High-Density Lipoprotein Regulates Calcification of Vascular Cells

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Abstract—Accumulating evidence has suggested the protective role of HDL in cardiovascular disease processes. Calcification is a common feature of atherosclerotic lesions and contributes to cardiovascular complications due to the loss of aortic resilience and function. Recent studies have suggested that vascular calcification shares several features with skeletal bone formation at the cellular and molecular levels. These include the presence of osteoblast-like calcifying vascular cells in the artery wall that undergo osteoblastic differentiation and calcification in vitro. We hypothesized that HDL may also protect against vascular calcification by regulating the osteogenic activity of these calcifying vascular cells. When treated with HDL, alkaline phosphatase activity, a marker of osteogenic differentiation of osteoblastic cells, was significantly reduced in those cells. Prolonged treatment with HDL also inhibited calcification of these cells, further supporting the antiosteogenic differentiation property of HDL when applied to vascular cells. Furthermore, HDL inhibited the osteogenic activity that was induced by inflammatory cytokines interleukin (IL)-1β and IL-6 as well as by minimally oxidized LDL. HDL also partially inhibited the IL-6–induced activation of signal transducer and activator of transcription 3 in calcifying vascular cells, suggesting that HDL may inhibit cytokine-induced signal transduction pathways. The inhibitory effects of HDL were mimicked by lipids extracted from HDL but not by HDL-associated apolipoproteins or reconstituted HDL. Furthermore, oxidation of HDL rendered it pro-osteogenic. Taken together, these results suggest that HDL regulates the osteoblastic differentiation and calcification of vascular cells and that vascular calcification may be another target of HDL action in the artery wall. (Circ Res. 2002;91:570-576.)

Key Words: vascular calcification • calcifying vascular cells • high-density lipoproteins • cytokines

Vascular calcification is a hallmark of atherosclerosis and has been linked to increased cardiovascular disease and mortality.1–4 Atherosclerotic calcification contains numerous factors associated with bone mineralization, including matrix vesicles, the bone-liver-kidney isozyme of alkaline phosphatase, bone morphogenetic protein-2, osteopontin, osteocalcin, and collagen.5 In addition, our laboratory and others have isolated osteoblast-like cells from the artery wall. These calcifying vascular cells (CVCs) spontaneously calcify in vitro and express osteoblastic differentiation markers such as alkaline phosphatase, osteopontin, osteocalcin, osteonectin, and collagen.6 We have previously found that osteoblastic differentiation and mineralization of these cells are enhanced by inflammatory factors such as minimally oxidized LDL (MM-LDL), tumor necrosis factor (TNF)-α, and leptin.7–9 Therefore, CVCs and factors that regulate their behavior may also regulate vascular calcification in vivo.

HDL plasma levels are inversely correlated with coronary artery disease.10–12 However, the mechanisms by which HDL exerts its protective effects are diverse. HDL is responsible for reverse cholesterol transport, ie, the removal of free cholesterol from blood vessels to the liver.13,14 It also possesses antioxidant and antiinflammatory properties.15 Several studies have demonstrated the ability of HDL to inhibit cytokine-induced responses in endothelial cells.16,17 Furthermore, studies by Navab et al18 and Parthasarathy et al19 have shown that HDL inhibits LDL oxidation and production of monocyte chemotactic proteins in an in vitro coculture model of artery wall cells. The antiinflammatory effects of HDL appear to be mediated in part by associated proteins, such as apolipoprotein (apo)A-I, which has lipid-binding properties, and the enzymes platelet factor acetyl hydrolase and serum paraoxonase (PON-1), which can eliminate the bioactivity of oxidized lipids.15,20

However, HDL can be modified to lose its protective abilities and to become proinflammatory.20 During the acute-phase response, a systematic reaction to tissue injury, HDL is converted to a proinflammatory molecule.21,22 In addition, overexpression of apoA-II in mice elicits a reduction in PON-1 levels and converts HDL to a proinflammatory molecule.23 HDL is susceptible to oxidation, and oxidized HDL has proinflammatory characteristics24 and has been localized to atherosclerotic plaques.25 Proinflammatory cytokines contribute to early atherogenesis,26–28 Interleukin (IL)-1, one of the most potent proinflammatory cytokines, acts on endothelial cells and smooth
muscle cells and is involved in atherogenesis. Recently, Subbanagounder et al. have demonstrated the accumulation of atherogenic oxidized phospholipids in cultures of human aortic endothelial cells treated with IL-1\(\beta\). IL-6 is another proinflammatory cytokine expressed in atherosclerotic plaques, and it mediates at least some of the inflammatory effects of atherogenic oxidized phospholipids. Reports suggest that the protective effects of HDL may regulate vascular calcification by directly inhibiting the osteogenic differentiation of vascular cells.

In the present study, we present the first evidence that HDL may also play an important role in regulating vascular calcification associated with atherosclerotic lesions. We demonstrate that human HDL inhibits the spontaneous osteogenic differentiation and mineralization of CVCs in vitro. In addition, HDL inhibits the osteogenic differentiation of CVCs induced by MM-LDL, IL-1\(\beta\), and IL-6. Furthermore, we found that minimally oxidized HDL (MM-HDL) enhances the osteogenic behavior of CVCs. Altogether, these results suggest that HDL may regulate vascular calcification by directly inhibiting the osteogenic differentiation of vascular cells.

Materials and Methods

Cell Culture

CVC clones were isolated from cultures of bovine smooth muscle cells harvested from bovine aortic medial explants and cultured as previously described. Recombinant human IL-1\(\beta\) was obtained from R&D Systems, Inc., and recombinant human IL-6 and recombinant human interferon-\(\gamma\) were obtained from Biosource International. Human apoA-I, apoA-II, and apoE were from Sigma Chemical Co, purified human PON-1 was a generous gift from Dr Bert La Du (University of Michigan Medical School, Ann Arbor), and reconstituted HDL was a generous gift from Dr Geeta Datta (University of Alabama at Birmingham). HDL pretreatment of CVCs was performed in DMEM containing 5% FBS (Hyclone) for 24 hours, followed by the addition of fresh HDL and media and the addition of cytokines or MM-LDL. Pretreatment with HDL (200 \(\mu\)g/mL) significantly inhibited calcification induced by IL-1\(\beta\) for 4 days (Figure 1A). Inhibition was also achieved even when HDL was present for only the first 24 hours of incubation, although to a lesser extent than when HDL was continuously present during the 4 days of incubation (Figure 1B). Furthermore, continuous incubation with HDL (200 \(\mu\)g/mL) also dramatically inhibited spontaneous calcification of CVC cultures, as shown by a \(^{45}\)Ca incorporation assay (Figure 1C). However, HDL did not inhibit nodule formation in the CVC cultures (data not shown). These results suggest that HDL inhibits the spontaneous osteoblastic differentiation of CVCs.

Western Blot Analysis

At the end of treatments, CVC cultures were rinsed twice with PBS and lysed in cold lysis buffer consisting of 50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 50 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 100 \(\mu\)mol/L Na$_2$VO$_3$, 0.1% Triton X-100, and 10 mmol/L HEPES, pH 7.4, along with protease inhibitor cocktail (Calbiochem). The lysate was then sonicated for 10 seconds and microcentrifuged, and the supernatant was used for Western blotting. Lysate (20 \(\mu\)g) was electrophoresed on 8% Tris-glycine gels (Novex). This was followed by transfer to nitrocellulose membranes, as previously described. The blots were probed with either antibody to native or phosphorylated signal transducer and activator of transcription 3 (STAT3, Santa Cruz Biotechnology) according to the manufacturer’s instructions. Detection of immunoreactive proteins was performed by chemiluminescence (Amer sham), and signals were quantified by densitometric analysis.

Statistical Analysis

Data are presented as mean\(\pm\)SD and were analyzed by using the Statview 4.5 program. All probability values were calculated by ANOVA and the Fisher protected least significant difference test. A value of \(P<0.05\) was considered significant.

Results

HDL Inhibits Spontaneous Osteogenic Activity of CVCs

CVCs undergo an in vitro differentiation process similar to that of preosteoblasts. This process involves nodule formation and the sequential upregulation of several osteoblast-specific genes, such as osteocalcin and Cbfa-1. Alkaline phosphatase, an early marker of osteoblastic differentiation, provides phosphate from organic sources for calcium phosphate mineral formation. HDL treatment caused a dose-dependent inhibition of alkaline phosphatase activity in CVCs at 4 days (Figure 1A). Inhibition was also achieved even when HDL was present for only the first 24 hours of incubation, although to a lesser extent than when HDL was continuously present during the 4 days of incubation (Figure 1B). Furthermore, continuous incubation with HDL (200 \(\mu\)g/mL) also dramatically inhibited spontaneous calcification of CVC cultures, as shown by a \(^{45}\)Ca incorporation assay (Figure 1C). However, HDL did not inhibit nodule formation in the CVC cultures (data not shown). These results suggest that HDL inhibits the spontaneous osteoblastic differentiation of CVCs.

HDL Inhibits Cytokine-Induced and MM-LDL–Induced Osteogenic Activity of CVCs

We previously found that CVC differentiation and mineralization are enhanced by several inflammatory agents such as TNF-\(\alpha\) and oxidized lipids and lipoproteins. More recently, we have found that CVC differentiation is also dose-dependently enhanced by IL-1\(\beta\) and IL-6. Treatment of CVCs with IL-1\(\beta\) or IL-6 for 2 or 4 days, respectively, caused a dose-dependent increase in alkaline phosphatase activity (Figures 2A and 2B). In contrast, treatment of CVCs with interferon-\(\gamma\) (1 to 10 ng/mL) for 4 days did not significantly affect alkaline phosphatase activity (data not shown). Mineralization was also enhanced in CVC cultures treated for 10 days with IL-1\(\beta\) or IL-6 (data not shown). Twenty-four–hour pretreatment with HDL (200 \(\mu\)g/mL) significantly inhibited alkaline phosphatase and calcification induced by IL-1\(\beta\)
(Figures 3A and 3B) and IL-6 (Figures 4A and 4B). Furthermore, the previously reported enhancement of alkaline phosphatase activity by the atherogenic lipoprotein MM-LDL was also blocked in the presence of HDL (Figure 5). Altogether, these results demonstrate that HDL can prevent the pro-osteoogenic differentiation effects of several inflammatory agents on CVCs.

Mechanism of HDL Effects on CVCs
Several different mechanisms have been proposed for the anti-inflammatory effects of HDL in different experimental systems.\textsuperscript{15} We found that spontaneous differentiation and calcification of CVCs, which correspond to the growth stages of preconfluence, confluence, condensing, and calcification,\textsuperscript{34} were associated with increased levels of STAT3 and its activated phosphorylated form, pSTAT3 (Figure 6A). Furthermore, the effects of IL-6 on CVC differentiation were associated with STAT3 activation. Treatment of CVCs for 15 minutes with IL-6 (50 ng/mL) caused a significant increase in the levels of pSTAT3 in those cells (Figure 6B). Pretreatment of CVCs for 24 hours with HDL (200 μg/mL) caused a 50% inhibition of IL-6–induced STAT3 activation (Figure 6B). This suggests that inhibition of cytokine-induced signaling in CVCs may be at least in part responsible for the inhibitory effects of HDL.

To determine which component(s) of HDL exerts its inhibitory effects on CVC differentiation, we examined the effects of lipids extracted from HDL. Results showed that total lipids extracted from HDL had effects similar to those of

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**Figure 1.** Effect of HDL on alkaline phosphatase activity and calcification in CVC cultures. A, CVCs were incubated for 4 days with HDL at the indicated concentrations in DMEM containing 5% FBS. Alkaline phosphatase activity was measured in total cell homogenates, and results from a representative of 3 experiments are shown as mean±SD of quadruplicate determinations. P<0.005 for all HDL-treated vs control (0) samples. B, CVCs were incubated for the first 24 hours only (HDL-Pre) or continuously (HDL) for 4 days with HDL (200 μg/mL) in DMEM containing 5% FBS. At the end of the 4-day incubation period, alkaline phosphatase activity was determined in total cell homogenates, and the results from a representative of 3 experiments are shown as mean±SD of quadruplicate determinations. P<0.05 for HDL-Pre and HDL-treated samples vs untreated control sample. C, CVCs were treated for 10 days with HDL (200 μg/mL) as described above, and then a 45Ca incorporation assay was performed. Results from a representative of 3 experiments are shown as mean±SD of quadruplicate determinations. P<0.01 for HDL-treated vs untreated control samples.

**Figure 2.** Effect of IL-1β and IL-6 on alkaline phosphatase activity in CVC cultures. CVCs were incubated with IL-1β (A) or IL-6 (B) for 2 or 4 days, respectively, at the indicated concentrations in DMEM containing 5% FBS. Alkaline phosphatase activity was measured in total cell homogenates, and results from a representative of 3 experiments are shown as mean±SD of quadruplicate determinations. P<0.001 for all IL-1β– and IL-6–treated samples vs control untreated (0) samples.
intact HDL, inhibiting both spontaneous and IL-1β-induced alkaline phosphatase activity in CVCs (Figure 7). Furthermore, reconstituted HDL and purified apoA-I, apoA-II, apoE, and PON-1 did not have effects similar to those of HDL. Treatment of CVCs with up to 200 μg/mL reconstituted HDL, 100 μg/mL purified apoA-I or apoA-II, 10 μg/mL apoE, or 4 μg/mL purified PON-1 did not inhibit the spontaneously increased alkaline phosphatase activity in those cells (data not shown).

Minimally Oxidized HDL Enhances CVC Osteogenic Activity

HDL can lose its protective capacity after modification by oxidation or after an acute-phase response. To examine the effect of oxidation on the antosteogenic effects of HDL, HDL was oxidized by dialysis against iron by the same approach that was used for minimal oxidation of LDL to form MM-LDL. We found that oxidized HDL was no longer antiosteogenic and was able to induce alkaline phosphatase activity (Figure 8A) and calcification (Figure 8B) in CVC cultures after 4 and 10 days of treatment, respectively.

Discussion

The present report suggests that HDL regulates calcification of vascular cells. CVCs treated with HDL had significantly lower alkaline phosphatase activity, an established marker of osteoblastic differentiation in osteoprogenitor cells, including CVCs, suggesting a block in the spontaneous osteoblastic differentiation process as these cells mature into osteoblast-like cells. HDL also blocked matrix calcification in CVC cultures, the late marker of differentiation, suggesting that HDL regulates early and late events in osteogenic differentiation. The inhibition by HDL did not affect nodule formation. In previous studies, we demonstrated that calcification and differentiation of CVCs appear to be under regulatory mechanisms different from those of nodule formation. This was evidenced by the decrease in nodule formation in face of increased differentiation and calcification when CVCs were treated with prodifferentiation agents such as dibutyryl cAMP or TNF-α. Furthermore, HDL blocked the calcification induced by inflammatory cytokines IL-1β and IL-6 as well as that induced by MM-LDL. In contrast, after oxidation, HDL not only lost its antiosteogenic effect but also induced...
calcification of CVCs. Altogether, these results demonstrate the ability of HDL to negatively regulate the calcification of vascular cells and may be an important determinant of vascular calcification in vivo. Interestingly, our data demonstrate that lipid components of HDL are responsible for its inhibitory effects on CVCs, whereas reconstituted HDL and purified apoA-I, apoA-II, apoE, and PON-1 did not have similar inhibitory effects on CVCs. Previous reports by other investigators have also demonstrated potentially antiatherogenic biological activity of HDL-associated lipids, including inhibitory effects of lysosphingolipids on endothelial cell apoptosis and inhibitory effects of specific phospholipids on endothelial cell adhesion molecule expression. These data suggest that HDL contains important lipids as well as proteins that may regulate cellular responses to inflammatory factors.

The ability of HDL to protect against vascular disease has been the subject of intense research. Epidemiological data suggest a protective effect of HDL against atherosclerotic disease. As a result, HDL-based intervention is considered a potential strategy against cardiovascular disease in humans. HDL may act by mediating reverse cholesterol transport and thereby preventing accumulation and oxidation of lipids and lipoproteins in the artery wall. Enzymes associated with HDL, platelet-activating factor acetyl hydrolase, and PON are able to directly act on oxidized phospholipids and lipoproteins and, hence, destroy their proinflammatory activity. HDL may also exert its antiinflammatory effects by impairing the response of vascular cells to inflammatory cytokines. Cockrell et al have shown that HDL inhibits IL-1β- or TNF-α-induced expression of endothelial cell adhesion molecules E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1. HDL may also facilitate the production of protective molecules such as NO by activating endothelial NO synthase and by preventing the inhibitory effects of oxidized LDL on that enzyme. Our data also suggest that HDL may prevent calcification of vascular cells and, hence, prevent calcification-induced vascular complications that would impair the proper functioning of the vessel wall.

The finding that inflammatory cytokines such as IL-1β, IL-6, and TNF-α induce osteogenic differentiation and mineralization of vascular cells suggests that inflammatory cytokines initiate or promote vascular calcification associated with atherosclerotic lesions by regulating the differentiation of CVCs. Hence, strategies that downregulate inflammation or upregulate antiinflammatory agents may prove beneficial in controlling vascular calcification.

One mechanism by which HDL may inhibit cytokine-induced osteogenic activity of vascular cells is interference with osteogenic signal transduction pathways. In the present studies, HDL partially inhibited STAT3 activation by IL-6. STAT3 and pSTAT3 levels increased in association with the differentiation and calcification of CVCs, suggesting that this signaling pathway may be involved in their osteogenic differentiation. The ability of HDL to interfere with other

Figure 5. Effect of HDL on CVC response to MM-LDL. CVC cultures were pretreated for 24 hours with HDL (200 µg/mL) in DMEM containing 5% FBS, followed by the addition of HDL (50 µg/mL) or control buffer. After 4 days, alkaline phosphatase activity was measured as previously described. Results from a representative of 3 experiments are shown as mean±SD of quadruplicate determinations. P<0.001 for control vs MM-LDL-treated samples and for MM-LDL- vs MM-LDL+HDL-treated samples; P<0.005 for control vs HDL-treated samples.

Figure 6. STAT3 and pSTAT3 levels in CVCs. A, CVC cultures were grown in DMEM containing 15% FBS to allow for rapid growth conditions. Cells were lysed and analyzed by Western blotting for levels of pSTAT3 or native STAT3, as described in the Materials and Methods section, at the following stages of growth: preconfluence (Pre), confluence (Con), condensing (Cond), and calcification (Calc). A representative of 2 experiments is shown, with each lane corresponding to a separate sample. B, CVC cultures were pretreated for 24 hours with HDL (200 µg/mL) in DMEM containing 0.1% BSA, followed by the addition of IL-6 (50 ng/mL). After 15 minutes, cells were lysed and analyzed by Western blotting for levels of pSTAT3 and STAT3. Bar graph shows densitometric analysis of the scanned autoradiograph normalized to STAT3 levels, expressed as the mean of duplicate samples.
signaling molecules has been demonstrated. Xia et al reported that HDL interrupts the sphingosine kinase signaling pathway that regulates TNF-induced responses in endothelial cells. Similarly, Nofer et al showed that HDL inhibits thrombin-induced platelet aggregation and fibrinogen binding by decreasing the production of phosphoinositide-derived second messengers. Therefore, HDL may exert its multitude of antiinflammatory effects by blocking cellular responses to inflammatory agents. Such widespread effects of HDL would likely require more than one downstream effector. One mechanism would be a generalized change in plasma membrane characteristics affecting structure and function of multiple membrane bound signaling receptors and effectors. Evidence for this concept comes from studies showing that HDL affects the structure and function of caveolae, which are important in facilitating and integrating a multitude of cellular responses and activities.

The present results also support the notion that HDL is itself susceptible to conversion from an antiinflammatory to a proinflammatory state. Van Lenten et al showed that HDL obtained during an acute-phase response lacks antiinflammatory properties and promotes endothelial-monocyte interactions in an in vitro assay. Similarly, Sharma et al reported that oxidized HDL induces proinflammatory effects on monocytes, including an increase in their binding to aortic endothelial cells. In the present study, oxidized HDL was no longer osteogenic and was able to induce alkaline phosphatase activity and calcification in cultures of CVCs.

In conclusion, the present study demonstrates the ability of HDL to inhibit the calcification of vascular cells. This effect appears to occur at least in part through blocking the effects of inflammatory factors such as cytokines and oxidized lipids. Future studies should further evaluate the effects of HDL on the complete inflammatory spectrum that regulates vascular calcification associated with atherosclerosis.

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