High-Density Lipoprotein Loses Its Anti-Inflammatory Properties During Acute Influenza A Infection

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Background—Viruses have been identified as one of a variety of potential agents that are implicated in atherogenesis.

Methods and Results—C57BL/6J mice were killed before or 2, 3, 5, 7, or 9 days after intranasal infection with 10^5 plaque-forming units (pfu) of Influenza A strain WSN/33. Peak infectivity in lungs was reached by 72 hours, and it returned to baseline by 9 days. No viremia was observed at any time. The activities of paraoxonase and platelet-activating factor acetylhydrolase in HDL decreased after infection and reached their lowest levels 7 days after inoculation. The ability of HDL from infected mice to inhibit LDL oxidation and LDL-induced monocyte chemotactic activity in human artery wall cell cocultures decreased with time after inoculation. Moreover, as the infection progressed, LDL more readily induced monocyte chemotaxis. Peak interleukin-6 and serum amyloid A plasma levels were observed at 2 and 7 days after inoculation. HDL apoA-I levels did not change. ApoJ and ceruloplasmin levels in HDL peaked 3 days after infection. Ceruloplasmin remained elevated throughout the time course, whereas apoJ levels decreased toward baseline after the third day.

Conclusions—We conclude that alterations in the relative levels of paraoxonase, platelet-activating factor acetylhydrolase, ceruloplasmin, and apoJ in HDL occur during acute influenza infection, causing HDL to lose its anti-inflammatory properties. (Circulation. 2001;103:2283-2288.)

Key Words artherosclerosis ■ infection ■ lipoproteins ■ mice ■ myocardial infarction

Established risk factors do not fully explain the risk of coronary heart disease. Several lines of evidence indicate that inflammation and, possibly, infection may play important roles in the initiation and progression of atherosclerosis. Pathological studies have demonstrated that atherosclerotic lesions are heavily infiltrated with cellular components associated with inflammation. Moreover, proinflammatory cytokines seem to be critical in early atherogenesis. Epidemiological studies suggest a correlation between the incidence of atherosclerosis and the presence of infectious diseases, both bacterial and viral, including Chlamydia pneumoniae, cytomegalovirus, and chronic bronchitis.

Death from cardiovascular diseases, particularly acute myocardial infarction, is more common in winter than summer. Meier and coworkers found strong relationships between acute respiratory infections and the risk of first-time acute myocardial infarctions. Influenza is probably the most common acute respiratory infection, and increased death rates from cardiovascular diseases were observed during influenza epidemics.

It has been demonstrated that C pneumoniae accelerates the progression of atherosclerosis in apoE-deficient mice. Infection elicits a cascade of host defenses that lead to the resolution of infection and protection against reinfection. However, that same cascade can also contribute to the development of local and systemic responses that compromise cardiovascular integrity. Our laboratory previously showed that during an acute phase response (APR) in humans after surgery and in rabbits after croton oil injection, HDL lost its protective enzymes paraoxonase (PON) and platelet-activating factor acetylhydrolase (PAF-AH), concomitant with a marked increase in its content of serum amyloid A (SAA) and ceruloplasmin. These changes resulted in a loss of the anti-inflammatory properties of HDL. In the present study, we found that infecting mice with influenza resulted in alterations of HDL similar to those observed in our APR rabbit model. After infection, the relative contents of PON, PAF-AH, apoJ, and ceruloplasmin in HDL varied as a function of time after infection. As a result, the ability of HDL to protect against LDL oxidation in vitro also varied. As demonstrated here, interleukin (IL)-6 and SAA levels also changed after infection with influenza.

A large number of parameters are altered in the APR, including those that are potentially thrombogenic, such as plasminogen activator inhibitor-1 and fibrinogen. Although the relative importance of each of these changes...
cannot be currently estimated, loss of the anti-inflammatory properties of HDL coupled with an increase in thrombogenic proteins during the APR after viral infection may collectively predispose to an increased risk for atherosclerotic clinical events.

Methods

Mice
Female C57BL/6J mice, 8 to 10 weeks old, were purchased from the Jackson Laboratory, Bar Harbor, Maine and maintained on Purina chow diet (Ralston Purina Co). Mice were bled under anesthesia by retro-orbital puncture, in accordance with protocols approved by the UCLA Animal Research Protection Committee.

Influenza Inoculation
Mice were anesthetized in a restraining chamber containing isoflurane (Fort Dodge Animal Health). A 23-g gavage needle was inserted distal to the nasal opening, and 10^5 plaque-forming units (pfu) of influenza virus strain A/WSN/33 in 50 μL of PBS was injected. The mice were retained in a supine position, and breathing was monitored to assure complete inhalation of virus dose without immediate aspiration. To ensure that saline vehicle did not introduce variations in the parameters measured, one set of mice was injected with the same volume of sterile saline alone and killed at the same time points. No differences were observed between mice receiving saline alone and mice killed at day 0 (data not shown).

Lipoproteins
Plasma LDL (d=1.019 to 1.063 g/mL) and HDL (d=1.063 to 1.210 g/mL) were isolated by sequential density ultracentrifugation using an airfuge.15

Western Blot Analysis
HDL (40 μg of cholesterol) from mice at each time point was loaded on each lane and subjected to SDS-PAGE14 using 4% to 20% gels; it was then applied to Hybond ECL Nitrocellulose membranes (Amer sham) in Tris-buffered saline. Chemiluminescent detection of proteins was performed using an ECL Western blotting kit (Amer sham). For the detection of ceruloplasmin, the primary antibody used was goat anti-human ceruloplasmin (Sigma), which we previously showed cross-reacted with rodent ceruloplasmin.10 The secondary antibody used was horseradish peroxidase–conjugated anti-goat IgG (Vector). To detect apoA-I, the primary antibody was purchased from Chemicon. The secondary antibody was horseradish peroxidase-conjugated anti-goat IgG (Vector). To detect apoJ, clusterin/apo-J primary antibody produced in goats was purchased from Biodesign. The secondary antibody was horseradish peroxidase-conjugated anti-goat IgG (Vector). To detect PON, the primary antibody used was generated in rabbits as described previously.15 The secondary antibody used was horseradish peroxidase–conjugated donkey anti-rabbit IgG (Amersham). To detect PAF-AH, the primary antibody used was rabbit anti-mouse antibody from Biodesign. The secondary antibody used was anti-goat IgG from Vector Labs. To detect apoA-I, the primary antibody used was rabbit anti-mouse antibody from Biodesign. The secondary antibody used was horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham). To detect PON, the primary antibody used was generated in rabbits as described previously.15 The secondary antibody used was horseradish peroxidase–conjugated donkey anti-rabbit IgG (Amersham).

Lipid Hydroperoxide Assay
Lipid hydroperoxide formation was measured using the Auerbach method.16 We found a highly significant correlation (r²=0.99) between values for lipid hydroperoxide levels determined by the Auerbach assay and F2-isoprostane concentrations using an ELISA from Cayman Chemical (data not shown).

Monocyte Chemotaxis Assay
Blood monocytes were isolated from a pool of healthy donors.17 Human aortic endothelial cells and smooth muscle cells were isolated as described previously.18 Cocultures were treated with LDL (250 μg/mL) in the absence or presence of HDL for 18 hours. Supernatants were collected and used to determine lipid hydroperoxides. Cocultures were subsequently washed, and fresh culture medium without additions was added and incubated for 8 additional hours. This allowed the monocyte chemotactic activity released by the cells after stimulation by the oxidized LDL to be collected. At the end of incubation, supernatants were collected from cocultures, diluted 40-fold, and assayed for monocyte chemotactic activity as described previously.18

Enzyme Activity Assays
PAF-AH activity was determined by the method described previously.19 PON activity was measured using paraoxon, as previously described.20

Cholesterol Efflux Assay
The abilities of HDL to promote cholesterol efflux from monocyte-macrophages before and after influenza infection were compared using the technique of de la Liera Moya et al.21

Other Procedures
Plaque assays were used to determine plasma and tissue viral titers.22 ELISA kits to measure IL-6 and SAA were obtained from Biosource. Lipoprotein cholesterol concentrations were determined using a Cholesterol-20 kit (Sigma). Statistical analyses were performed using ANOVA.

Results

Infectivity Titers in Lung and Plasma During Influenza A Infection
Figure 1 shows the results of infecting mice with influenza. Peak infectivity titers in lung were achieved by 3 days after inoculation. By 9 days, lung virus levels had returned to baseline. No viremia was detected at any time point during the course of infection.

Loss of HDL Enzyme Activities During Influenza A Infection
HDL was isolated from plasma at each time point, and PON and PAF-AH activities were determined. PON activity in HDL decreased with time after inoculation, reaching a nadir by 7 days (Figure 2A). The time course for HDL PAF-AH activity was similar to that observed for PON (Figure 2B). PAF-AH activity declined after 2 days and reached its lowest level by 7 days. HDL isolated from plasma by fast-performance liquid chromatography showed the same changes in PON activity observed with HDL prepared with an airfuge or standard ultracentrifuge (data not shown).

Figure 1. Infectious time course in plasma and lungs after infection of mice with influenza A. A total of 42 mice were divided into 6 groups of 7 mice each and intranasally inoculated with 10^6 pfu of influenza A strain WSN/33. After indicated number of days, each group was killed, and viral titers in plasma (■) and lung tissue (△) were determined by plaque assay. Results are mean±SD of 3 separate experiments.
Loss of HDL Protection Against LDL Modification During Influenza A Infection

In the experiment shown in Figure 3A, lipid hydroperoxide levels were measured in supernatants from cells treated with LDL in the presence of HDL from mice after influenza infection. HDL isolated from mice 2 to 5 days after infection suppressed the level of lipid hydroperoxides generated by artery wall cells in the presence of LDL (although less so than those from 3 to 5 days after infection). However, HDL from mice 7 to 9 days after infection did not. Figure 3B shows that the level of protection against LDL-induced monocyte chemotactic activity afforded by HDL at each time point was inversely correlated with the level of lipid hydroperoxides generated. Control HDL (day 0) and HDL isolated 2 to 5 days after infection inhibited the LDL-induced increase in monocyte chemotactic activity; however, HDL from days 3 and 5 was less effective. HDL from mice 7 and 9 days after influenza A infection did not prevent the LDL-induced increase in monocyte chemotactic activity.

LDL Induces More Monocyte Chemotactic Activity After Influenza A Infection

In Figure 4, LDL was isolated from the plasma of control mice and from mice at each time point after influenza infection, and its ability to stimulate artery wall cells to induce monocyte chemotactic activity was determined. LDL from mice 5 to 9 days after inoculation induced a significantly greater level of monocyte chemotactic activity than did control LDL.

Expression of Acute Phase Reactants During Influenza A Infection

It has been reported that during an APR, the levels and composition of plasma lipoproteins are altered. We previously showed that during an APR induced by surgery in humans or by croton oil injection in rabbits, HDL exhibits a marked increase in SAA protein, with a concomitant loss in PON and PAF-AH activities. IL-6, a principal mediator of the APR, is known to affect the hepatic synthesis of a number of apolipoproteins and acute phase reactants, including...
To determine if these markers of the APR were altered with influenza infection, we measured SAA and IL-6 levels in the plasma of mice before and after viral infection. Both IL-6 (Figure 5A) and SAA (Figure 5B) showed 2 peak concentrations during the course of infection: at day 2 and at day 7. The viral titer sharply increased on the third day after infection, at which time the levels of IL-6 and SAA decreased. As the viral titer decreased, the levels of IL-6 and SAA rebounded before returning to baseline by day 9 (compare Figures 1 and 5).

ApoJ is an acute phase reactant that was previously shown to protect LDL against oxidation by human artery wall cells. 25 As shown in Figure 6A, HDL apoJ levels increased dramatically 2 days after infection, peaked on the third day, and then returned toward baseline. Ceruloplasmin, another acute phase reactant shown to increase in HDL during an APR and to promote LDL oxidation,10 increased in HDL by 2 days after influenza infection and remained elevated throughout (Figure 6B). Changes in PON protein levels mirrored the changes in PON activity, decreasing after influenza infection to a nadir 7 days after infection and then returning to baseline by the 9th day (Figure 6C). In data not shown, apoA-I levels were determined; they were unchanged after influenza infection, whereas apoB levels increased from days 2 to 7 after infection and returned to baseline by day 9. HDL cholesterol modestly declined after infection; it reached a nadir after 3 days, peaked at 7 days, and returned to baseline by 9 days (data not shown).

Because ceruloplasmin in HDL remained elevated from days 2 through 9, the elevation in ceruloplasmin was independent of apoA-I and HDL-cholesterol levels. As shown in Figure 7, the ability of HDL to promote cholesterol efflux from macrophages increased dramatically 2 days after infection and returned to baseline by 5 days after infection.

Figure 5. Plasma levels of IL-6 and SAA after infection of mice with influenza A. A total of 42 mice were divided into 6 groups of 7 mice each and intranasally inoculated with 10^5 pfu of influenza A strain WSN/33. After indicated number of days, IL-6 (A) and SAA (B) concentrations were determined by ELISA as described in Methods. *P<0.05 vs no additions (N/A) vs various LDL treatments. HPF indicates high-powered field.

**Discussion**

Numerous epidemiological studies have associated HDL with an inverse risk for coronary artery disease. This “protective” effect of HDL may be due in part to an inhibition of the oxidative modification of LDL.18 Under certain conditions, such as an APR, the normally antioxidant nature of HDL is altered.10 We observed in the present study that over the course of infection with influenza A, HDL lost its anti-inflammatory properties (Figure 3). By 2 to 3 days after infection with influenza A, there was a loss in the activities of PON and PAF-AH, enzymes that can catalyze the hydrolysis of the biologically active lipids in mildly oxidized LDL.26,27 HDL was less able to protect against LDL-induced monocyte chemotactic activity 3 days after infection and was least protective 7 to 9 days after infection (Figure 3). PON and PAF-AH activities were lowest 7 days after infection but were not significantly different from baseline by 9 days (Figure 2).

ApoJ levels in HDL increased 2 days after infection, peaked on the third day, and then returned toward baseline afterward (Figure 6A). It was during these 2 to 3 days after infection that HDL was most effective in promoting cholesterol efflux from foam
cells and indicated that it might have a function in cellular cholesterol homeostasis in both normal and pathological situations, such as during viral infections. Ceruloplasmin levels increased after infection but remained elevated throughout (Figure 6B). Ehrenwald and colleagues demonstrated that ceruloplasmin is a potent oxidant, and we showed previously that ceruloplasmin markedly enhanced LDL oxidation by artery wall cells. We can conclude from these observations that the relative levels of apoJ, PON, PAF-AH, and ceruloplasmin in HDL are important in determining the anti-inflammatory activity of HDL.

As shown in Figure 4, LDL more readily induced monocyte chemotactic activity 5 days after infection, and this persisted through day 9. On the basis of our previous work and the data presented here, it is likely that these changes in LDL were secondary to the changes in HDL described above. The increased ability of LDL after influenza infection to induce artery wall cells to produce monocyte chemotactic activity may be a reflection of the reduced ability of HDL after influenza infection to destroy “seeding molecules” in LDL. Memon et al recently reported that after injection with bacterial lipopolysaccharide, zymosan, or turpentine, LDL contained increased amounts of conjugated dienes and lipid hydroperoxides, as well as lysophosphatidylcholine. Considering these and our own observations, we suspect that the changes in LDL seen by Memon et al were secondary to changes in HDL induced by the APR. These modifications in HDL may have evolved to provide an oxidative environment to promote host defense in combating viral infection.

Hajjar recently asked if oxidized lipoproteins and infectious agents are in “collusion to accelerate atherogenesis.” The data in the present article suggest that this may be so. The absence of viremia at all time points measured indicates that changes in HDL function were not due directly to the virus but instead reflected changes induced by a systemic response. The APR induces a large number of changes, both local and systemic, expressed as fever, activation of clotting, complement, and kinin-forming pathways, as well as an alteration of plasma lipoproteins. The loss of the anti-inflammatory nature of HDL, coupled with an increase in thrombogenic proteins and an increased susceptibility of LDL to oxidation during the APR after viral infection, may predispose to an increased risk for atherosclerotic clinical events.

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