma was assessed by SDS-PAGE and Western blotting. Again as in Figure 2, some non–PEG-rHDL was present in the PEG-rHDL–infused mice, which served as an internal control. This along with the comparison with mice infused with rHDL clearly demonstrated the increased half-life of PEG-rHDL (Figure 3C higher Mr band=PEG-HDL versus lower Mr band=non–PEG-HDL). Using densitometry, we found that PEG-rHDL had a markedly reduced clearance rate and an ≈7-fold prolonged half-life (t1/2=48.7 hours for PEG-rHDL versus t1/2=7.7 hours for rHDL, and t1/2=7.7 hours for non–PEG-rHDL internal control, n=5/group, P<0.001; Figure 3C and 3D). We also noted some intermediate bands in that reacted with the anti-apoAI antibody in the mice infused with PEG-rHDL (Figure 3C, right). It is possible that these could be partially degraded pegylated apoAI molecules because it has previously been reported that apoAI within HDL particles can be cleaved in vivo.38 In addition, the nonspecific band detected was not a by-product of PEG-rHDL preparation or a modification because this band was also detected in plasma from mice that received a saline infusion (Figure 1).
PEG-rHDL Is More Efficient in Reducing Hematopoietic Stem and Progenitor Cell Proliferation and Monocytosis

To test the idea that pegylation of rHDL would extend its half-life and thus promote a more prominent antiatherogenic effect, we infused rHDL or PEG-rHDL into WTD-fed Apoe−− mice. We and others have shown that hypercholesterolemic Apoe−− mice develop monocytosis that seems to contribute to accelerated atherosclerosis.26,40 and we showed that rHDL infusion could reduce monocyte levels in this mouse model.26 Thus, monocyte counts could potentially act as a biomarker for the in vivo efficacy of rHDL infusions. To detect a potential difference in the effectiveness of rHDL versus PEG-rHDL, we infused mice with 2 doses of 40 mg rHDL or PEG-rHDL protein/kg body weight separated by 1 week. This dosing schedule was chosen, as we had previously shown that 40 mg/kg of rHDL was a submaximal dose, having a modest effect on inhibiting monocytosis in WTD-fed Apoe−−.26 At the 40 mg/kg dose in the present study, rHDL infusion had no effect on the count of total white blood cells, monocytes, or neutrophils 7 days after the second infusion (Figure 4A and 4B). In contrast, PEG-rHDL significantly reduced these parameters relative to rHDL or the vehicle control. Our previous studies showed that effects of rHDL on monocyte counts reflected decreased proliferation of HSPCs and myeloid progenitors in the bone marrow.26 Consistent with its effect of blood poiesis, monocyte counts, and atherosclerosis.

PEG-rHDL Is More Effective in Inhibiting Atherosclerosis and Stabilizing Lesions

We initially conducted a dose–response study using rHDL infusions in Apoe−− mice fed a WTD for 4 weeks to ascertain the amount of rHDL to use in our comparison with PEG-rHDL. Our goal was to find a dose of rHDL that failed to significantly improve atherosclerotic lesions. We administered 2 infusions of rHDL (40, 80, or 120 mg protein/kg body weight) separated by 1 week. Doses of rHDL at 80 and 120 mg/kg, but not 40 mg/kg, significantly reduced the area of Oil Red O staining of the aortic arch compared with mice treated with vehicle (Online Figure SII). Therefore, we decided to compare rHDL with PEG-rHDL at a dose of 40 mg/kg.

We examined the impact of rHDL or PEG-rHDL at a dose of 40 mg/kg on the development of atherosclerosis in the hypercholesterolemic Apoe−− mice. We observed no changes in total plasma or HDL cholesterol levels measure 7 days after the second infusion (Online Figure SIII). Infusion of PEG-rHDL but not rHDL at equal doses (40 mg/kg) significantly reduced the area of Oil Red O staining of the aorta, indicating a reduction in aortic lipid content (Figure 5A). In comparison with pilot experiment (Online Figure SII), these results may suggest that PEG-rHDL administered at 40 mg/kg has an efficacy similar to rHDL at 80 mg/kg. Assessment of lesion formation in the proximal aorta showed no change in the overall lesion area (Figure 5B), which is consistent with a number of previous reports.30 However, we observed a significant reduction in the macrophage content and cellularity of proximal aortic lesions of PEG-rHDL–treated mice, which was accompanied by a reduction in necrotic core size (Figure 5C through 5E). Lesions of PEG-rHDL–treated mice also showed increased collagen (Figure 5F). Taken together, these results suggest that PEG-rHDL effectively remodeled lesions to what is considered a more stable phenotype, similar to that reported with rHDL infusions in humans with peripheral vascular disease and animal models where HDL levels were increased.7,19,22–24

Discussion

We have developed a novel formulation of HDL prepared by targeted pegylation of HDL particles. Although our initial studies focused on pegylation of lipid-poor apoAI, attempts to scale up preparations by increasing temperature or PEG concentration were unsuccessful because of formation of multi-pegylated apoAI species that were inefficient in cholesterol efflux assays. However, pegylation of plasma HDL or rHDL led to the formation of functional, N-terminally, monopegylated apoAI, likely because key amino acid residues were protected by binding to lipids. After injection of this preparation, the half-life of apoAI was increased ≈7-fold. Most importantly, PEG-rHDL was significantly more effective than control rHDL in reducing myelopoiesis, monocyte counts, and atherosclerosis.
The apparent increase in the efficiency of PEG-rHDL in reducing myelopoiesis and atherosclerosis was likely related to the increased half-life of apoAI in the preparation. Because the half-life of apoAI in HDL particles is several-fold more than that of HDL lipids, it is likely that apoAI is recycled through several generations of HDL particles, probably being released by lipases that act on HDL, followed by lipidation of apoAI via ATP-binding cassette transporter A1 in various tissues. However, we do not expect pegylation of rHDL to alter or enhance its cholesterol efflux ability on a molar basis compared with native rHDL. We speculate that compared with native apoAI, monopegylated apoAI may be less susceptible to glomerular filtration and degradation in kidney to increase its therapeutic concentration in the circulation, desirable properties for apoAI and HDL. Although pegylation does not lead to conformational changes of the polypeptide chains in most cases, it has the potential to reduce the immunogenicity of the target protein.

Various formulations of cholesterol acceptors intended to promote cholesterol efflux from macrophage foam cells and reverse cholesterol transport in vivo have been studied. In addition to rHDLs, infusions of synthetic amphipathic peptides complexed with phospholipids have been investigated. However, on a molar basis, the ability of synthetic peptides to promote cholesterol efflux from macrophage foam cells is much less than rHDL. Infusion of a large amount of the peptide–phospholipid complexes is needed to achieve effective concentrations, which may increase the potential toxicity, for example, in the kidney after glomerular filtration. In addition, the synthetic amphipathic peptides contain an artificial amino acid sequence, which has the potential to induce adverse immune responses.

Various strategies have been studied as a means of reducing the renal clearance of apoAI. This included recombinant trimeric apoAI, which showed an increased circulation time when injected into animals either as lipid-free or as lipidosome forms. However, this approach has some intrinsic limitations. Cost-effective large-scale manufacturing of recombinant proteins free of adverse

![Figure 4. Effects of reconstituted high-density lipoprotein (rHDL) or polyethylene glycol (PEG)-rHDL on leukocytosis and hematopoietic stem and multipotent progenitor cells (HSPC) proliferation associated with apolipoprotein E (apoE) deficiency. Apoe−/− mice fed a Western-type diet for 8 weeks (n=10/group) were given a single infusion of rHDL or PEG-rHDL (40 mg/kg) weekly for 2 weeks. Seven days after the second infusion, (A) peripheral blood white blood cells (WBC), (B) neutrophil and monocyte counts, as well as (C) bone marrow (BM) progenitor cell abundance and (D) proliferation were determined by flow cytometry. Data are presented as mean±SEM (n=10/group). *P<0.05 vs saline. CMP indicates common myeloid progenitor; and GMPs, granulocyte-macrophage progenitor.](http://circres.ahajournals.org/)
foreign components remains a challenge, particularly for apoAI with expected therapeutic doses higher than most recombinant protein drugs. In addition, trimeric apoAI, as a complex of recombinant fusion proteins of human apoAI and the trimerization domain of human tetranectin, has the potential to be recognized as nonself protein and to induce adverse immune responses. The lipidated trimeric apoAI also seems to be less efficient in promoting cholesterol efflux from macrophage foam cells relative to lipidated recombinant apoAI monomers, at equal molecular mass; however, trimeric apoAI preparations still retain many of the known anti-inflammatory functions of native apoAI.

Our study has several limitations. We compared the efficacy of rHDL and PEG-rHDL at a submaximal dose, which was chosen to assess a potential benefit of the pegylated preparation. For synthetic HDL preparations, the ability to use a lower dose represents a major advantage, both in terms of cost and convenience of preparations and also in terms of potential toxicities. We showed a beneficial effect of PEG-rHDL on aortic atherosclerosis as revealed by Oil Red O staining, consistent with reduced macrophage foam cells content. However, lesion area in the proximal aorta was not reduced, similar to some previous studies using rHDL infusion strategies. Nonetheless, macrophage content, lesion cellularity, and necrotic core size were decreased and collagen content was increased, consistent with lesion stabilization.

Figure 5. Quantification of aortic atherosclerosis lesions. ApoE−/− mice fed a Western-type diet for 8 weeks were infused with reconstituted high-density lipoprotein (rHDL) or polyethylene glycol (PEG)-rHDL 40 mg/kg weekly for 2 weeks. Seven days after the second infusion, the mice were euthanized and lesion characteristics were analyzed. A, The percentage of the aortic arch stained with Oil Red O staining. B, Lesion area in the proximal aorta. C, Macrophage content as assessed by F4/80 staining in the proximal aorta. D, Cellularity of the lesions in the proximal aorta determined by nuclei/area. E, Necrotic core size from hematoxylin and eosin (H&E)-stained sections of the proximal aorta. F, Collagen staining in the proximal aorta by picrosirius red. Data are presented as mean±SEM (n=10/group). *P<0.05 vs saline.
suppress HSPC proliferation and monocytosis. This indicates that monocyte and leukocyte counts may be useful biomarkers to assess the effectiveness of different HDL-based therapies in vivo. Together, the improved pharmacokinetics and enhanced antiatherogenic activity of PEG-rHDL suggest that it may be a useful preparation, with certain advantages such as lower dosing and decreased potential for toxicity.

Several recent clinical and genetic studies have led the generic HDL hypothesis to be called into question. However, we believe this hypothesis should be refined to emphasize HDL functionality especially its ability to promote macroparticle cholesterol efflux and reverse cholesterol transport. Although recent studies of HDL raising with niacin have not shown clinical benefit, the macrophage cholesterol efflux potential of HDL from subjects treated with niacin is at best marginally improved. HDL raising with dalcetrapib also did not produce clinical benefit, but lipoprotein changes may have been suboptimal because of incomplete cholesteryl ester transfer protein inhibition. Variants in some genes associated with increased HDL, and reverse cholesterol transport will not influence the production of apoAI or HDL. Finally, a large body of preclinical evidence strongly supports the concept that interventions to increase macrophage cholesterol efflux and reverse cholesterol transport, as is likely to occur with HDL infusions, are associated with reduced atherosclerosis.

Whether rHDL infusions succeed in improving clinical outcomes remains to be proven. If such therapies do prove successful, targeted N-terminal pegylation of various forms of apoAI or related peptides, which may be accomplished by initial introduction into a lipid-containing HDL particle before pegylation, may represent a novel method for extending the effectiveness, reducing the dose and decreasing toxicity of the therapeutic HDL preparation.

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References
Novelty and Significance

What Is Known?

- Raising high-density lipoprotein (HDL) by direct infusion of reconstituted HDL seems to be a promising therapeutic approach to treat cardiovascular disease.
- However, the relatively short half-life of the current preparations may require frequent dosing.
- Reconstituted HDL for clinical use has been difficult to manufacture and is expensive, and toxicity has limited dosing and usefulness.

What New Information Does This Article Contribute?

- We developed a method for efficient, N-terminal monopegylation of HDL, which greatly extends the in vivo half-life of HDL and increases its antatherogenic properties.
- This extends the concept of protein pegylation to pegylation of a particle, with beneficial results.
- The use of pegylated HDL may reduce the dosing requirement for reconstituted HDL preparations, with greater efficacy and reduced potential for side effects.

Directly raising HDL levels by reconstituted HDL is thought to be a promising approach in the treatment of cardiovascular disease. However, reconstituted HDL is cleared fairly quickly from the circulation, limiting its bioavailability. We describe a technique to effectively monopegylate the N terminus of apolipoprotein A-I in HDL particles which extends its in vivo half-life. Pegylation of HDL particles can be applied to holo-HDL, reconstituted HDL and possibly peptide/lipid-complexes, increasing efficacy, decreasing dosage, and potentially reducing side effects. This approach could be applied to the treatment of atherosclerosis and myeloproliferative neoplasms.
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Pegylation of HDL Decreases Plasma Clearance and Enhances Anti-atherogenic Activity
Andrew J. Murphy (PhD), Samuel Funt (MD), Darren Gorman, Alan Tall* (MD), Nan Wang* (PhD).

Materials and Methods

Animals
C57BL6/J or Apoe−/− mice were from Jackson Laboratory. For atherosclerosis studies, Apoe−/− mice were fed a Western type diet (WTD) (TD88137; Harlan Teklad) for the indicated period of time. Where indicated, vehicle (saline), rHDL, or PEG-rHDL was injected at the indicted dose into the mice via the tail vein. Purified human ApoA-I or rHDL (CSL-111) was provided by CSL Behring AG, Bern, Switzerland; CSL-111 is comprised of human apoA-I and phosphatidylcholine from soybean in a ratio of 1:150. The Columbia University animal ethics committee approved this study.

Pegylation of apoA-I or HDL
Holo-HDL was purified from human plasma as previously described using KBr density gradients1. Human ApoA-I, HDL or rHDL were pegylated with M-PEG-ALD of MW 20000 or 40000. M-PEG-ALD was purchased from JenKem Technology USA (Allen, TX). After fully equilibrated to room temperature from -20°C storage and dissolved in an aliquot of 50 mM sodium acetate, pH 5.5, 10 mM sodium cyanoborohydride solution, M-PEG-ALD was immediately mixed at an indicated molar ratio with apoA-I, HDL or rHDL reconstituted in the same solution with gentle agitation. The final concentration of apoA-I, HDL or rHDL was ranging from 3 to 6 mg/ml. The mixture was incubated at 4°C for 16 to 72 hours. At the end of incubation, the reaction was quenched by addition of an aliquot of 1M Tris solution to the mixture to make the final concentration 100 mM Tris. The pegylated apoA-I, HDL or rHDL were subjected to SDS-PAGE and Coomassie Brilliant Blue staining for evaluation of the pegylation efficiency. The unmodified control was processed similarly without M-PEG-ALD. For cholesterol efflux assays or infusion of the pegylated or non-pegylated apoA-I, HDL or rHDL preparations into mice, same amounts of quenched, inactivated M-PEG-ALD were added to the non-pegylated apoA-I, HDL or rHDL preparations and the pegylated or non-pegylated preparations were dialyzed.
against phosphate buffered saline for final formulation. To determine the molecular mass of pegylated human apoA-I, we isolated pegylated apoA-I with a modified protocol of a method previously reported\(^2\). Briefly, pegylated apoA-I preparation was subjected to SDS-PAGE and the unfixed, unstained PEG-apoA-I band was excised from the gel after its location in the gel was estimated using a sample run in the same gel in parallel. After passive elution from the gel strip, the pegylated apoA-I sample was repeatedly diluted with phosphate buffered saline and concentrated with Amicon Centrifugal filters. The molecular mass of PEG-apoA-I was determined by MALDI-TOF.

**Plasma clearance**

An aliquot of unmodified or pegylated apoA-I, HDL or rHDL at the indicated dose was injected into the mice via tail vein. At the indicated time point, an aliquot of blood was collected from the mice. The blood samples were subjected to SDS-PAGE and Western analysis with anti-human apoA-I antibodies. Native or PEG-apoA-I was quantified by densitometry analysis with ImageJ.

**Cholesterol efflux**

Cholesterol efflux from mouse peritoneal macrophages or THP-1 cell derived macrophage like cells was performed as described previously\(^1\). Briefly, the cells were cholesterol loaded by incubation with the indicated amount of acetyl-LDL containing \([^{3}\text{H}]\)cholesterol for 16 hours in the presence of 1 μM TO901317. The cells were then washed and cholesterol efflux was initiated by addition of indicated amount of cholesterol acceptors before the media and cells were collected for analysis. Cholesterol efflux was expressed as the percentage of the radioactivity released from the cells into the medium relative to the total radioactivity in cells plus medium.

**Analysis of blood leukocytes**

Total white blood cell count in freshly collected mouse blood was performed using hematology cell counter (Oxford Science Inc.). Monocytes and neutrophils were identified from whole blood as previously described\(^3\). Blood was collected from the tail into EDTA lined tubes and immediately placed on ice. All following steps were performed on ice. RBCs were lysed and WBCs were centrifuged, washed and resuspended in flow-buffer (Hanks balanced salt solution + 0.1% BSA w/v, 5mM EDTA). Cells were stained with a cocktail of antibodies against CD45-Alexa Fluor
Monocytes were identified as CD45<sup>hi</sup>CD115<sup>hi</sup> and neutrophils were identified as CD45<sup>hi</sup>CD115<sup>lo</sup>Ly6-C/G<sup>hi</sup> (Gr-1).

**Hematopoietic Stem Cells.** Hematopoietic stem and progenitor cells from the BM were analyzed by flow cytometry as previously described<sup>3</sup>. BM was harvested from femurs and tibias by flushing with ice cold PBS. Spleen was minced into small pieces, gently rubbed against the mesh in cell strainer and flushed with PBS. The cell suspension was centrifuged at 500 g for 5 mins, supernatant aspirated and the cells were resuspended in RBC lysis buffer (for 5 min). Lysis was stopped by adding excess amount of flow buffer and the cell suspension was once again centrifuged, aspirated and single cell suspension was prepared by gently pipetting up and down with 200 µl of flow buffer. This was followed by incubation with a cocktail of antibodies against lineage-committed cells (B220, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, Ly6-C/G: All FITC, eBioscience), Sca1-Pacific Blue and ckit-APC Cy7. HSPCs were identified as lin<sup>−</sup>, Sca1<sup>+</sup> and ckit<sup>+</sup> (LSK) while the hematopoietic progenitor subsets were separated by using antibodies to CD16/CD32 (FcγRII/III) and CD34. CMPs were identified as lin<sup>−</sup>, Sca1<sup>−</sup>, ckit<sup>−</sup>, CD34<sup>int</sup>, FcγRII/III<sup>int</sup>, GMPs as lin<sup>−</sup>, Sca1<sup>−</sup>, ckit<sup>−</sup>, CD34<sup>int</sup>, FcγRII/III<sup>int</sup>. Cell cycle analysis was performed using DAPI (Sigma) in cells that had been stained with the above markers and then incubated in cytofix/cytoperm buffer (BD Biosciences).

Flow cytometry was performed using an LSRII running FACS DiVa software. Flow cytometry data was analyzed using FlowJo software (Tree Star Inc.).

**Quantification of aortic atherosclerosis lesions**

The mice were euthanized and perfusion fixed with 10% buffered formalin via the left ventricle for 5 minutes. The lesions located in the aorta and aortic sinuses were analyzed using Oil Red O staining. To measure lesions in the aorta, the whole aorta, including the ascending arch, thoracic, and abdominal segments, was dissected, gently cleaned of adventitial tissue, cut longitudinally, stained with Oil Red O (Sigma, St. Louis, MO) followed by washing and mounting on a silicone coated dish. Aortas were viewed on an Olympus SZX16 dissecting microscope with a 0.8X objective and images were captured using a Spot Insight Mosaic camera. Quantification of ORO staining was performed off-line using Adobe Photoshop CS5 and presented as the percentage of the total surface area of the aorta. Lesion area also was quantified by morphometric analysis.
of H&E stained sections of aortic root as described\textsuperscript{4}. Paraffin-embedded serial sections were prepared from the aortic root and average lesion size was determined from six sections per mouse. Collagen staining was performed using picrosirius red as per the manufactures instructions (PolySciences, Inc). Macrophage staining was performed by staining with antibody against F4/80 (Abcam).

**Statistical analysis**

One-way ANOVA with post-hoc analysis and \( t \) tests were performed using GraphPad Prism version 5.00 for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com) or STATVIEW 5.0 (Abacus Concepts, Inc). The post-test was Fisher’s PLSD and the threshold for significance was \( p = 0.05 \). Data shown are mean ± SEM.
Figure I. Chow-fed C57BL6/J mice were given a single infusion of saline or PEG-rHDL (60 mg/kg). 24 hours post infusion western blot analysis with an anti-apoA-I antibody was performed on total plasma. Lane 1 = saline, lane 2 = PEG-rHDL. N.S = non-specific binding of the anti-body to an unknown plasma protein.
Figure II. Apoe−/− mice fed a WTD for 4 weeks were infused with rHDL at 40, 80 or 120 mg/kg weekly for two weeks. 7 days post the second infusion the percentage of the aortic arch stained with Oil-Red was quantified. *P<0.05, ***P<0.001 vs Saline, n=8, data presented as means ± SEM.
Figure III. Apoe−/− mice fed a WTD for 8 weeks were infused with rHDL or PEG-rHDL 40mg/kg weekly for two weeks. 7 days post the second infusion (A) Total plasma cholesterol and (B) HDL plasma cholesterol levels were analyzed. Data are presented as mean ± SEM, n=10/group.
References:


