Reconstituted High-Density Lipoprotein Stimulates Differentiation of Endothelial Progenitor Cells and Enhances Ischemia-Induced Angiogenesis

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Background—Plasma high-density lipoprotein (HDL) levels have an inverse correlation with incidence of ischemic heart disease as well as other atherosclerosis-related ischemic conditions. However, the molecular mechanism by which HDL prevents ischemic disease is not fully understood. Here, we investigated the effect of HDL on differentiation of endothelial progenitor cells and angiogenesis in murine ischemic hindlimb model.

Methods and Results—Intravenous injection of reconstituted HDL (rHDL) significantly augmented blood flow recovery and increased capillary density in the ischemic leg. rHDL increased the number of bone marrow–derived cells incorporated into the newly formed capillaries in ischemic muscle. rHDL induced phosphorylation of Akt in human peripheral mononuclear cells. rHDL (50 to 100 μg apolipoprotein A-I/mL) promoted differentiation of peripheral mononuclear cells to endothelial progenitor cells in a dose-dependent manner. The effect of rHDL on endothelial progenitor cells differentiation was abrogated by coadministration of LY294002, an inhibitor of phosphatidylinositol 3-kinase. rHDL failed to promote angiogenesis in endothelial NO−deficient mice.

Conclusions—rHDL directly stimulates endothelial progenitor cell differentiation via phosphatidylinositol 3-kinase/Akt pathway and enhances ischemia-induced angiogenesis. rHDL may be useful in the treatment of patients with ischemic cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2007;27:813-818.)

Key Words: high-density lipoproteins — endothelial progenitor cells — angiogenesis — mouse — collateral

Plasma high-density lipoprotein (HDL) levels have an inverse correlation with incidence of ischemic heart diseases as well as other atherosclerosis-related ischemic conditions. However, the molecular mechanism by which HDL prevents ischemic diseases is not fully understood. Atheroprotective functions of HDL are thought to be attributed to the ability of HDL to uptake cellular cholesterol from peripheral organs and to mediate the transport of excess cholesterol to the liver. In addition to the conventional role of HDL, recent studies revealed that HDL has various favorable effects on endothelial cells. Angiogenesis, the process of postnatal neovascularization, plays a critical role in the pathogenesis of several human diseases, including ischemic heart disease, peripheral artery disease, cancer, diabetic microvascular disease, and rheumatoid arthritis. Accumulating evidence suggests that circulating endothelial progenitor cells (EPCs) significantly contribute to angiogenesis. Moreover, it was demonstrated that transplantation of EPCs or mobilization of EPCs from bone marrow could augment angiogenesis.

Here, we investigated the effects of reconstituted HDL (rHDL) on differentiation of EPCs and angiogenesis. The results suggest that rHDL may be useful in therapeutic angiogenesis.

Methods

Reconstituted HDL
Discoidal rHDL was prepared as described previously. Briefly, HDLs were obtained from samples of expired human plasma (Gribbles Pathology) by sequential ultracentrifugation (1.07<d<1.21 g/mL). The HDLs were delipidated, and apolipoprotein A-I (apoA-I) was isolated by anion exchange chromatography. Discoidal rHDLs containing apoA-I as their sole protein constituent and 1-palmitoyl-2-oleoyl phosphatidylcholine as their sole lipid were prepared by the cholate dialysis method. The molar ratio of 1-palmitoyl-2-oleoyl phosphatidylcholine:apoA-I was 100:1.

Mouse Hindlimb Ischemia Model
Wild-type C57BL/6 and C3H/He mice were purchased from SLC Japan (Hamamatsu, Japan). Endothelial NO synthase (eNOS)−/− mice were purchased from Jackson Laboratory (Bar Harbor, Me). Unilateral hindlimb ischemia was induced...
in 30- to 35-week-old male mice by resecting the right femoral and saphenous artery. PBS (control) or rHDL (containing 0.2 mg apoA-I in rHDL/body suspended in 0.3 mL PBS) were injected intravenously twice per week, starting 1 week before surgery (n=10 for each group). Hindlimb blood perfusion was measured using laser Doppler perfusion imager system (Moor Instruments) as described previously. The laser Doppler perfusion imager system incorporates a helium–neon laser to generate a beam of light that sequentially scans a 12×12 cm tissue surface to a depth of 1 mm. During the scanning procedure, blood cells moving through the vasculature shift the frequency of incident light according to the Doppler principle. Laser Doppler perfusion imager was used to record perfusion of both right and left limbs every week. Excess hairs were removed by depilatory cream from the limb before imaging, and mice were placed on a heating plate at 40°C to minimize temperature variation. Color-coded images were recorded. Analyses were performed by calculating the average perfusion for each (ischemic and nonischemic) foot. To account for variables, including ambient light and temperature, calculated perfusion was expressed as a ratio of right (ischemic) to left (untreated) limb as used by many laboratories.7–9

C3H/He mice, which show moderate spontaneous recovery in this model,10 were used to determine whether rHDL can augment angiogenesis with a minimized number of mice. The mice were returned to the animal facility on recovery from surgery and were monitored on a daily basis by experienced personnel for medical complications or pain. If the mice appeared to experience pain, leperophen maleate was administered at a dose of 0.01 to 0.06 mg/kg via subcutaneous injection. No mouse displayed severe necrosis, irreversible pain, or other complications or pain. If the mice appeared to experience pain, the mice were euthanized. The adductor muscle was randomly selected, and CD31 and GFP double-positive cells were counted. Capillary density was expressed as the number of capillaries per square millimeter. The number of capillaries per muscle fiber was also determined.

Immunofluorescence double staining was performed as described previously.14 To identify the endothelial-like cells arising from the bone marrow, the frozen sections were stained with an anti-CD31 antibody (clone MEC13.1; BD Pharmingen) and anti-GFP antibody (Molecular Probes). Human umbilical vein endothelial cells and human aortic smooth muscle cells were purchased from SANKO JUNAYAKU were used as negative and positive controls for eNOS expression. The endothelial progenitor cells (EPCs) were quantified by the standard method described by Asahara et al.16 Adherent cells were incubated with 10 μg/mL acetylated low-density lipoprotein (LDL) labeled with 1,1'-dioctadecyl-3,3,3,’3’-tetramethylindocarbocyanine perchlorate (DiI–Ac–LDL; Biomedical Technologies Inc) for 4 hours. Cells were washed in PBS, fixed with 2% paraformaldehyde, and counterstained with fluorescein isothiocyanate–labeled lectin from Bandeiraea simplicifolia (BS-lectin; Sigma). Cells that were positive for both DiI–Ac–LDL and fluorescein isothiocyanate–BS-lectin were identified as EPCs, as described previously.14 Two independent investigators evaluated the number of EPCs per well by counting 4 randomly selected fields.

Bone Marrow Reconstitution
Green fluorescent protein (GFP) mice, which are transgenic mice (C57BL/6 background) that ubiquitously express enhanced GFP, were a generous gift from Dr Masaru Okabe (Osaka University, Japan). Bone marrow transplantation (BMT) was performed as described previously.12 Eight-week-old male wild-type mice were lethally X-irradiated with a total dose of 9.5 Gy (MBR-1520RB; Hitachi). One day later, the recipient mice received unfractionated bone marrow cells (3×10^6) by tail vein injection. Seven weeks after the BMT, hindlimb ischemia was induced in the recipient mice. The reconstitution rate of the peripheral leukocytes was 75% to 85% as determined by flow cytometry.13

Histological Analyses
The adductor muscles were harvested at 4 weeks after the surgery. Capillaries were identified by positive staining for CD31 and their morphology.6 The adductor muscle was randomly selected, and capillaries were counted. Capillary density was expressed as the number of capillaries per square millimeter. The number of capillaries per muscle fiber was also determined.

Cell Culture
Peripheral mononuclear cells (MNCs) were isolated from peripheral blood of healthy human volunteers by density-gradient centrifugation with HISOPAQUE-1077 (Sigma). MNCs were cultured at a density of 4×10^6 cells per a fibronectin/gelatin-coated well in a 24-well dish in 0.5 mL endothelial basal medium (EBM) (Clonetics) supplemented with 1 μg/mL hydrocortisone, 3 μg/mL bovine brain extract, and 20% FBS. MNCs were stimulated with 100 ng/mL human recombinant vascular endothelial growth factor (VEGF; R&D Systems) or rHDL for the indicated time. Expression of eNOS by the adherent cells was evaluated by RT-PCR analysis and immunocytochemistry as described previously.15 Total RNA was isolated from the cells at 7 days with the use of RNAzol reagent (TEL-TEST). Reverse transcription was performed with random hexamer primers and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (ReverTraAce-a; TOYOBO). The PCR primers were as follows: eNOS, 5′-GCTGCGCACCTTCACCTCCT-3′ (sense) and 5′-GGCTGCAAGCTTGTCTCAA-3′ (anti-sense); GAPDH, 5′-ACCACAGTCATCCATAC-3′ and 5′-TCCACCACCTGTTGCTGTA-3′. For immunocytochemistry, cells were fixed in 4% paraformaldehyde. After being permeabilized with 0.5% Nonidet P-40 in PBS, the cells were stained with an anti-eNOS monoclonal antibody (clone 3; BD Pharmingen) and an Alexa 488–conjugated anti-mouse Ig secondary antibody (Molecular Probes). Human umbilical vein endothelial cells and human aortic smooth muscle cells were purchased from SANKO JUNAYAKU were used as negative and positive controls for eNOS expression. The endothelial progenitor cells (EPCs) were quantified by the standard method described by Asahara et al.16 Adherent cells were incubated with 10 μg/mL acetylated low-density lipoprotein (LDL) labeled with 1,1'-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI–Ac–LDL; Biomedical Technologies Inc) for 4 hours. Cells were washed in PBS, fixed with 2% paraformaldehyde, and counterstained with fluorescein isothiocyanate–labeled lectin from Bandeiraea simplicifolia (BS-lectin; Sigma). Cells that were positive for both DiI–Ac–LDL and fluorescein isothiocyanate–BS-lectin were identified as EPCs, as described previously.14 Two independent investigators evaluated the number of EPCs per well by counting 4 randomly selected fields.

Immunoblotting
MNCs were cultured in EBM with rHDL for the indicated time and lysed with lysis buffer containing 150 mmol/L NaCl, 20 mmol/L Tris–HCl, pH 8.0, 10 mmol/L NaF, 1 mmol/L Na3VO4, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitor cocktail (Sigma). The cell lysates (20 μg/lane) were analyzed by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Hybond-P; GE Healthcare Bioscience). The membrane was incubated with an anti-Akt polyclonal antibody (Akt) (1:500; Cell Signaling Technology) and an anti–phospho-Akt (Ser473) polyclonal antibody (p-Akt; 1:500; Cell Signaling Technology). Antibody binding was detected with horseradish peroxidase–conjugated rabbit Ig (1:2000; Chemicon) and enhanced chemiluminescence system (GE Healthcare Bioscience).

Statistics
All data are expressed as the mean value±SEM. Blood flow recovery in the ischemic hindlimb was compared between the two groups by repeated-measures ANOVA. Statistical comparisons of means were performed by ANOVA followed by Student t test. P<0.05 was considered to be statistically significant.

Results
rHDL Promoted Collateral Development in Murine Hindlimb Ischemia Model
To evaluate the angiogenic effect of rHDL, hindlimb ischemia was induced in C3H/He mice, which were treated with intravenous injection of PBS or rHDL (0.2 mg apoA-I/body)
twice per week starting 1 week before the surgery (n=10 for each group). In the control mice treated with PBS, the blood flow of the ischemic leg recovered gradually and reached half the blood flow of the untreated leg at 4 weeks (Figure 1). rHDL significantly augmented blood flow recovery in the ischemic leg (blood flow ratio at 4 weeks: control 0.53 ± 0.07, rHDL 0.81 ± 0.10; P<0.05).

Collateral formation was also evaluated by the capillary density of the ischemic hindlimb muscle harvested at 4 weeks after the surgery. Double immunofluorescent study revealed that most of the CD31-positive cells were also stained by BS-lectin (Figure 2A). Although CD31 could be expressed by inflammatory cells, few CD31-positive cells were stained for CD45. Thus, we estimated the capillary density in the ischemic muscles by anti-CD31 immunostaining (Figure 2B). Consistent with the measurement by laser Doppler imaging, anti-CD31 immunostaining revealed that rHDL significantly increased the number of histologically detectable capillaries in the ischemic leg (control 254±26/mm²; rHDL 474±41/mm²; P<0.05; Figure 2C). The number of capillaries in a fiber was also significantly increased. On the other hand, there was no statistical difference in the capillary density of the nonischemic leg (left leg) between the PBS-treated group (190±23/mm²) and the rHDL-treated group (206±23/mm²), suggesting that rHDL does not function to enhance angiogenesis in nonischemic tissue.

**rHDL Enhanced Contribution of Bone Marrow–Derived Cells to Neovascularization**

Effect of rHDL on bone marrow–derived EPCs was investigated by inducing hindlimb ischemia in bone marrow chimeric mice, in which bone marrow–derived cells were genetically labeled by GFP. Seven weeks after BMT, hindlimb ischemia was induced in the recipient. Peripheral leukocytes (75% to 85%) had been reconstituted as determined by flow cytometry. The mice were treated with intravenous injection of PBS (control) or rHDL twice per week. At 4 weeks after the surgery, bone marrow–derived cells could be detected in the ischemic muscle. Anti-CD31 immunostaining readily detected bone marrow–derived endothelial-like cells that were positive for GFP and CD31 (Figure 3A). rHDL significantly increased the number of bone marrow–derived endothelial cells among total endothelial cells (CD31+GFP+/CD31; control 6.4±2.0%; rHDL 13.7±2.3%; P<0.05; Figure 3B). On the other hand, CD31 and GFP double-positive cells were not detected in the nonischemic hindlimb (data not shown).

**rHDL Promoted Differentiation of EPCs In Vitro**

Effects of rHDL on differentiation of EPCs were investigated in vitro. Human MNCs differentiated into adherent endothelial-like cells, which expressed eNOS (Figure 4A), VEGF-receptor 2 and CD31 (data not shown). The adherent cells were positive for FITC-conjugated BS-lectin and DiI–Ac-LDL (Figure 4B). rHDL significantly increased the number of double-positive cells at 7 days in a dose-dependent manner (Figure 4C). The effect of rHDL was comparable to that by VEGF. rHDL had no effect on the number of adherent cells at 1 day. The number of double-positive cells was significantly increased by rHDL at 7 days, but not at 3 days (Figure 4C).
4D), suggesting that rHDL promotes differentiation and proliferation of EPCs.

rHDL Stimulated Phosphatidylinositol 3-Kinase/Akt Signaling Pathway to Regulate EPC Differentiation

We investigated the molecular mechanisms by which rHDL promotes differentiation of EPCs. Western blotting revealed that rHDL (100 μg/mL) rapidly activated Akt in MNCs (Figure 5A). Moreover, pharmacological inhibition of phosphatidylinositol 3-kinase (PI3K)/Akt pathway with LY294002 (10 μmol/L) abrogated the beneficial effects of rHDL on EPCs differentiation (Figure 5B). PD98059 (10 μmol/L), an inhibitor of mitogen-activated protein kinase-1, had no effect on EPC differentiation. Together, these results suggest that rHDL promotes differentiation of EPCs via PI3K/Akt pathway, at least in part.

rHDL Failed to Promote Blood Flow Recovery in eNOS+/− Mice

To investigate the role of eNOS, which is activated by PI3K/Akt, in enhancement of blood flow recovery by rHDL, we evaluated the effects of rHDL in eNOS+/− mice. These mice lack the ability to dilate the vessel via NO production in the endothelial cells.6,9 Blood flow recovery was impaired severely in eNOS+/− mice, as reported previously.6,9 rHDL had no beneficial effects on blood flow recovery in eNOS+/− mice (Figure 6A). Anti-CD31 immunostaining revealed that rHDL treatment did not increase the number of capillaries in the ischemic leg (Figure 6B).

Discussion

In this study, we found that intravenous injection of rHDL enhanced blood flow recovery and increased the number of histologically detectable capillaries in ischemic muscles. Angiogenic effect of rHDL was associated with enhanced contribution of bone marrow–derived cells to neovascularization. rHDL promoted differentiation of peripheral MNCs into endothelial-like cells via PI3K/Akt pathway. rHDL failed to promote blood flow recovery when eNOS was genetically ablated, suggesting an essential role of eNOS in angiogenic effects of rHDL.

Numerous epidemiologic studies revealed that a low plasma level of HDL is a major risk factor for ischemic heart disease.1 Recent clinical trials suggested that the increase in
HDL may account for the clinical benefits of fibrate therapy to retard the progression of coronary atherosclerosis and reduce ischemic heart disease events in patients with low HDL levels. Moreover, it was reported that a recombinant apoA-I Milano/phospholipid complex (ETC-216) administered intravenously produced a significant reduction in coronary plaque burden as measured by intravascular ultrasound. These data suggest that strategies targeting HDL would be promising to treat patients with ischemic heart disease.

The atheroprotective actions of HDL are thought to be attributed to the ability of HDL to uptake cellular cholesterol from the periphery and to mediate the transport of excess cholesterol to the liver. Recent studies reported that HDL has various direct effects on endothelial cells. However, relatively little is known about the effects of HDL on new vessel formation.

The PI3K/Akt signal transduction pathway is one of the main signal routes that coordinate complex events leading to changes in cell metabolism, cell growth, cell movement, and cell survival in various cell types, including endothelial cells. Growth factors, cytokines, and insulin, as well as attachment of cells to the extracellular matrix, stimulate the recruitment of PI3K to the plasma membrane. HDL was reported previously to bind scavenger receptor class B type I or G-protein–coupled S1P receptors, leading to PI3K activation and downstream activation of Akt kinase and mitogen-activated protein kinase. Our findings provide evidence that rHDL activates PI3K/Akt in MNCs/EPCs that promotes their differentiation into endothelial-like cells.

EPCs have been shown to contribute to neovascularization in ischemic hindlimb. Many reports demonstrated that transplantation of EPCs augments ischemia-induced angiogenesis. Increase in the number of circulating EPCs may mediate the therapeutic effects of angiogenic cytokines, such as VEGF or granulocyte–macrophage colony-stimulating factor. 3-Hydroxy-3-methylglutaryl–coenzyme A reductase inhibitors, or statins, have been reported to promote EPC differentiation via the PI3K/Akt pathway and augment collateral development in ischemic tissues. Recently, Tso et al reported that rHDL increased the number of EPCs and enhanced progenitor-mediated endothelium repair in mice. Here, we found that rHDL stimulated EPC differentiation via PI3K/Akt pathway and increased EPC contribution to angiogenesis. Together, these results suggest that rHDL can augment the functions of EPCs to maintain vascular homeostasis and promote collateral development, thus exerting cardiovascular protective effects.

In this study, we provided mouse genetic evidence that eNOS mediates the angiogenic effect of rHDL. rHDL has been shown to upregulate eNOS expression at protein level, P3K/Akt activates eNOS by phosphorylation. It was demonstrated that the impaired neovascularization in eNOS−/− mice is related to a defect in progenitor cell mobilization. It is likely that rHDL promotes ischemia-induced angiogenesis via activation of eNOS in circulating EPCs as well as pre-existing endothelial cells.

Clinical studies revealed that a low plasma level of HDL is associated with impaired endothelial function and a decrease in EPC number. Clinical trials of therapeutic angiogenesis also revealed that patients with low HDL level tend to be refractory to angiogenic therapies. Thus, intravenous injection of rHDL would be clinically promising in patients with ischemic diseases, who have a low level of HDL and decreased number of EPCs.

In conclusion, our findings suggest a previously unappreciated effect of rHDL on EPCs and angiogenesis. rHDL may hold a therapeutic potential to treat patients with ischemic diseases.
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Disclosures

None.

References


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