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.

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 disease 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)–deficient (eNOS–/–) mice were purchased from Jackson Laboratory (Bar Harbor, Me). Unilateral hindlimb ischemia was induced...
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 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. 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 MMLV reverse transcriptase (ReverTra-Ace-a; TOYOBO). The PCR primers were as follows: eNOS, 5'-GCTGCCAGGCCTCTCACCTC-3' (sense) and 5'-GGCTGCAGCCCTTTGGCTCTCAA-3' (anti-sense); GAPDH, 5'-ACACAGCTTCATGCCATCAC-3' and 5'-TCCACACCCTGGTGGCTGA-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 JUNYAKU) 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. 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 vs control). The blood flow of the ischemic hind limb was expressed as the ratio to that of the uninjured limb.

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 4D), suggesting that rHDL promotes differentiation and proliferation of EPCs.
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 kinases, extracellular signal regulated kinase-1, and extracellular signal regulated kinase-2, had no effect on EPC differentiation. Together, these results suggest that rHDL promotes differentiation of peripheral MNCs 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.5,6 Blood flow recovery was impaired severely in eNOS−/− mice, as reported previously.5,6 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.3 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 the differentiation of EPCs toward the endothelial lineage.

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.

**Figure 5.** rHDL promotes EPC differentiation via PI3K/Akt pathway. A, Cells were harvested at the time points indicated. Western blotting against phosphorylated Akt (p-Akt) and Akt was performed (n=3). B, MNCs were incubated with rHDL (100 μg/mL), LY294002 (10 μmol/mL), or PD98059 (10 μmol/mL) for the time indicated. *P<0.05 vs rHDL (n=4).

**Figure 6.** rHDL fails to improve blood flow recovery in eNOS−/− mice. Hindlimb ischemia was induced in male 8-week-old eNOS−/− mice. PBS (control) or rHDL (0.2 mg apoA-I/body; 0.3 mL) was injected intravenously twice per week, starting 1 week before surgery (n=4 for each group). A, Blood flow recovery was monitored weekly by a laser Doppler perfusion imager. B, The ischemic muscles were harvested from eNOS−/− mice 4 weeks after surgery. Capillary density was measured by anti-CD31 immunostaining.
Sources of Funding
This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

Disclosures
None.

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Arterioscler Thromb Vasc Biol. published online February 1, 2007;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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