High Density Lipoprotein–Induced Angiogenesis Requires the Activation of Ras/MAP Kinase in Human Coronary Artery Endothelial Cells

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Objective.—Plasma high density lipoprotein (HDL) levels have been shown to be inversely correlated with coronary artery disease, but the mechanisms of the direct protective effect of HDL on endothelial cells (ECs) are not fully understood. In this study, we investigated the role of the HDL-mediated promotion of angiogenesis in human coronary artery ECs (HCECs).

Methods and Results.—We developed an in vitro model of HCEC tube formation on a matrix gel. We optimized the maximum dose of HDL required to induce tube formation in initial experiments, in which the dose response showed that the maximum effective dose of HDL was 100 μg/mL. PD98059, an inhibitor of p42/44 mitogen-activated protein kinase (MAPK) activity, but not SB203580, an inhibitor of p38 MAPK activity, suppressed HDL-induced tube formation. Dominant-negative Ras N17 inhibited HDL-induced tube formation. HDL activated Ras according to a ras pull-down assay, and this effect was inhibited by pertussis toxin. Moreover, HDL activated phospho(p)-p42/44 MAPK, whereas Ras N17 blocked HDL-induced pp42/44 MAPK.

Conclusions.—These results indicate that HDL induced a potent signal through a Ras/MAPK pathway mediated by a pertussis toxin–sensitive G-protein coupled receptor to the angiogenic phenotype in HCECs. (Arterioscler Thromb Vasc Biol. 2003;23:802-808.)

Key Words: high density lipoprotein ▪ Ras ▪ mitogen-activated protein kinase ▪ human coronary artery endothelial cells

High-density lipoproteins (HDL) are a heterogeneous group of small, dense lipoproteins that can be isolated from plasma at a density of 1.063 to 1.21 g/mL. A low HDL level is one of the strongest predictors of coronary risk. The native correlation between coronary heart disease and plasma HDL cholesterol has been attributed to the ability of HDL to take up cellular cholesterol from the periphery and to mediate the transport of excess cholesterol to the liver.

HDL is an important endothelial mitogen. The proliferative effect of HDL on endothelial cells seems to require extracellular calcium. HDL also seems to induce protein kinase C–dependent phosphorylation of a 27-kDa protein. Although apoptosis of endothelial cells has been demonstrated in many cardiovascular diseases, including atherosclerosis, HDL protects endothelial cells from tumor necrosis factor–α–induced apoptosis. HDL has thus far been implicated in a variety of endothelial behaviors, including proliferation, apoptosis, prostaglandin synthesis, and NO synthesis, but not angiogenesis.

Angiogenesis, the process of postnatal neovascularization, is a critical component of several human diseases, including ischemic heart disease, cancer, diabetic microvascular disease, rheumatoid arthritis, and psoriasis. Moreover, angiogenesis is believed to be mediated by the proliferation, migration, and remodeling of fully differentiated endothelial cells. Activation of Ras is involved in the activation of mitogen-activated protein kinase (MAPK), which can play a pivotal role in cell proliferation. Although recent reports have suggested that Ras might be a target for an effective angiogenic therapy, it is unclear whether Ras plays a direct role in HDL-induced signal transduction. We thought that it would be important to evaluate the role of Ras-mediated signal transduction and its relationship to control of the angiogenic phenotype by HDL. Therefore, the precise role of HDL in angiogenesis is likely to be important. We investigated the role of HDL in angiogenesis in an in vitro model of human coronary artery endothelial cell (HCEC) tube formation on a matrix gel and explored the possibility that HDL may induce angiogenesis in HCECs.

Methods

Materials

The following antibodies and reagents were generously provided as indicated or purchased: a specific inhibitor of MEK kinase,
PD98059, 2-[(2'-amino-3'-methoxyphenyl) oxanaphthalen-4-1 (New England BioLabs); the p38 MAPK inhibitor SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) 1H-imidazole, sphingosine-1-phosphate (S1P), and pertussis toxin (PTX) (Sigma); and phospho-p42/44 MAPK (Thr202/Tyr204) antibody and p42/44 MAPK antibody (Cell Signaling Technology).

Cell Culture
HCECs were purchased from Clonetics. HCECs were cultured in media supplemented with 5% FBS, penicillin/streptomycin, and endothelial cell growth supplement (Takara Co) at 37°C under 5% CO₂. In the experiments, HCECs supplemented with 5% FBS but without endothelial cell growth supplement were used.

Generation of Fusion Protein and Transfection
The expression vector pCMV-RasN17 (dominant-negative type) was purchased from Clontech. In mock transfection control samples, the pEGFP (enhanced green fluorescent protein) vector (Clontech) was used. The pEGFP-RasN17 expression vector was constructed by fusion of the coding sequence of EGFP to the S' end of the Ras coding sequence. HCECs were transfected using the lipofectamin without endothelial cell growth supplement were used.

Preparation of HDL and LDL
Blood from healthy volunteers was collected into chilled tubes (4°C) and the plasma was immediately separated by centrifugation and collected. HDL (1.063<d<1.21 g/mL) and LDL (1.019<d<1.063 g/mL) were then purified by sequential ultracentrifugation, dialyzed against PBS, and filter-sterilized. HDL and LDL purified from the healthy volunteers was pooled before use in the experiments.

Preparation of Protein Extract, Immunoblotting
Preparation of Protein Extract, Immunoblotting

Ras Pull-Down Assay
A Ras pull-down assay was performed using a Ras activation assay kit (Upstate Biotechnology) according to the manufacturer's instructions. After 1 day in serum-free culture medium, cells were preconditioned with or without PD98059 for 30 minutes and stimulated with HDL for the indicated number of minutes at 37°C and 5% CO₂. The procedure for cell lysis and Western blot analysis of signaling proteins on Immobilon-P membranes (Millipore Corp) has been described previously. Horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system (Amersham) were used for detection, and the band intensity was quantified by digital image analysis.

Statistical Analysis
Results are given as the mean±SEM. The significance of differences between mean values was evaluated by unpaired t test. A value of P<0.05 was considered significant.
Results

HDL But Not LDL Induces Angiogenesis

We first examined the ability of HDL to stimulate and stabilize tube formation and used LDL as a negative control, with HCECs cultured on Matrigel. As shown in Figures 1A through 1H, HDL but not LDL dose-dependently led to the formation of a capillary-like structure on the Matrigel surface; this proangiogenic effect was maximal after 8 hours. We optimized the dose for the maximum dose required to induce tube formation in initial experiments in which the dose-response showed that the maximum effective dose of HDL was 100 μg/mL.

HDL-Induced Angiogenesis Is Inhibited by p42/44 MAPK Inhibitor But Not p38 MAPK Inhibitor

Activation of the Ras effector p42/44 MAPK affects cellular proliferation and cell migration.13,14 These changes occur in response to several mitogenic growth factors. Because Ras acts through a p42/44 MAPK pathway to exert these phenotypic effects, we used the p42/44 MAPK inhibitor PD98059, which is highly selective for p42/44 MAPK.15 We then tested the effect of this inhibitor on tube formation and optimized the dose of the inhibitor for the minimum dose required to inhibit activation in initial experiments, in which the dose-response showed that the minimum effective dose of PD98059 was 10 μmol/L (not shown). As shown in Figures 1G and 1H, the inhibition of p42/44 MAPK activation suppressed HDL-induced tube formation. We next sought to examine the role of the pathway for p38 MAPK in regulating tube formation. Rousseau et al15a demonstrated that treatment with the p38 MAPK inhibitor SB203580 inhibits VEGF-induced migration and actin reorganization. These data directly implicated p38 as a critical modulator of migration. In other studies on the PDGF receptor, Ras has been reported to

Figure 2. Endothelial tube formation was induced by S1P and inhibited by PTX. Shown are representative pictures of HCECs plated on Matrigel for 18 hours. HDL was used as a positive control. A, Control; B, HDL 100 μg/mL; C, PTX 100 ng/mL; D, S1P 0.01 μmol/L; E, S1P 0.02 μmol/L; F, S1P 0.1 μmol/L; G, S1P 0.2 μmol/L; H, S1P 1 μmol/L; I, HDL 100 μg/mL + PTX 100 ng/mL; and J, S1P 1 μmol/L + PTX 100 ng/mL. Graph shows tube formation as a percentage of that in the control (n = 3, mean ± SE). *P < 0.05 vs control.

Figure 3. EGFP-RasN17–transfected HCECs showed inhibited tube formation. Shown are representative pictures of HCECs plated on Matrigel for 18 hours after transfection. A through E, EGFP-transfected cells; F through J, EGFP-RasN17–transfected cells. Graph shows angiogenesis as a percentage of that in the control (n = 3, mean ± SE). *P < 0.05 vs EGFP-transfected cells.
be required for the stimulation of p38 MAPK. In this system, the inhibition of p38 MAPK activation did not affect tube formation (not shown).

**S1P Induced Angiogenesis, and This Effect Is Blocked by PTX**
Next, we examined the effect of S1P, which is a carrier of bioactive lipids of HDL, on tube formation, because it activates MAPK and has an antiapoptotic effect. As shown in Figure 2, although S1P induced tube formation in a dose-dependent manner, this effect was blocked by PTX, an inhibitor of G/G protein function, suggesting that S1P or HDL-induced tube formation is mediated by PTX-sensitive G-protein coupled receptors (GPCRs).

**Dominant-Negative Ras Blocks HDL-Induced Angiogenesis**
All of the above results showed that the effect of HDL is related to mitogenic factors. Because HDL intrinsically induces small G-protein and MAPK-dependent proliferation and cell cycle progression in vascular smooth muscle cells, we produced an expression vector encoding EGFP fused to Ras and investigated Ras distribution on serum-stimulated endothelial cells. Activation of Ras is involved in the activation of mitogen-activated protein kinase, which can play a pivotal role in cell proliferation. Although EGFP-transfected HCECs showed HDL-induced tube formation, EGFP-RasN17-transfected cells did not (Figures 3 and 4).

**HDL-Induced Ras Activation**
To examine the direct effect of HDL and PTX on Ras activation, we used a Ras pull-down assay. When HDL activates Ras, the activated Ras should bind to Raf-1 RBD and should be detectable by anti-Ras antibody after immunoprecipitation. HDL (100 μg/mL) led to the time-dependent activation of Ras, as shown in Figure 5A. HDL-induced Ras activation was inhibited by PTX (Figure 5B).

**PD98059 Inhibitor Blocks HDL-Induced Phospho-p42/44 MAPK**
To investigate the effect of PD98059 inhibitor on HDL-induced p42/44 MAPK activity, we examined the phosphorylation of MAPK. Treatment of cells with 100 μg/mL of HDL led to the time-dependent phosphorylation of p42/44 MAPK. Maximal stimulation occurred after HCECs had been incubated for 10 minutes (Figure 6A). As shown in Figure 6B, 10 μmol/L of PD98059 inhibitor did not affect basal levels of pp42/44 MAPK and blocked 100 μg/mL HDL-induced pp42/44 MAPK.

**HDL-Induced pp42/44 MAPK Activity Is Blocked by Dominant-Negative Ras**
To investigate signal transmission from HDL to MAPK, the effect of HDL on Ras was studied. For this purpose, we examined the phosphorylation of p42/44 MAPK in dominant-negative Ras-transfected HCECs. Overexpression of dominant-negative Ras inhibited HDL-induced pp42/44 MAPK activity (Figure 6C).

**Discussion**
Although some studies have shown that HDL has direct protective effects on the endothelium, relatively little is known about the mechanisms of the angiogenic effects of HDL on ECs. Therefore, we investigated the impact of MAPK activation on HDL-mediated signal transduction and...
the induction of angiogenesis in HCECs. Our results identified Ras as a key player in the angiogenic action of HDL. This is the first comprehensive analysis of the role of MAPK in the HDL-induced modulation of an angiogenic effect.

In atherosclerosis, the development of angiogenesis seems to have both beneficial and deleterious effects. Whereas increased angiogenesis may be a favorable sign in the healing of ischemic tissues such as in myocardial infarction and necrosis of the lower extremities, progressive angiogenesis is a primary atherosclerotic lesion that has been considered to cause plaque expansion, plaque vulnerability, and the risk of significant disease complications, such as plaque rupture and vascular thrombosis. HDL-induced angiogenesis may also have both beneficial and deleterious effects. Although it is not clear whether HDL in its relation to angiogenesis induces the progression of atherosclerosis, HDL in fact reduces atherosclerosis because one of its main functions is to uptake cholesterol from peripheral tissues.

Although recent reports have suggested that the inhibition of Ras might be an effective antiangiogenic therapy, it has been unclear whether Ras plays a direct role in HDL-induced signal transduction. However, several lines of evidence suggest that Ras signal transduction may be critical in regulating endothelial cell function, because Ras may modulate mitogenesis, endothelial cell motility, and cell survival as well as organization and differentiation into a vessel. These actions are all critical to angiogenesis and may be induced by HDL. We thought that it would be very important to evaluate the role of Ras-mediated signal transduction and its relationship to the control of the angiogenic phenotype by HDL. While our experiments were in progress, Meadows et al investigated the role of Ras in vascular endothelial growth factor (VEGF)-induced angiogenesis. Although they found that VEGF potently induces Ras activation and that this step is essential for the stimulation of VEGF-induced angiogenesis, we examined whether HDL induces MAPK activation through a Ras pathway and the potential for HDL-induced angiogenesis. Because Ras regulates p42/44 MAPK activation and the inhibition of p42/44 MAPK activity using PD98059 had an antiangiogenic effect, the inhibitory effect may be attributable to an antiproliferative effect of PD98059. Moreover, the inhibition of MAPK activation by dominant-negative src kinase abrogates the apoptosis-suppressive and proangiogenic effect of VEGF. Nofer et al reported that HDL induced VSMC proliferation, cell cycle progression,
cyclin D1 expression, and activation of Raf-1/MEK-1/MAPK in cells preincubated with pertussis toxin, indicating the involvement of trimeric G-protein. The strong and specific mitogenic effect of HDL should be considered when HDL stimulates angiogenesis in the endothelium. In addition to the MAPK pathway, HDL is known to activate several other kinases, such as Akt. Based on Kimura’s report, we calculated that 100 μg/mL of HDL significantly induced tube formation. They separated HDL and LDL from freshly isolated human plasma using sequential ultracentrifugation, which is the same method that we used. Therefore, both HDL and LDL in our experiments should contain S1P. We observed that 100 μg/mL of HDL significantly induced tube formation. Based on Kimura’s report, we calculated that 100 μg/mL of HDL contains 0.018 μmol/L of S1P (Figure 2). With regard to tube formation, 100 μg/mL of HDL (245%) had an effect comparable to that of 1 μmol/L of S1P (230%). Although 0.02 μmol/L of S1P significantly promoted tube formation, the respective value was only 130%. The tube formation induced by 100 μg/mL of HDL may be partly attributable to S1P. On the other hand, 100 μg/mL of LDL did not induce tube formation, because the concentration of S1P was only ~0.007 μmol/L. Because HDL- or S1P-induced tube formation was completely blocked by PTX, signals of HDL- or S1P-induced tube formation seem to be mediated by PTX-sensitive GPCRs. In addition, Kimura et al reported that 100 μg/mL of HDL contained S1P and HDL activated phospho-p42/44 MAPK and induced cytoprotective action through PTX-sensitive GPCRs. To reconcile the observations presented here with those in previous reports, we propose the model depicted in Figure 7. Although we did not specify the subtype of S1P receptor involved in the actions of HDL, we confirmed that human coronary endothelial cells express EDG-1 and EDG-3 by reverse transcriptase–polymerase chain reaction (Miura et al, unpublished data, 2002). Therefore, the present data indicate that the signaling pathway in HDL-induced tube formation is through PTX-sensitive GPCRs and may be partly through receptors for S1P, such as EDG-1 and EDG-3. Our model assumes that lipoprotein induces angiogenic signals through PTX-sensitive GPCRs.

HDL signaling through cholesterol ester donation is an exciting possible route to the induction of angiogenesis. The scavenger receptor SR-BI mediates the selective cellular uptake of cholesterol from HDL. Consistent with this idea is the observation that HDL maintains the appropriate cellular localization of eNOS by replenishing endothelial cellular membranes with cholesterol esters in an SR-BI–dependent fashion. Although it is now possible to conclude that HDL improves endothelial function, ie, angiogenesis, fundamental questions regarding the relationship between HDL and Ras remain unanswered. More detailed investigations will be required to determine if HDL-induced activation of eNOS plays a role in angiogenesis along with Ras/MAPK activation.

In conclusion, the present results demonstrate that HDL induces angiogenesis and that Ras/MAPK is involved in this process. Thus, HDL is associated with intrinsic angiogenic activity. Whether or not this is antiatherogenic, the strong and specific angiogenic effect of HDL deserves attention in the development of therapeutic strategies to elevate plasma levels of these lipoproteins.

Acknowledgments

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References

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