Enhanced Delivery of Rapamycin by V156K-apoA-I High-Density Lipoprotein Inhibits Cellular Proatherogenic Effects and Senescence and Promotes Tissue Regeneration

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Although rapamycin (rapa) is a fungicide, it is now believed to possess the capacity to extend mammalian life span. Because rapamycin is insoluble in water, its study in the aqueous phase has been limited. We therefore solubilized rapamycin in isotonic buffer using reconstituted high-density lipoprotein containing V156K-apolipoprotein A-I (V156K-rHDL). Rapamycin (final concentration, 0.1 mg/mL) was solubilized in rHDL containing either wild-type (WT) or V156K-apoA-I (1 mg/mL of protein) prepared using the sodium cholate dialysis method. V156K-rHDL containing rapamycin (V156K-rapa-rHDL) had a slightly larger particle size than rapamycin-loaded WT-rHDL (WT-rapa-rHDL). V156K-rapa-rHDL exhibited enhanced antioxidant ability, cholesteryl ester transfer protein inhibitory activity, and anti-atherosclerotic activity. Treatment with V156K-rapa-rHDL resulted in attenuation of senescence in human cells with increased cell survival and enhancement of tissue regenerative activities in zebrafish model compared with WT-rapa-rHDL or rHDL alone.

Key Words: apoA-I—V156K—Rapamycin—High-density lipoprotein—Rejuvenation.

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RAPAMYCIN is a fungicide but has recently been reported to promote longevity (1). It evinces immunosuppressive effects (2), antitumor activity (3), and antiinflammatory activity in drug-eluting stents (4). Nevertheless, there have been practical limitations in its physiological study due to its very poor water solubility and bioavailability (5). To maximize rapamycin’s research applications, it is advantageous to dissolve the compound in a physiological and isotonic solution.

Apoplipoprotein (apo) A-I is the principal protein component of high-density lipoprotein (HDL) and is known to play a crucial role in the reverse cholesterol transport pathway through its antioxidant and antiinflammatory activities (6,7). Healthy HDL has atheroprotective effects with potent antioxidant and antiinflammatory activities (8). Furthermore, the Rye group (9) recently reported that lipid-free or lipid-bound apoA-I increases β-cell insulin secretion. Due to these findings, apoA-I and reconstituted HDL (rHDL) are now widely believed to constitute an emerging therapeutic target for the treatment of coronary artery disease using a process referred to as HDL therapy (10,11). V156K-apoA-I has recently been developed by site-directed mutagenesis by our research group (12) and has been shown to have more potent antioxidant and antiinflammatory activities in vivo and in vitro (13) as well as an in vivo antiatherosclerotic effect (14).

In addition to HDL therapy, our research group recently reported that the efficiency of viral gene delivery and the stability of adenovirus (Ad) were significantly enhanced when Ad is incorporated into rHDL containing wild-type (WT) or V156K-apoA-I in cell culture and zebrafish models (15). The report raised the possibility of rHDL’s use as a vehicle, formulated for viral or gene and drug delivery. In addition, other research groups have reported that HDL can serve as a delivery system for hydrophobic biomolecules, including anticancer (16), antifungal (17), and antiviral drugs (18); each has been incorporated into liposomes or rHDL particles.

In the current study, we solubilized rapamycin in isotonic solution using reconstituted HDL (rHDL) containing wild-type apoA-I or V156K-apoA-I to maximize the therapeutic application of drugs insoluble in water. After incorporation of rapamycin into rHDL, functional and structural properties were characterized using several antioxidant and antisenescence assays in vitro and in vivo. Furthermore, tissue regeneration activity was tested in a zebrafish model to probe the association between antisenescence activity and facultative regeneration.
Rapamycin was purchased from LC laboratories (Cat# R-5000; Woburn, MA). Pamitoyloleoylphosphatidylcholines (POPC; 1 g) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol (C8667) and sodium cholate were obtained from Sigma (St. Louis, MO).

**Purification of WT and V156K-apoA-I**

WT and V156K-apoA-I were expressed in the BL21 (DE3) *Escherichia coli* expression system from the pET30a(+) expression vector (Novagen, Madison, WI), then purified using Ni²⁺-column chromatography to at least 95% purity as previously described. Synthesis of rHDLs Containing Rapamycin

Discoidal rHDLs containing rapamycin were prepared by the sodium cholate dialysis method, with a slight modification (19), using initial molar ratios of POPC: cholesterol: apoA-I: rapamycin: sodium cholate of 95:5:1:3:150. After attempts to dissolve various concentrations of rapamycin in the rHDL, 0.1 mg of rapamycin and 1 mg of apoA-I in 1 mL of rHDL were shown to be the optimal amounts for solubilization in Tris-buffered saline (10 mM Tris/140 mM NaCl/1 mM ethylenediaminetetraacetic acid [pH 8.0]).

Characterization of Rapamycin-rHDL

The size distributions of rHDL with or without rapamycin were determined by 8%–25% native polyacrylamide gradient gel electrophoresis (Pharmacia Phast System; Amersham Pharmacia, Uppsala, Sweden) and comparison of the results to standard globular proteins. The number of apoA-I molecules per rapamycin-loaded rHDL particle was determined by cross-linking with bis-sulfosuccinimidyl suberate (BS3) using the method described by Staros (20). Reaction products were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on precast 8%–25% gradient gels (Pharmacia, Uppsala, Sweden) and comparison of the results to standard globular proteins. The number of apoA-I molecules per rapamycin-loaded rHDL particle was determined by cross-linking with bis-sulfosuccinimidyl suberate (BS3) using the method described by Staros (20). Reaction products were then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on precast 8%–25% gradient gels (Pharmacia, Uppsala, Sweden). Cholesteryl Ester Transfer Protein Inhibition Assays

Reconstituted HDL (rHDL), as a cholesteryl ester (CE) donor containing apoA-I and cholesteryl oleate, was synthesized in accordance with the method described by our research group (19) with trace amounts of [³H]-cholesteryl oleate (TRK886, 3.5 μCi/mg of apoA-I; GE Healthcare). Human HDL₃ (0.05 mL) and LDL (0.05 mL and 0.25 mg/mL) were used as cholesteryl ester transfer protein (CETP) and CE-acceptor sources, respectively. Each equally diluted rHDL containing rapamycin or rHDL alone was used as a CETP inhibitor. The extent of CETP inhibition was calculated as follows:

\[
\%\text{inhibition} = 100 \times \left\{1 - \frac{\text{sample (cpm)} - \text{blank (cpm)}}{\text{control (cpm)} - \text{blank (cpm)}}\right\},
\]

where “sample” is rHDL containing rapamycin (as an inhibitor source) and “control” is without inhibitor.

Acetylation of LDL

The acetylation of LDL (acLDL) was performed using saturated sodium acetate and acetic anhydride according to a previously described method (25). After acetylation and subsequent dialysis, acLDL protein content was determined and acLDL particles filtered through a 0.22-µm filter (Millex; Millipore, Bedford, MA) prior to use.
Antiatherosclerotic Assays

THP-1 cells, a human monocyte cell line, were obtained from the American Type Culture Collection (ATCC, #TIB-202; Manassas, VA) and maintained in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum. To compare antiatherosclerotic activity using a cellular model, rapa-rHDL–induced inhibition was tested against macrophage LDL uptake as previously reported by our research group (26).

Differentiated adherent macrophages were then rinsed with warm phosphate-buffered saline (PBS) and incubated with 400 μL of fresh RPMI-1640 medium containing 1% fetal bovine serum, 50 μL of acLDL (50 μg of protein in PBS), and 50 μL of each rapa-rHDL preparation (final concentration, 0.3 or 3.3 μM of apoA-I and 1 or 10 μM of rapamycin) for 48 hours at 37°C in a humidified incubator. After incubation, the cells were stained with Oil red O solution (0.67%) to visualize the lipid species in the cell. Culture medium (0.2 mL) was then analyzed by the thiobarbituric acid reactive substances assay to evaluate changes in levels of oxidized species with a malondialdehyde (MDA) standard.

In order to confirm the effects of acLDL treatment as visualized by Oil red O staining, we compared fluorescence intensity of NBD-labeled oxLDL in macrophages. After a 48-hour incubation, the cells were washed three times with PBS and fluorescence (Ex = 488 nm, Em = 535 nm) detected using an LS55 spectrofluorometer with WinLab software equipped with a plate reader (L2250140). Light and fluorescent micrographs were captured with a Nikon Eclipse TE2000 microscope (Tokyo, Japan). Quantification of cellular fluorescence was carried out via computer-assisted morphometry using Image Proplus software (version 4.5.1.22; Media Cybernetics, Bethesda, MD).

In addition, expression levels of apoA-I were compared in cell lysates via Western blot analysis using antihuman apoA-I antibody (Ab7613; Abcam, Cambridge, UK), according to a previously published protocol (27). Protein was quantitated by Bradford assay (Bio-Rad, Hercules, CA) before loading of equal amounts of protein (25 μg/lane) from each lysate into 13% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. The relative band intensity was compared by band scanning with a Gel Doc XR (Bio-Rad) gel electrophoresis gels. The relative band intensity was compared by band scanning with a Gel Doc XR (Bio-Rad) using Quantity One software (version 4.5.2) after normalization to the band intensity of β-actin.

Antisenescence Assays

Primary human dermal fibroblasts (HDFs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM: Life Technologies; Gaithersburg, MD). HDFs were plated in DMEM at 1 × 10^5 cells per 100-mm culture plate and cultured at 37°C in a 5% CO₂–humidified incubator as described in our previous report (26,27). HDFs were exposed at passage 9 (approximately 40% confluence) to the indicated concentrations of rapamycin or rHDLs containing rapamycin (final concentrations, 1 mM of rapamycin and 0.3 μM apoA-I) for 30 days with subculture to passage 18.

Human umbilical vein endothelial cells were cultured in endothelial cell basal medium-2 (EBM-2, CC-3156) containing several growth factors and supplements (EGM-2, SingleQuots, and CC-4176 [containing hydrocortisone, hEGF, fetal bovine serum, VEGF, hFGFB, R3-IGF-1, ascorbic acid, heparin, and gentamicin/amphotericin-B]) purchased from Lonza Walkersville, Inc. (Walkersville, MD). After subculture to passage 10, human umbilical vein endothelial cells were treated with rHDL alone or rHDL containing rapamycin (final concentrations, 1 μM of rapamycin and 0.3 μM apoA-I).

The extent of aging and cellular SA-β-gal activity was compared as previously described (28). Cells were fixed for 5 minutes in 3% paraformaldehyde in PBS, washed three times in PBS, and incubated in SA-β-gal staining solution (40 mM citric acid/phosphate [pH 6.0], 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂, and 1 mg/mL of 5-bromo-4-chloro-3-indolyl-X-galactosidase) for 16 hours at 37°C. The percentage of blue cells was calculated under phase contrast light microscopy.

Zebrafish

Zebrafish and embryos were maintained according to standard protocols. Zebrafish maintenance and experimental procedures using zebrafish were approved by the Committee of Animal Care and Use of Yeungnam University (Gyeongsan, Korea). The fish were maintained in a system cage at 28°C during treatment under a 10:14 hour light:dark cycle.

Microinjection of Zebrafish Larvae

Larvae at 2 days postfertilization were individually microinjected using a pneumatic picopump (PV820; World Precision Instruments, Sarasota, FL) equipped with a magnetic manipulator (MM33; Kantec, Bensenville, IL) with a pulled microcapillary pipette using device (PC-10; Narishigen, Tokyo, Japan). To minimize bias, the injections were performed at the same position on the yolk abdomen. Just prior to injection, oxLDL and each rHDL (12.5 ng of protein) was mixed with or without rapamycin (1.25 ng) in 50 nL. Following injection, live embryos were observed at 48 hour under a stereomicroscope (Motic Cam 168; Hong Kong) and imaged using a Motic Cam2300 CCD camera.

Imaging of Reactive Oxygen Species

After injection with rHDL, changes in the levels of reactive oxygen species in the larvae were imaged by dihydroethidium (DHE; cat # 37291, BioChemika) staining as previously described (29). Images were obtained by...
fluorescence microscopy (Ex = 588 nm and Em = 605 nm) on a Nikon Eclipse TE2000 instrument (Tokyo, Japan). In order to avoid bias, red fluorescence was measured in the trunk area away from the injection site.

**Fin Regeneration**

The effects on wound healing of rHDL particles containing rapamycin were determined using a zebrafish model, according to previously published methods (30, 31). After treatment with rapa-rHDL, increases in reactive oxygen species in the injected area were imaged.

Experimental zebrafish, approximately 12 weeks old, were anesthetized by submerging in 2-phenoxyethanol (Sigma P1126) in a system water (1:1000 dilution). For fin regeneration studies, the zebrafish were anesthesized and the tail fins cut with a scalpel proximal to the proximal branch point of the dermal rays within the fin. After amputation, 10 μL of each rapa-rHDL preparation containing either WT- or V156K-apoA-I (7.5 μg of apoA-I and 717 ng of rapamycin) was injected into tail muscle near the urostyle (n = 9 for each group). Following injection, fish consumed their normal diet and were observed in a 28°C system incubator. Images of fin regeneration were taken at 24-hour intervals from live zebrafish up to 148 hours post-injection under a stereomicroscope (Motic SMZ 168; Hong Kong) and imaged using a Motic Cam2300 CCD camera.

**Data Analysis**

All data are expressed as the mean ± standard deviation of at least three independent experiments with duplicate samples. Two-group comparisons were carried out by independent t tests using SPSS (version 14.0; SPSS, Inc., Chicago, IL). Statistical significance was defined as p < 0.05.

**RESULTS**

**Synthesis of rHDL Particles Containing Rapamycin**

WT- and V156K-rHDL could be efficiently synthesized in the presence of rapamycin. After incorporation of rapamycin into rHDL with various rapamycin amount, 0.1 mg/mL of rapamycin were dissolved well in rHDL (POPC:cholesterol:apoA-I:rapamycin [95:5:1:3]) containing WT or V156K (1 mg/mL of protein). By polyacrylamide gradient gel electrophoresis, the major band of V156K-rHDL containing rapamycin (V156K-rapa-rHDL) had a slightly larger particle size (approximately 101 Å) than WT-rHDL containing rapamycin (WT-rapa-rHDL; approximately 99 Å), as shown in Figure 1A, whereas V156K-rHDL without rapamycin had a smaller particle size (approximately 99 Å) than WT. In addition, a new, larger band appeared in V156K-rapa-rHDL (approximately 140 Å; indicated by the arrowhead) compared with WT-rapa-rHDL, suggesting that the particle size of V156K-rapa-rHDL might be increased during incorporation of rapamycin. Bis-sulfosuccinimidyl suberate (BS3-) cross-linking revealed that rHDL containing rapamycin had up to four molecules of apoA-I in the particle, as shown in Figure 1B, whereas two apoA-I were present in rHDL without rapamycin. This suggests that a greater number of apoA-I molecules are required to solubilize rapamycin and incorporate rapamycin into rHDL.

**Antioxidant Activity**

FRA was carried out to assess differences in antioxidant activity between rHDL particles incorporating WT- or V156K-apoA-I, determining that in the absence of rapamycin, each had similar antioxidant ability; specifically, there was an approximately fourfold increase from the initial value, as
shown in Figure 2A. Although rapamycin alone did not show detectable activity, V156K-rapa-rHDL and WT-rapa-rHDL had significantly enhanced FRA (up to a 10- or 6-fold increase, respectively, from the initial value). This result suggests that the in vitro reductive ability of rHDL was maintained irrespective of the presence of rapamycin.

A comparison of electromobility in agarose gels suggests that more oxidized LDL (oxLDL, lane O) evinces faster mobility than native LDL (lane N; Figure 2B), likely due to increased negative charge and reduced size from apo-B fragmentation in oxLDL. All LDL under presence of Cu\(^{2+}\), which were treated rHDL with or without rapamycin, moved much slower than oxLDL alone (lane O) as shown in Figure 2B. The LDL even exhibited mobility similar to native LDL devoid of cupric ion (lane N), suggesting that WT- and V156K-rHDL, with or without rapamycin (containing final 20 \(\mu\)M), had strong antioxidant ability against the cupric ion–mediated LDL oxidation. However, treatment of LDL with rapamycin alone demonstrated faster electromobility, suggesting that rapamycin itself does not possess notable antioxidant activity; this was confirmed separately by our finding that high dosage with rapamycin (final concentration, 20 \(\mu\)M) elicited little antioxidant effect. This result suggests that incorporation of rapamycin

Figure 2. Antioxidant abilities of reconstituted high-density lipoprotein (rHDL) with or without rapamycin. A. Ferric ion–reducing ability of Wild-type- or V156K-apoA-I-rHDL with or without rapamycin. In 500 \(\mu\)L of the reaction mixture, each rHDL contained 1 \(\mu\)M of apoA-I (final concentration) and 2 \(\mu\)M of rapamycin (final concentration). B. Relative electrophoretic mobility profiles of low-density lipoprotein (LDL; 15 \(\mu\)g of protein) samples from copper-mediated oxidation in the presence of each rHDL type (final concentration, 10 \(\mu\)g/mL). Lane N, native LDL (without Cu\(^{2+}\)); lane O, oxidized LDL (final 10 \(\mu\)M of Cu\(^{2+}\)).

Figure 3. Cholesteryl ester (CE) transfer protein inhibition of reconstituted high-density lipoprotein (rHDL). Data are shown as the mean ± standard deviation from three independent experiments performed in duplicate. CE transfer from \(^{3}H\)-HDL (50 \(\mu\)g of apoA-I, 30000 CPM) to human low-density lipoprotein (50 \(\mu\)g of protein) by human HDL\(_3\) (25 \(\mu\)g of protein) was inhibited by rHDL containing rapamycin.

B

\[\text{rapamycin alone}\]
into rHDL might therefore exert an additive effect toward the antioxidative activity of rHDL.

**CETP Inhibition Activity**

In the absence of rapamycin (rHDL alone), V156K-rHDL had more potent CETP-inhibitory activity than WT-rHDL, as shown in **Figure 3A**. Treatment of V156K-rHDL (final concentration, 1.7 μM apoA-I) resulted in 32% inhibition of CETP activity, which was associated with human HDL-3, whereas WT-rHDL showed 11% inhibition. At the same protein concentration, treatment with V156K-rapa-rHDL (containing 5 μM rapamycin) effected 20% inhibition, whereas WT-rapa-rHDL (the same concentrations of apoA-I and rapamycin) had near loss of inhibitory capacity (approximately 3% inhibition). Rapamycin dissolved in ethanol alone did not show detectable CETP-inhibitory activity over the concentration range from 16 nM to 10 μM (data not shown).

V156K-rapa-rHDL (3.5 μM protein) showed potent inhibitory activity at 10 μM rapamycin (up to 29% inhibition), as shown in **Figure 3B**, whereas the same concentration of WT-rapa-rHDL (3.5 μM protein) evinced no activity. This suggests that incorporation of rapamycin into V156K-rHDL particles caused no loss of CETP-inhibitory activity even at high concentration of rapamycin (**Figure 3B**).
Inhibition of acLDL Uptake

Rapamycin itself did not inhibit macrophage uptake of acLDL, as shown in Figure 4A (image 5). In the presence of acLDL, treatment with WT-rHDL (image 1) and V156K-rHDL (image 2) elicited a lesser degree of Oil red O staining than PBS treatment, indicating that rHDL could prevent acLDL uptake.

In the presence of acLDL, cells exposed to rHDL containing rapamycin (images 2 and 4) demonstrated approximately 45% less Oil red O staining (by area) than rapamycin alone (Figure 4B), suggesting that incorporation of rapamycin into rHDL is additive in preventing the uptake. Determination of macrophage oxidized species content also revealed that WT-rapa-rHDL and V156K-rapa-rHDL–treated cells showed less malondialdehyde (MDA) production (Figure 4C), indicative of lower oxidized species release into the cell medium. Production of oxidized species by interaction between acLDL and macrophages has previously been reported (32). In the presence of acLDL, WT-rHDL–treated cells evinced a 26% increase in MDA levels relative to V156K-HDL–treated cells, as shown in Figure 4C. V156K-rapa-rHDL–treated cells showed a 28% decrease in MDA levels compared with WT-rapa-rHDL–treated cells, suggesting that V156K-rHDL and V156K-rapa-rHDL possessed antioxidant activity in the cell; incorporation of rapamycin into rHDL enhanced this beneficial effect.
As observed with Oil red O staining, uptake of NBD-cholesterol–labeled oxLDL was substantial (image 1); cotreatment with rHDL inhibited this uptake (Figure 5). Of the rHDL particles assayed, WT-rapa-rHDL and V156K-rapa-rHDL showed strongest inhibitory ability at less than 39% and 42% uptake, respectively, compared with the acLDL-treated control. Treatment with WT-rHDL (image 3) or V156K-rHDL (image 5) inhibited uptake of acLDL approximately 17% or 21% compared with that of acLDL (Figure 5B). This result also confirmed that incorporation of rapamycin into rHDL particles may therefore contribute additively to prevent oxLDL uptake.

**Antisenescence Activity**

In HDFs without rapamycin, WT-rHDL treatment resulted in an increased number of SA-β-gal–positive cells compared with V156K-rHDL at the same passage number, indicating that V156K-rHDL was more effective in suppressing cellular senescence (Figure 6A). At passage 17, WT-rHDL– and V156K-rHDL–treated cells demonstrated 70% and 49% of SA-β-gal–positive cells, respectively. However, at the same passage, WT-rapa-rHDL and V156K-rapa-rHDL treatment resulted in 52% and 29% of SA-β-gal positive cells, respectively, with a rapamycin-dependent increase in cell survival (1 μM rapamycin, final concentration; bottom of Figure 6B). V156K-rapa-rHDL–treated cells showed 5.6-fold and 2.8-fold higher cell survival than groups exposed to V156K-rHDL or rapamycin alone, suggesting that incorporation of rapamycin and V156K-rHDL was more advantageous to cellular replicative capacity. Because irreversible loss of replicative capacity is a typical marker for cellular senescence, this result correlates well with the findings depicted in Figure 6A. V156K-rapa-rHDL treatment significantly decreased the number of SA-β-gal–positive cells detected relative to treatment with rapamycin alone, suggesting that V156K-apoA-I and rapamycin might additively prevent aging (Figure 6).

In human umbilical vein endothelial cells, the proportion of SA-β-gal–positive cells increased passage dependently under PBS treatment (Figure 7A). At passage 13, there was no significant difference in the extent of senescence between cells treated with PBS– or WT-rHDL alone, although V156K-rHDL treatment resulted in 15% fewer SA-β-gal–positive cells than PBS treatment (61% and 72% of the cells displayed positive staining, respectively). However, V156K-rapa-rHDL–treated cells manifested the lowest level of SA-β-gal positivity (15%), whereas cells treated with WT-rapa-rHDL or rapamycin alone were 20% or 36% positive for the SA-β-gal, respectively. Furthermore, at passage 13, the SA-β-gal–stained area (in blue) was remarkably decreased in the presence of rHDL containing rapamycin; cells treated with rHDL alone showed a darker blue intensity (Figure 7B). These results strongly suggest the potential for an additive effect in the suppression of senescence during the incorporation of rapamycin into rHDL particles.

**Antinflammatory Activities of rHDL**

Zebrafish larvae injected with oxLDL alone showed a sixfold larger region of DHE staining than PBS injected (Figure 8), indicating the highest production of reactive oxygen species by oxLDL. However, coinjection of oxLDL and vitamin C induced an 83% decrease of the stained area,
suggesting that the combined presence of these antioxidants could reduce oxidative damage. In the presence of oxLDL, rapamycin- (1.2 ng) injected larvae showed a 52% reduction in staining, indicating that rapamycin could also prevent larval oxidative damage. This result is in good agreement with the mild inhibitory ability of rapamycin against cupric ion–mediated LDL oxidation, as shown in Figure 2B. In the presence of oxLDL, V156K-rapa-rHDL or WT-rapa-rHDL injection caused a 74% or 69% reduction in stained area relative to that seen with oxLDL alone.

**Fin Regeneration**

When zebrafish were fed a plain diet, injection of rapamycin or vehicle alone (8 μL of EtOH) did not induce detectable fin regeneration up to 72 hours postinjection; most zebrafish had died by this point, suggestive of possible ethanol-mediated toxicity. At 120 hours postinjection, however, rHDL containing rapamycin induced notable regeneration of fins, although the V156K-rapa-rHDL–injected group showed a 1.6-fold increase in regenerative capacity relative to that injected with WT-rapa-rHDL (Figure 8). The zebrafish tolerated the 120-hour experimental period well, suggesting that HDL-mediated rapamycin delivery did not cause toxicity. DHE staining for reactive oxygen species production revealed that vehicle- (EtOH) injected zebrafish evinced the most pronounced oxidative stress (Figure 9). At 72 hours postinjection, rapamycin-injected zebrafish showed an 80% reduction in DHE staining intensity, suggesting that rapamycin could relieve oxidative stress, although no fin regeneration was detected. These results suggest that rapamycin in rHDL could enhance tissue regeneration relative to rapamycin alone.

**DISCUSSION**

In the present study, we have demonstrated a method for solubilization of rapamycin in physiological solution using rHDL that may facilitate development of a new drug delivery vehicle. It has been reported that rapamycin is a potential extender of mammalian life span. Because cardiovascular disease and type 2 diabetes are age-related diseases, chronic metabolic disease progression with senescence may be linked to target of rapamycin signaling.

HDL therapy using plasma apoA-I, apoA-I mutants, and apoA-I mimetics is an emerging therapeutic tool for regression of atherosclerotic plaques and enhancement of healthy blood vessels (10,11). Because rHDL and rapamycin have...
RAPAMYCIN IN RHDL

We have investigated the possibility of a therapeutic enhancement by rapamycin of rHDL’s benefits, especially those issuing from exposure to V156K-apoA-I HDL, compared with treatment with rapamycin alone. V156K-rapa-rHDL showed increased particle size (Figure 1A) with enhanced reductive capacity (Figure 2A) and antioxidant activity in vitro and in vivo (Figures 2B and 7). Compared with rapamycin alone, WT- or V156K-rHDL particles containing rapamycin exhibited enhanced inhibition of CETP activity (Figure 3), uptake of LDL (Figures 4 and 5), antisenescence activity (Figures 6 and 7), and facultative tissue regeneration activity (Figure 9).

Regarding longevity and lipoprotein metabolism, the I405V variant of CETP is associated with healthy aging (33). Homozygous subjects with a single nucleotide polymorphism at CETP codon 405 that alters isoLeucine to valine exhibit much lower plasma CETP concentrations and activity. Furthermore, I405V is associated with slower memory decline and a lower incidence of dementia and risk of Alzheimer’s disease (34).

CETP mediates reciprocal transfer and exchange of cholesteryl ester and triglyceride between lipoproteins, especially HDL and apoB-containing lipoproteins (35). It is well known that higher CETP activity in HDL particles can cause their reduction in CE and increase in TG (36). These TG-enriched HDL particles are proinflammatory in nature (37), and CETP activity is increased with aging (23). We previously reported that plasma and lipoproteins from elderly subjects had significantly increased CE-transfer activity compared with young subjects, especially when HDL$_3$ was used as a CETP source. Plasma CETP has been established as an independent atherogenic factor (38,39), and variants of CETP are associated with healthy aging and longevity (40,41).

High HDL cholesterol levels are frequently observed in healthy elderly members of the population (42,43). HDL-C levels and HDL particle size and composition are influenced by CETP activity. Elevated CETP activity can reduce the size of HDL particles; this is directly correlated with impairment of HDL’s beneficial functions. Likewise, CETP inhibition by V156K-rapa-rHDL is predicted to antagonize antiatherosclerotic and antiaging capability.

The current study provides a new method to solubilize rapamycin using V156K-apoA-I–reconstituted HDL with a
different molar ratio of phospholipids to apoA-I. This rapamycin encapsulation method may apply to other water-insoluble agents with pharmaceutical activity. Furthermore, in addition to enhanced drug delivery, the efficiency of cellular absorption is increased via the scavenger receptor (SR-B1) and/or apoA-I receptors in various cells.

Our group has previously reported a gene delivery vehicle comprising a proteoliposome (PL) containing V156K and adeno virus (Ad), which showed significantly enhanced stability and Ad infection ability in vitro and in vivo compared with WT-PL-Ad (15). In combination with the current results, the apoA-I/phospholipid complex, especially the V156K variant, appears to serve as a better therapeutic agent or viral delivery vehicle than those presently in use owing to the ability of peripheral cells to acquire HDL core components.

In conclusion, incorporation of rapamycin into rHDL containing apoA-I, especially its V156K variant, can promote cell survival and suppress senescence through enhanced antioxidant ability. The enhanced solubility of rapamycin in rHDL particles may be applicable to the development of numerous types of therapeutics. Rapamycin and rHDL possess independent therapeutic activities, and the beneficial activities of rHDL were not impaired by incorporation of rapamycin. New agents can likely be developed from rapa-rHDL to treat aging, cardiovascular, and brain disease as HDL can penetrate the blood–brain barrier. These same protocols can be applied to solubilize other hydrophobic drugs for a wide range of therapeutic applications.

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