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A point mutant of apolipoprotein A-I, V156K, exhibited potent anti-oxidant and anti-atherosclerotic activity in hypercholesterolemic C57BL/6 mice

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Abbreviations: HCHF, high cholesterol high fat; NBD-cholesterol, 2-(N-7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino-23,24-bisnor-5-cholen-3β-ol; POPC, palmitoyloleoyl phosphatidylcholine; TBARS, thiobarbituric acid reactive substances; WT, wildtype

Abstract

In our previous study, two point mutants of apolipoprotein A-I, designated V156K and A158E, revealed peculiar characteristics in their lipid-free and lipid-bound states. In order to determine the putative therapeutic potential of these mutants, several *in vitro* and *in vivo* evaluations were conducted. In the lipid-free state, V156K showed more profound antioxidant activity against LDL oxidation than did the wildtype (WT) or A158E variants in an *in vitro* assay. In the lipid-bound state, V156K-rHDL showed an enhanced cholesterol delivery activity to HepG2 cells in a time-dependent manner, as compared to WT-rHDL, A158E-rHDL, and R173C-rHDL. We assessed the physiological activities of the mutants in circulation, using hypercholesterolemic mice (C57BL6/J). Palmitoyloleoyl phosphatidylcholine (POPC)-rHDL preparations containing each of the apoA-I variants were injected into the mice at a dosage of 30 mg of apoA-I/kg of body weight. Forty eight hours after injection, the sera of the V156K-rHDL injected group showed the most potent antioxidant abilities in the ferric acid removal assay. The V156K-rHDL- or R173C-rHDL-injected mice showed no atherosclerotic lesions and manifested striking increases in their serum apo-E levels, as compared to the mice injected with WT-rHDL or A158E-rHDL. In conclusion, V156K-rHDL exhibited the most pronounced antioxidant activity and anti-atherosclerotic activity, both *in vitro* and *in vivo*. These results support the notion that HDL-therapy may prove beneficial due to its capacity to induce accelerated cholesterol excretion, as well as its enhanced antioxidant and anti-inflammatory effects and lesion regression effect.

Keywords: antioxidants; apolipoprotein A-I; atherosclerosis; lipoproteins, HDL; mutant proteins

Introduction

A growing body of evidence suggests that HDL may exert atheroprotective effects (Gordon *et al*., 1977; Linsel-Nitschke and Tall, 2005), due to its anti-oxidative and anti-inflammatory properties (Barter *et al*., 2004) via the augmentation of reverse cholesterol transport. The functional virtues of HDL appear to be profoundly dependent on apolipoprotein (apo) composition and on the conformation of primary apolipoproteins, including apoA-I and apoA-II (Schultz et al., 1993). Over decades, several sets of apoA-I variants, with different function and structural correlations of HDL, have been generated and characterized, and their clinical potentials have been assessed in animal and human models. This essential field is generally referred to as HDL-therapy (Newton and Krause, 2002; Brewer, 2004). Among these mutants, intravenous injections of rHDL containing R173C-apoA-I (apoA-I_{Milano}) have been shown to have potent regression activity in a human phase II clinical trial, although the mechanism by which this

remedy was affected has yet to be clearly elucidated (Nissen *et al*., 2003). A series of *in vivo* studies with R173C-apoA-I resulted in improvements in the pharmaceutical potential of HDL-therapy, via the use of reconstituted HDL (rHDL) with apoA-I variants (Shah *et al*., 1998; Chiesa *et al*., 2002).

Recently, we described the unique structural and functional properties of V156K and A158E (Han *et al*., 2005), during which we discovered that the two amino acids in helix 6 are essential to both the structure and function of rHDL. Our study also indicated that various parameters of the two mutants were markedly different in the lipid-free and lipid-bound states, thereby suggesting that the mutants might play different physiological roles in the circulation. We then prepared four kinds of rHDL with palmitoyloleoyl phosphatidylcholine (POPC) using apoA-I and its point mutants; wildtype (WT), V156K, A158E, and R173C, and attempted to further evaluate their *in vivo* therapeutic potential, as recently reported (Cho *et al*., 2006). WT-rHDL and R173C-rHDL were used as negative and positive controls (Nissen *et al*., 2003), respectively, and both of these harbored an extra 6 propeptide amino acids within their N-terminal regions, as did V156K and A158E. From the previous report, we determined that V156K and R173C promote anti-inflammatory function *in vivo* and antioxidant activities *in vitro*. In this report, the potent antioxidant activities of these variants were examined at lower concentrations using an *in vitro* system, as well as a cellular system that was able to detect the delivery of HDL-cholesterol into hepatic cells. In order to obtain further information regarding the antioxidant and anti-atherogenic potential of the V156K mutant, both *in vitro* and *in vivo* assays were conducted with hypercholesterolemic C57BL/6 mice.

Materials and Methods

Recombinant apolipoproteins and synthesis of rHDL

Wildtype proapoA-I (WT) and its mutants, V156K, A158E, and R173C, were generated via PCR based on a site-directed mutagenesis kit (Staragene, La Jolla, CA). Proteins were expressed and purified using the pET30 expression system (Novagen, Madison, WI) coupled with Ni^{2+}-nitrilotriacetic acid column chromatography. Reconstituted HDL (rHDL) was prepared with the purified apolipoproteins (at least 95% purity) via sodium cholate dialysis at a molar ratio of 95:5:1:150, POPC:cholesterol:apoA-I: sodium cholate, as described previously by our research group (Han *et al*., 2005). The relevant apolipoproteins and rHDLs were characterized in a previous report (Han *et al*., 2005).

Antioxidant assay against LDL-oxidation

In order to determine the anti-oxidative ability of LDL, fresh human LDL (0.05 ml, 108 μg/ml) in PBS (pH 7.4) was incubated for 4 h at 37°C with final 5 μM of $CuSO_4$ solution, in the presence of the indicated concentrations of apolipoproteins. Following incubation, the extent of LDL-oxidation was assessed via the measurement of the generated thiobarbituric acid reactive substances (TBARS) at 517 nm (Blois, 1958). In order to verify the spectroscopic data, the oxidized samples were subjected to electrophoresis on 0.5% agarose gel to compare their electromobilities (Noble, 1968). The migration of each lipoprotein (VLDL, LDL, HDL_2, and HDL_3) is known to depend on its intact charge and size. The gels were dried and the bands were stained with 0.125% Coomassie Brilliant Blue.

Measurement of cholesterol delivery into HepG2 cells

Hepatic carcinoma cell (HepG2) was cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, and 1% (v/v) penicillin/streptomycin antibiotics. Cells were incubated in a chamber with an air-humidified atmosphere containing 5% CO_2 at 37°C. An aliquot of rHDL containing 22-(*N*-7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-23,24-bisnor-5-cholen-3 β-ol (NBD-cholesterol, Molecular Probe N-1148, 70 μg of NBD-cholesterol/1 mg of apoA-I) was added to 12-well plates (4×10^5 cell/well) at a final concentration of 25 μM in the absence of acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, in order to allow for cholesterol esterification and the detection of fluorescence. After the designated incubation time, the cell in the plate was washed with PBS and counted with a Victor^2 microplate reader (Perkin-Elmer) from the top reading (Ex = 488 nm, Em = 535 nm) without cell disruption. The parallel plate, which was incubated on a cover glass, was observed via confocal microscopy (Zeiss LSM510 Meta, Ex = 488 nm, Em = 533 nm) in order to compare the fluorescence intensity. The confocal visualization of NBD-cholesterol uptake was conducted as previously described (Dagher *et al*., 2003; Kwak *et al*., 2005).

In vivo test using hypercholesterolemic mice

The experimental design of the *in vivo* test was identical to our previous report (Cho *et al*., 2006). In brief, normal male C57BL/6J mice (22-24 *g* of body weight) were obtained at 5-weeks of age from the Jackson Laboratory (Bar Habor, ME). The mice were maintained at 22 ± 1°C with 55 ± 5% humidity under a 12-h light-dark cycle. After 23 weeks of the *ad libitum* consumption of a CRF-1 diet (Oriental Yeast, Tokyo, Japan) containing high cholesterol and high fat (HCHF, 0.5% cholesterol/15% lard/0.1% of

sodim cholate), the mice were randomly divided into 7 groups (*n* = 7-12 animals per group) and injected with rHDL in 0.3 ml of PBS with 30 mg of apoA-I/kg of body weight, as described in Table 1. After two injections given at 24-h intervals, the mice were anesthetized via enflurane inhalation, and sacrificed 48 h after the initial injection. During the entirety of the experimental period, the animals were cared for in accordance with the principles for the use of animals for research provided by the committee of the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea).

Immunodetection of serum apolipoproteins and PLTP

Forty eight h' post-injection, blood samples were obtained from the retroorbital plexus through heparinized microhematocrit capillaries (Sigma, St. Louis, MO) and collected in EDTA-treated tubes. In order to compare the expressional levels of apolipoproteins in serum, the pooled sera of each group were diluted to equal concentrations of approximately 30-fold. The equally diluted serums were then electrophoresed via SDS-PAGE and analyzed by Western blot analysis (Eun *et al.*, 2004) using apo-B antibody (Chemicon, #AB742, Temecula, CA), apo-E antibody (BD Sciences #610449, Franklin Lakes, NJ), and phospholipid transfer protein (PLTP) antibody (Abcam #7735, Cambridge, UK). Relative band intensities were compared via band scanning using a Gel Doc® XR (Bio-Rad, Hercules, CA) with Quantity One software, version 4.5.2.

Ferric reducing ability of serum assay

In order to compare antioxidant ability of mouse serum among the groups, ferric reducing ability of serum (FRAS) was determined as previously described (Benzie and Strain, 1996). The FRAS reagents were freshly prepared via the mixing of 25 ml of 0.2 M acetate buffer (pH 3.6) with 2.5 ml of 10 mM 2,4,6, tripyridyl-s-triazine (TPTZ, Sigma #T1253), and 2.5 ml of 20 mM $FeCl_3$ $6H_2O$ solution. The antioxidant activities of the sera were estimated from the increase in absorbance induced by the generated ferrous ions. Freshly prepared FRAS reagent (300 μl) was mixed with 10 μl of mouse serum and 30 μl of water. Absorbance readings were obtained at 593 nm every 20 s over a 10 min period.

Atherosclerotic lesion analysis

Aortic lesion formation was evaluated via a previously described method (Paigen *et al*., 1987) with the slight modifications described by Gulledge *et al.* (2003). After the mice were exsanguinated, their hearts were perfused through the left ventricle with 30 ml of 4% paraformaldehyde and fixed for at least 24 h. The isolated upper heart/aortic sinus was then embedded in Tissue-Tek OCT compound (Fisher Scientific, Pittsburgh, PA), and frozen. The frozen tissue blocks were placed on a cryotome model AS620 (Shandon, Pittsburgh, PA), and 10-μm serial sections of the ascending aorta were collected on 3-aminopropyltriethoxysilane-coated slides until the aortic sinus appeared. The sectioned slides were stained with oil red-O and hematoxylin to visualize artherosclerotic lesions in accordance with the standard protocol (Paigen *et al*., 1987).

Table 1. Experimental design of rHDL injection into hypercholesterolemic mice (C57BL/6).*, ‖

group	Diet	Injected rHDL	n	Body weight at injection† (g)	Blood cholesterol concentration before injection (mg/dl)	Injected amount of protein†
C	Chow‡	saline	7	35.2 ± 1.9	130 ± 28	Saline 0.3 ml
S	HCHF§	saline	8	45.3 ± 6.4	221 ± 10	Saline 0.3 ml
WT	HCHF	Wildtype	8	41.2 ± 4.1	215 ± 20	30 mg/kg of body weight
V	HCHF	V156K	11	44.6 ± 4.3	188 ± 37	30 mg/kg of body weight
A	HCHF	A158E	12	42.8 ± 3.7	199 ± 17	30 mg/kg of body weight
R	HCHF	R173C	7	40.2 ± 4.5	213 ± 19	30 mg/kg of body weight

*Data were expressed as mean ± S.D. †The injection was done twice times 0 and 24 h in 0.3 ml of PBS. ‡Chow was CRF-1 diet from Oriental Yeast (Tokyo, Japan). §HCHF diet containing 0.5% cholesterol/15% lard/0.1% sodium cholate. ‖ Experimental design and data were adopted from our previous report (Cho *et al*., 2006)

Statistical analysis

All data were expressed as the means ± S.D. Data was evaluated via one-way Analysis of Variance (ANOVA) using SPSS, and the differences between the means were assessed via Duncan's multiple-range test. Statistical significance was defined as $P < 0.05$.

Miscellaneous

LDL ($1.019 < d < 1.063$) was isolated via sequential ultracentrifugation (Himac CP-90α, Hitachi, Tokyo, Japan), at the Instrumental Analysis Center, Yeungnam University, from human serum after density adjustment via the addition of potassium bromide (Havel *et al*., 1955). After centrifugation, the LDL was dialyzed extensively against 10 mM Tris-HCl/5 mM EDTA/140 mM NaCl, at a pH of 7.4, for 24 h in order to remove the KBr. Protein concentration was determined using a Lowry protein assay, with the modifications described by Markwell *et al*. (1978) or using Bradford assay reagent (BioRad, Seoul, Korea) with BSA as a standard. The removal of endotoxin from POPC-rHDLs was verified via testing with a limulus amebocyte lysate kit (BioWhittacker cat. no. 50-647U, Walkersville, MD) in accordance with the manufacturer's suggestions.

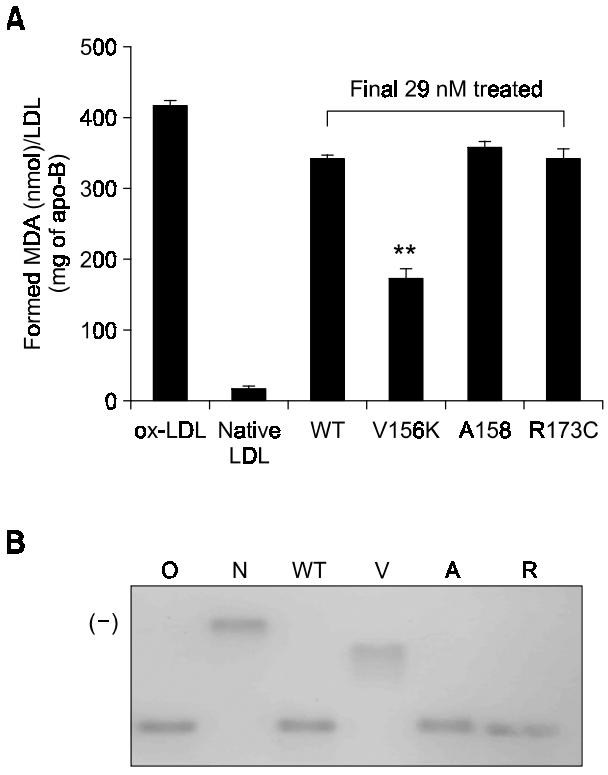

Figure 1. Anti-oxidant activity of apoA-I and its variants in lipid-free state. (A) Thiobarbituric acid reactive substance (TBARS) assay for the detection of the malondialdehyde (MDA) formed in LDL when apolipoprotein was administered at a final concentration of 29 nM in the presence of 5 μM of $CuSO_4$. **, $P < 0.01$. ox-LDL, oxidized LDL (final 5 μM $CuSO_4$ treated). (B) Comparison of the electrophoretic mobility of LDLs (0.05 ml, 108 μg/ml), which were 29 nM of each apolipoprotein was treated (0.5% agarose). Lane O, oxidized LDL (final 5 μM $CuSO_4$ treated); lane N, native LDL (without $CuSO_4$); WT, wildtype-treated LDL; V, V156K-treated LDL; A, A158E-treated LDL; R, R173C-treated LDL.

Results

Antioxidant activity against copper-mediated LDL oxidation

HDL has been observed to exert a profound antioxidant effect against LDL oxidation, a quality which has been identified as one of HDL's primary benefits with regard to the attenuation of the progress of atherosclerotic lesion formation. In the TBARS assay, all apolipoproteins (final 290 nM) exhibited strong antioxidant activity with at least an 85% inhibition of malondialdehyde (MDA) generation against Cu^{2+}-mediated LDL oxidation, whereas probucol treatment (final 5 μM), used as a positive control, resulted in a 78% inhibition (data not shown). When 29 nM of the protein was used for treatment, all proteins lost their activity, resulting in an inhibition rate of less than 10%, with the exception of V156K, which evidenced an inhibition rate of approximately 50%. In order to confirm the results of TBARS, the reaction mixtures were subjected to migration on 0.5% agarose gel in order to compare electromobility. More oxidized LDL moved faster to the bottom of the gel than did the less oxidized or native LDL, as the oxidized LDL (ox-LDL) has an increased negative charge and a reduced size as the result of apo-B fragmentation (Bedwell *et al*., 1989). As shown in Figure 1B, when the 29 nM of apoA-I treated LDL was applied, all LDL moved to the bottom, to the same position as was observed with the oxidized LDL, with the exception of the V156K-treated LDL. This indicates that all proteins lost their activity at lower concentrations. The V156K-treated LDL proved recalcitrant to move down, thus suggesting that V156K evidenced potent antioxidant activity under identical concentrations. This result was very consistent with the results of the TBARS assay (Figure 1A).

V156K-rHDL showed the strongest cholesterol delivery activity

In order to facilitate the excretion of cholesterol from the circulation, the major beneficial role of HDL

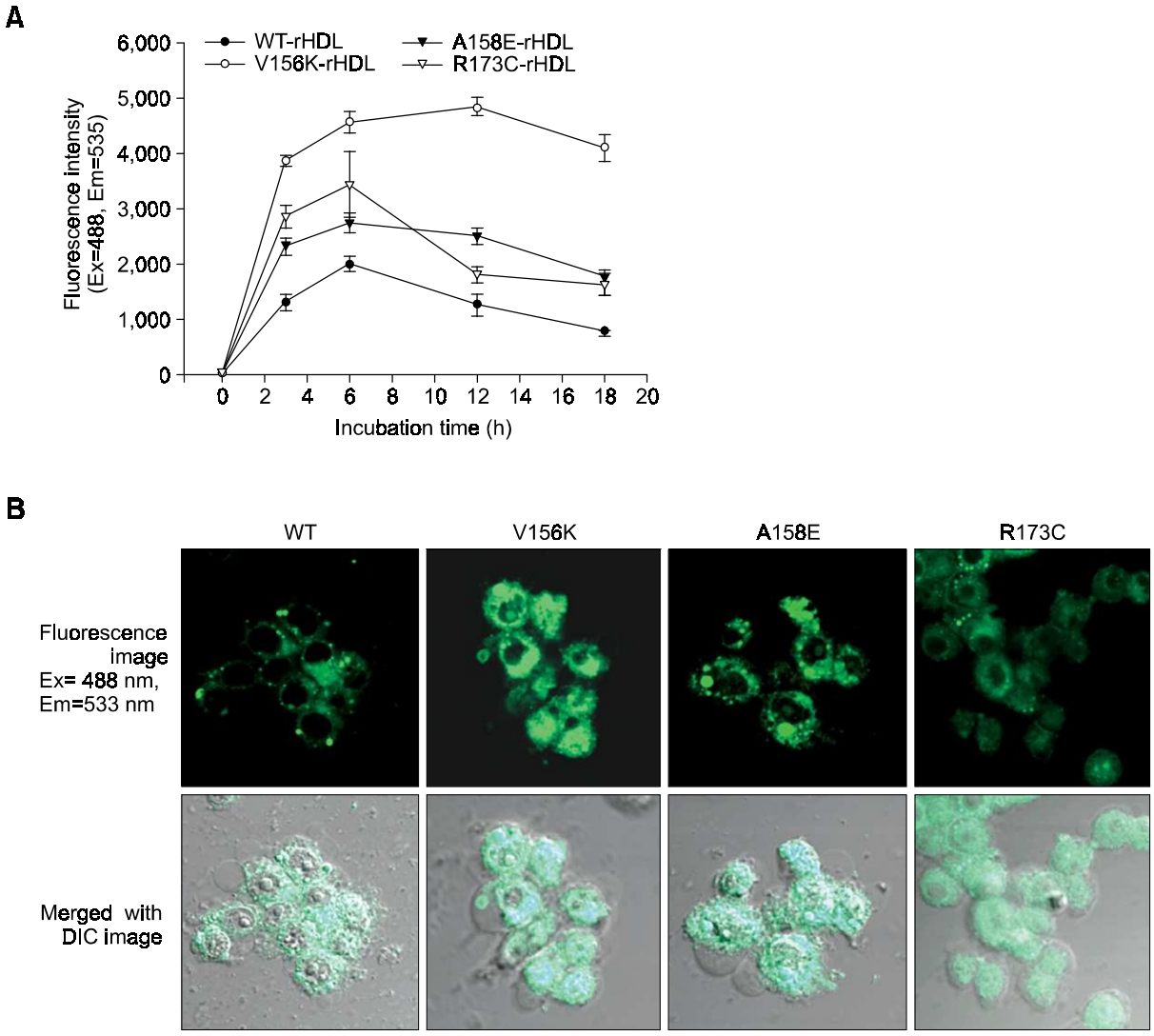

Figure 2. Measurement of cholesterol delivery activity into HepG2 cell. After the administration of rHDL containing NBD-cholesterol (70 μg of NBD-cholesterol per 1 mg of apoA-I) to cultured cells and incubation at 37°C in an atmosphere containing 5% CO_2. (A) Detected fluorescence signal at designated time after the removal of medium and cell rinsing by optical microplate reader (Ex = 488 nm, Em = 535 nm). Data were averaged from three independent assays. (B) Confocal scanning microscopic images of the rHDL-treated HepG2 cells (Ex = 488 nm, Em = 533 nm). During the 18 h incubation period, the NBD-cholesterol was delivered to the inside of the cells and esterified in cytosol for detection.

involves the delivery of cholesterol into hepatic tissue from the peripheral cells via reverse cholesterol transport (Glomset, 1968). In order to evaluate this cholesterol delivery activity, NBD-cholesterol was used as a fluorescent marker for the cholesterol analogue located in the core of the rHDL. After the treatment of the fluorescence-labeled rHDL to HepG2 cells, V156K-rHDL evidenced superior delivery efficiency to other mutant-rHDL variants, in a time-dependent manner. During the incubation period, V156K-rHDL evidenced almost two-fold higher delivery efficiency than was seen with A158E-rHDL, and almost three-fold higher delivery efficiency as compared to the WT-rHDL, as shown in Figure 2A. R173C-rHDL evidenced more potent activity than A158E-rHDL until 6 h of incubation time. In the early stages of incubation, at approximately 6 h, all rHDLs evidenced maximum cholesterol delivery activity, with the exception of V156K-rHDL, which achieved maximum activity at 12 h of incubation. Fluorescent microscopic examination supported the observation that the V156K-rHDL-treated cells exhibited the strongest fluorescence signal, and these signals were also detected in the cytosol, as shown in Figure 2B.

The R173C-rHDL-treated cells evidenced much weaker intensity, closer to that of WT- rHDL or A158E-rHDL, because the microscopic observation was conducted at 18 h.

Change of apolipoprotein expressional levels

In order to compare the changes in the levels of apolipoprotein expression as the result of the injection of each rHDL, mice serum apolipoproteins were analyzed via Western blot. Although this difference was not significant between groups, each rHDL-injected mouse group evidenced slightly increased mouse apoA-I expressional levels as compared to saline-injected mice (group S, data not shown). The mice consuming the HCHF-diet evidenced apo-B_{48} level two-fold that of the normal chow-fed group (top panel of Figure 3), thereby indicating that serum LDL levels had been increased by up to two-fold during the long-term HCHF-diet consumption period. Interestingly, the groups of mice injected with WT-rHDL (lane WT) or A158E-rHDL (lane A) evidenced significantly increased apo-B levels, of up to 2.6 and 3.2-fold that seen in the saline-injected group (lane S), whereas the V156K-rHDL (lane V) or R173C-rHDL (lane R) injected groups evidenced no significant increases in apo-B levels. Immunodetection using a mouse apo-E specific antibody showed that HCHF-diet consumption (group S) induced a marked reduction of apo-E (32 kDa) levels in serum at 48 h' post-injection, as was shown in the middle panel of Figure 3 (lane S). V156K-rHDL or R173C-rHDL injection, however, caused a remarkable increase of apo-E, by up to 2.5-fold (lane V), whereas the WT-rHDL or A158E-rHDL injected groups evidenced minimized apo-E expressional levels. HCHF-fed mice (group S) manifested notably reduced PLTP bands as compared to the normal chow-fed group, which suggests that PLTP may exert anti-atherogenic effects in murine models. The intensity of the PLTP band increased distinctly, reaching levels of approximately 2.1-2.4 fold that in the V156K-rHDL injected (lane V) and R173C-rHDL injected (lane R) mice, as compared to the levels measured in the WT-rHDL or A158E-rHDL-injected groups.

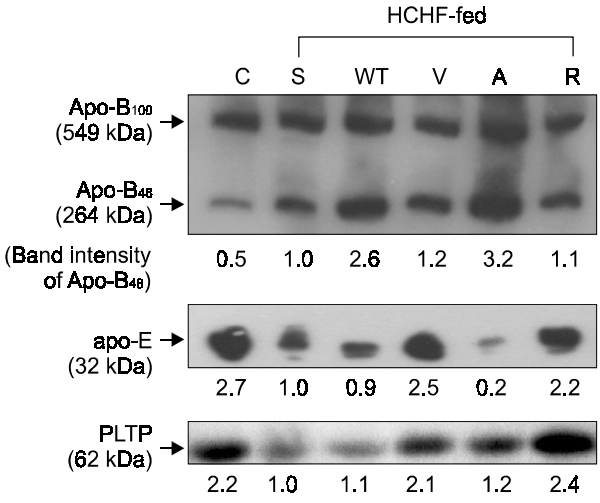

Figure 3. Immunodetection of apolipoproteins in mice serum. Western blot analysis with mouse apo-B antibody (264 kDa for apoB_{48}), apo-E (32 kDa) antibody, and PLTP (62 kDa) antibody. The lower numbers indicate the relative band intensity in comparison with lane S (saline-injected group). In V156K-rHDL and R173C-rHDL injected mice, the expressional levels of serum apo-E and PLTP were increased significantly (lanes V and R). Lane C, saline-injected mice normal chow-fed mice; lane S, saline-injected HCHF-fed mice; lane WT, WT-rHDL injected HCHF-fed mice; lane V, V156K-rHDL injected HCHF-fed mice; lane A, A158E-rHDL injected HCHF-fed mice; lane R, R173C-rHDL injected HCHF-fed mice.

V156K-rHDL infused serum evidenced the strongest antioxidant ability

The FRAS assay is known to be a rapid and reproducible method for determining the molar concentration of the antioxidant present (Benzie and Strain, 1996). We conducted the FRAS assay with mice sera in order to compare antioxidant effects between the groups. The V156K-rHDL-infused serum evidenced the most profound antioxidant activity in terms of serum ferric removal ability, as shown in Figure 4. Up to 10 min of incubation, V156K-injected serum evidenced dramatic increases in absorbance at 593

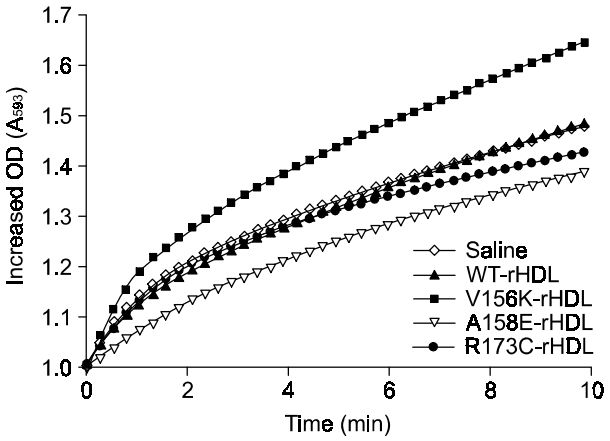

Figure 4. Comparison of antioxidant ability from ferric reducing ability of serum (FRAS). Up to 10 min of incubation, V156K-injected serum evidenced dramatic increases in absorbance at 593 nm (A_{593}) of up to 64% more than the initial values. However, the WT-rHDL, R173C-rHDL, and A158E-rHDL injected mice evidenced increases of 48%, 42%, and 38%, respectively, more than each of their initial values. The data were expressed as the means ± SD from three independent measurements with duplicate samples.

nm (A_{593}) of up to 64% more than the initial values. However, the WT-rHDL, R173C-rHDL, and A158E-rHDL injected mice evidenced increases of 48%, 42%, and 38%, respectively, more than each of their initial values (Figure 4), thus indicating that those mice evidenced weaker anti-oxidative ability than did V156K-rHDL in the blood. This result is quite consistent with the results of the *in vitro* antioxidant assay (Figure 1), in which V156K was determined to evidence the most potent antioxidant activity against copper-mediated oxidation.

Atherosclerotic lesions disappeared in the V156K-rHDL and R173C-rHDL mice

After 23 weeks of HCHF-feeding, relatively thick lesions were detected in the aortic sinus sections from the saline-injected mice, as evidenced by the oil-red O staining images shown in Figure 5. Although atherosclerotic lesions were not observed throughout the entirety of the sinus region, the oil red O-stained region was thickened and had obviously accumulated locally in the aortic sinus, as was also observed in the saline-injected group. The V156K-rHDL-injected group evidenced no such accumulated lesions, such that the lesions had almost completely disappeared. This result was similar to what had been observed with the R173C-rHDL injected group. These results could be compared with those of the WT-rHDL or A158E-rHDL-injected groups, which evidenced much weaker inhibition activity. Although the statistical significance of lesion size distribution was not determined in the current study, the obtained data correlate well with the expressional levels of serum apo-E data acquired via immunodetection (Figure 3). Apo-E is abundantly expressed by macrophages in both human and rabbit atherosclerotic plaques (Rosenfeld *et al*., 1993). Our results indicate that immobilized apo-E located inside the cores of foam cells might be secreted into the blood stream simultaneously with lesion removal in the V156K-rHDL or R173C-rHDL-injected groups.

Discussion

HDL-cholesterol levels are known to be associated inversely with reductions of cardiovascular risk, and this has been corroborated by the results of a number of epidemiologic studies (Assmann and

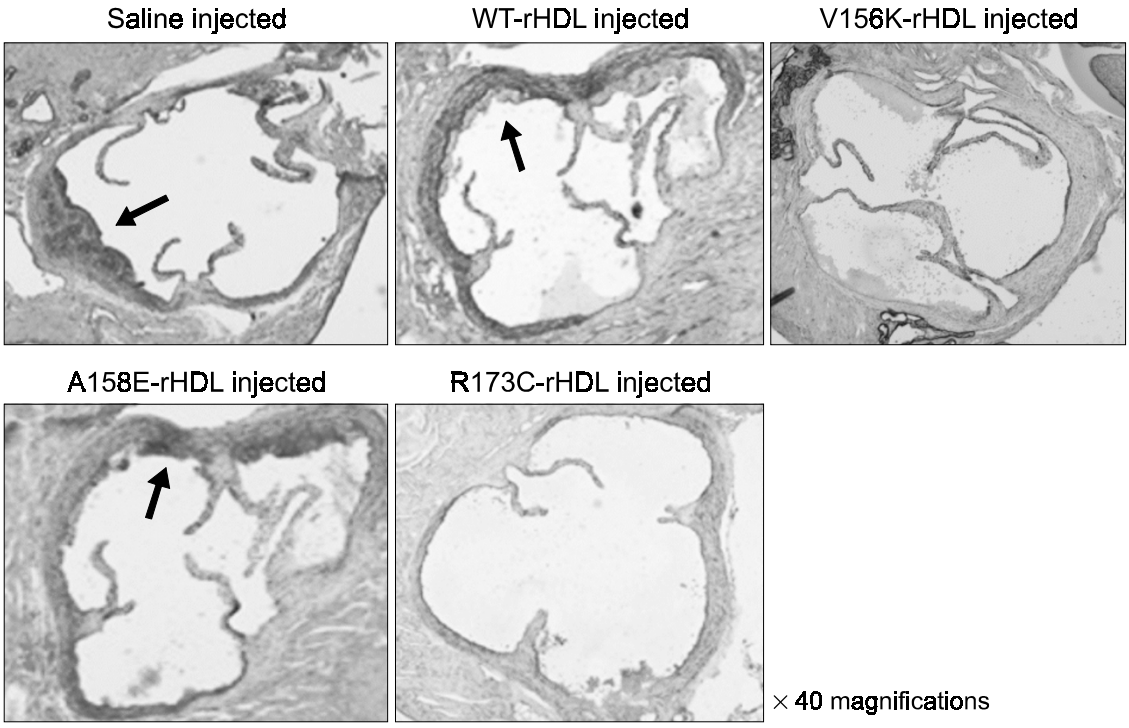

Figure 5. Representative photomicrograph of cross-sectioned mouse aortic sinus and visualization of atherosclerotic lesions. Representative sections were stained with oil red O and counterstained with hematoxylin, allowing for the visualization of the lipid area, which was observed as a red region (×40 magnification). After 23 weeks of consumption of an HCHF-diet, a locally thickened fatty streak lesion was detected in the saline-injected group. The lesion was almost undetectable in the V156K-rHDL and R173C-rHDL injected groups, as compared with the other rHDL-injected groups.

Schulte, 1992), although the exact mechanism underlying HDL's anti-atherogenic potential remains uncertain. One of the widely-accepted explanations is that inhibitory activity against LDL oxidation is a firmly established virtue of HDL, representing a potent anti-atherogenic factor. This antioxidant activity is known to impart a variety of beneficial cellular effects, i.e., prevention of oxidized-LDL uptake by macrophages, the inhibition of endothelial cell adhesion molecule expression, and the promotion of cholesterol efflux from macrophages in atherosclerotic lesions. In this report, the two antioxidant assays *in vitro* (Figrue 1) and *in vivo* (Figure 4) confirmed that V156K had the most profound antioxidant activity among the wildtype and mutants, both in the lipid-free (Figure 1) and rHDL states (Figure 4). The antioxidant activity of the apoA-I variants, apoA-I_{Milano} and apoA-I_{Paris}, in the lipid-bound state has been fairly well established (Bielicki and Oda, 2002), but the same activity of apoA-I in the lipid-free state has yet to be definitively elucidated. Our current results show that the antioxidant activity of HDL derives, at least in part, from apoA-I itself without phospholipid interaction. However, Pownall and Ehnholm (2005) emphasized that phosphatidylcholine is an active agent in HDL-therapy, rather than apoA-I or its variants. Our current data, however, suggest that POPC evidences different therapeutic activity depending on its apoA-I variant as a HDL constituent. Thus our results show that apoA-I has potent antioxidant activity in its lipid-free state, as shown in Figure 1, and this represents one of the more notable beneficial functions of HDL.

The hepatic uptake of plasma HDL-derived cholesterol and cholesteryl ester into the liver for excretion into the bile (Acton *et al*., 1996) has been identified as an essential pathway for the maintenance of cholesterol homeostasis. The delivery measurement (Figure 2A) indicates that the delivery activity of V156K-rHDL lasted longer than was seen with the other HDL variants, thereby suggesting that V156K has better protein stability in cell culture media and superior interaction ability with a putative HDL-receptor, such as scavenger receptor B-I (Acton *et al*., 1996), of the HepG2 cell.

It has been reported that apo-E is expressed abundantly by macrophages in human and rabbit atherosclerotic plaques (Rosenfeld *et al*., 1993) and they are co-localized inside of fatty streak lesions, especially the necrotic core, because the macrophage is the principal source of apo-E in the lesion. As shown in the middle panel of Figure 3, the V156K-rHDL and R173C-rHDL-injected groups evidenced remarkable increases in apo-E levels in the circulating blood, whereas the A158E-rHDL-injected group evidenced a band intensity 12-fold weaker (lane A of Figure 3). In comparison with the observed changes in the levels of apo-A-I, this dramatic increase in serum apo-E levels allows for speculation that the immobilized apo-E located within the core of the foam cell may be released into the bloodstream simultaneously with lesion regression.

Another interesting point is the increase of serum PLTP in the V156K-rHDL or R173C-rHDL- injected group, which was almost two-fold of the WT-rHDL or A158E-rHDL-injected groups, as shown in the bottom panel of Figure 3. Serum PLTP performs crucial functions in HDL remodeling and the maintenance of HDL levels via the transfer of phospholipids from remnant lipoproteins. The relationship between PLTP and CHD remains a matter of some controversy, i.e., high PLTP activity has been identified as a risk factor for CHD (Schlitt *et al*., 2003) but PLTP mass also appears to be a protective factor against CHD (Yatsuya *et al*., 2004). A study of 1,102 CHD patients indicated that the concentration of serum PLTP was significantly inversely related with the risk of cardiovascular disease (Yatsuya *et al*., 2004). This finding shows that immuno-detected PLTP masses increased in cases of V156K-rHDL or R173C-rHDL injection, thereby suggesting that increases in PLTP mass might be correlated with anti-atherosclerotic processes. These results can be interpreted such that V156K-rHDL, like R173C-rHDL may constitute a good candidate for HDL-therapy, whereas WT-rHDL and A158E-rHDL may not be effective HDL-therapy agents.

With regard to the regression effect of V156K-rHDL and R173C-rHDL, the regression effects appeared relatively rapid despite the low dosage of protein injection as compared to the previous reports of Shah *et al.* (1998, 2001). The remarkable regression effects were observed from 18 injections of 40 mg/kg of apoA-I_{Milano}/phosphatidylcholine (Shah *et al*., 1998) and a single injection of 400 mg/kg of apoA-I_{Milano}/dipalmitoyl phosphatidylcholine (DPPC) (Shah *et al*., 2001). However, it is hard to directly compare the current results with the results of the Shah group, as there are several different aspects inherent to the experimental design, including the model mouse (normal C57BL/6 vs apo-E deficient mice), the type of phospholipid in rHDL (POPC vs DPPC), protein dosage (30 mg/kg vs 400 mg/kg of body weight), and injection times. Normal C57BL/6 mice were relatively resistant to the progression of fatty streak lesion formation, as compared to the apo-E deficient mice; they evidenced relatively smaller and locally thickened lesions despite the consumption of the 0.5% cholesterol-diet for 23 weeks, as shown in Figure 5. However, apo-E deficient mice showed very thick lesion after only 9 weeks of consumption of regular mouse chow (Zhang, 1992).

Similarly, in addition, Kaul *et al*. (2003) reported that the intramural delivery of ETC-216, apoA-I_{Milano} and phospholipid complex, with low dosage (0.22 mg/kg of body weight) into domestic swine resulted in a striking regression effect. Although the delivery method and injection times are not the same as are listed in our current report, this result demonstrated a profound regression effect in non-transgenic animal receiving only a single administration. These results allow speculating that normal animals may have a different mechanism of atherosclerotic lesion formation and removal as the result of HDL-therapy than is exploited in apo-E- deficient mice.

Our current findings can be summarized as follows: potent antioxidant activity, cholesterol-excretion ability, and fatty-streak lesion regression effects were detected for V156K-apoA-I in both the lipid-free and lipid-bound states in cellular and animal models. From the standpoint of therapeutic potential, several coincidental points were found between V156K and R173C as compared to WT and A158E. V156K and R173C in the lipid free-state evidenced a superior antioxidant activity against copper-mediated LDL oxidation and cholesterol delivery activity into HepG2 cells as compared to WT and A158E. The injection of V156K-rHDL or R173C-rHDL induced enhancements of anti-oxidant activity and atherosclerotic lesion removal effects with increases in serum apo-E and PLTP.

In conclusion, our results with blood infusions of V156K-rHDL supported the benefit of HDL-therapy hypothesis (Newton and Krause, 2002), which posits that rHDL exerts an anti-atherosclerotic effect via the acceleration of reverse cholesterol transport, in addition to its anti-oxidant, anti-inflammatory, and anti-atherosclerotic effects.

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