Influenza Infection Promotes Macrophage Traffic Into Arteries of Mice That Is Prevented by D-4F, an Apolipoprotein A-I Mimetic Peptide

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Background—We reported that HDL loses its antiinflammatory properties during acute influenza A infection in mice, and we hypothesized that these changes might be associated with increased trafficking of macrophages into the artery wall. The present study tested this hypothesis.

Methods and Results—D-4F, an apolipoprotein A-I mimetic peptide, or vehicle in which it was dissolved (PBS) was administered daily to LDL receptor–null mice after a Western diet and after influenza infection. D-4F treatment increased plasma HDL cholesterol and paraoxonase activity compared with PBS and inhibited increases in LDL cholesterol and peak levels of interleukin-6 after infection. Lung viral titers were reduced by 50% in mice receiving D-4F. Injection of female mice with male macrophages, which were detected with real-time polymerase chain reaction to measure the male Sry gene, revealed a marked increase in macrophage traffic into the aortic arch and innominate arteries after infection that was prevented by administration of D-4F.

Conclusions—We conclude that loss of antiinflammatory properties of HDL after influenza infection in mice is associated with increased arterial macrophage traffic that can be prevented by administration of D-4F. (Circulation. 2002;106:1127-1132.)

Key Words: atherosclerosis ■ infection ■ lipoproteins ■ interleukins

During flu season, hospital admissions increase greatly. Patients are not admitted with influenza infection but are admitted with acute exacerbations of a variety of chronic diseases, including atherosclerosis. Several studies suggest that acute respiratory-tract infections contracted shortly before the onset of an acute myocardial infarction played a role in the clinical event.1–3

We recently reported that after influenza infection in mice, HDL lost its antiinflammatory properties.4 As a result, the ability of HDL to protect against LDL oxidation in vitro was lost. Because the single most important determinant of plaque vulnerability to rupture is the intensity of monocyte/macrophage infiltration into the plaque,5–8 we hypothesized that alterations in HDL during an acute-phase response might predispose to increased risk for atherosclerotic clinical events by increasing monocyte/macrophage trafficking into arteries.

Patel and coworkers9 previously described an in vivo model that demonstrated homing of peritoneal macrophages labeled with fluorescent microspheres to atherosclerotic plaques. Kim et al10 recently developed a highly sensitive method based on the polymerase chain reaction (PCR) to quantitatively track DNA-marked monocytes in mice. In the present study, using a combination of the techniques of Patel et al9 and Kim et al,10 we observed that during influenza infection in LDL receptor–null mice after a Western diet, there was a dramatic increase in macrophage infiltration into the aortic arch and innominate artery that was prevented by daily injection of an apolipoprotein A-I (apoA-I) mimic peptide, D-4F.11

Methods

Mice

Mice used in these studies were 8 to 10 weeks old (Jackson Laboratory, Bar Harbor, Maine). Female LDL receptor–null mice
on a C57BL/6J background were maintained on a Western diet (Teklad; diet No. 88137) containing 0.15% cholesterol to induce fatty streak formation. After 2 weeks, animals were switched to Purina chow diet (Ralston Purina Co). Mice were fasted for 16 hours before being bled as described, and core body temperatures were monitored (Vicks Comfort-Flex Digital Thermometer; Proctor & Gamble Co).

**Influenza Inoculation**
Mice were inoculated nasally with influenza virus strain A/WSN/33 as previously described.

**Lipoproteins, Cocultures, Monocyte Chemotaxis**
Lipoproteins, cocultures, and monocytes were prepared and monocyte chemotaxis assays were performed as described.

**Preparation of T Cells**
Jurkat clone E6-1 cells were obtained from ATCC and maintained in RPMI 1640 medium with 2 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, and 1.0 mmol/L sodium pyruvate, plus 10% FBS. Cells were activated for 6 hours by addition of *Phaseolus vulgaris* leukophytohemagglutinin (1 μg/mL; Sigma) and phorbol myristate acetate (5 ng/mL; Sigma). Cells were washed 3 times in PBS, fixed with 1% paraformaldehyde for 2 hours at 4°C, and again washed 3 times before use.

**Synthesis and Administration of ApoA-I Mimetic Peptide**
In preliminary studies, we found that injection of human apoA-I prevented macrophage trafficking into the arteries of mice much less than did injection of an apoA-I mimetic peptide, D-4F. D-4F was synthesized as described. Mice were injected intraperitoneally with 20 μg of D-4F in 200 μL PBS or PBS alone daily, or PBS with 50 μg serum albumin. No difference was seen with PBS alone or PBS plus serum albumin. A peptide containing the same number of D-amino acids as in D-4F but in a scrambled sequence did not bind lipids and was used as a control in some in vitro studies.

**Histological Analysis**
At the time the mice were euthanized, lungs were perfused with 10% buffered formalin through the trachea. Microscopic analysis of complete sections of entire lungs revealed 3 different patterns of inflammation: perivascular chronic inflammatory cell infiltrates; acute pneumonia (infiltrates of neutrophils) in alveolar spaces; and lymphoid hyperplasia, circumscribed interstitial aggregates of lymphoid cells. Each of these 3 patterns was graded as to severity by a blinded observer.

**Macrophage Preparation**
Peritoneal macrophages were prepared as described by Patel et al. Forty-eight hours before euthanasia, female LDL receptor-null mice were injected with 10^6 peritoneal macrophages from male donors in a volume of 200 μL saline via tail vein. After euthanasia, the mice were exsanguinated, the heart was perfused, and the aortic arch including the innominate artery was dissected and removed. Tissue DNA was extracted with a Qiagen DNeasy Tissue Kit (Qiagen).

**Real-Time PCR to Quantitatively Track DNA-Marked Macrophages**
DNA was quantified by real-time PCR on an ABI Prism 7700 Sequence Detector (Taqman, Perkin-Elmer Applied Biosystems) according to Kim et al. Sense and antisense primers used to amplify the Sry gene and the fluorescent probe were identical to those reported by Kim et al. As an external standard, β-globin was cloned with a sense primer corresponding to nucleotides 2998 to 3019 (5’-CATGGGTATGCGGAAGTGAAG-3’) and an antisense primer corresponding to nucleotides 3051 to 3070 (5’-CAAGTGAATTAAAGCATCTG-3’). The probe for β-globin corresponded to nucleotides 3021 to 3046 (5’-TCCATGGCAAGAGTTGCTAC-3’). The quantity of male DNA in recipient female aortic arch and innominate artery was calculated and normalized to the amount of β-globin. To evaluate the effects of cytokines on arterial macrophage trafficking, 200 ng each of mouse recombinant tumor necrosis factor-α and interleukin (IL)-6 (Biosource) was injected intraperitoneally in a total volume of 0.5 mL saline containing 1% BSA 30 minutes after injection of macrophages.

**Other Procedures**
Paraoxonase activity was measured as previously described. Plaque assays were used to determine lung viral titers. Paraoxonase activity was measured as previously described. Plaque assays were used to determine lung viral titers.
IL-10, and granulocyte-macrophage colony–stimulating factor (GM-CSF) were measured with ELISA kits (Biosource). Lipoprotein cholesterol concentrations were determined with a Cholesterol-20 kit (Sigma). Ouchterlony and Dot Blot analyses were used for detection of plasma IgG and IgM antibodies to D-4F. Statistical significance was determined with model I ANOVA, and significance was defined as a value of $P < 0.05$.

Results

D-4F Treatment Inhibits the Inflammatory Response of the Lung to Influenza Infection, Maintains Body Temperature, and Improves Sickness Behavior

All 3 major classes of lung inflammation were more severe in control mice (Figure 1). Only control mice, not D-4F–treated mice, demonstrated lymphoid hyperplasia (Figure 1C). In contrast to humans, core body temperature falls after influenza infection in mice of most strains. In control mice, core body temperatures fell 2 days after influenza infection. In mice treated with D-4F, however, there was no significant change in core body temperatures over the course of the experiment (data not shown). Consistent with the fall in core body temperature, control mice remained huddled together after influenza infection, whereas mice that received D-4F did not (data not shown).

D-4F Treatment Suppresses the Cytokine Response to Influenza Infection

In mice treated with D-4F, peak IL-6 levels in lungs 7 days after inoculation were half those of control mice (Figure 2A). D-4F treatment suppressed plasma IL-6 concentrations at all time points measured (Figure 2B). Neither IgG nor IgM antibodies to D-4F were detected (data not shown).

D-4F Treatment Alters LDL and HDL During Influenza Infection

The acute-phase response includes alterations of plasma lipoproteins. D-4F treatment significantly inhibited the increase in LDL cholesterol observed 9 days after inoculation.
additions, N/A, hatched bar) or were incubated with 250 μg/mL mouse HDL for 18 hours. Line on x axis, LDL + HDL, represents cultures receiving both LDL and HDL, and numbers on x axis above this line represent day after inoculation on which HDL was collected. Supernatants were collected, cocultures were subsequently washed, and fresh culture medium 199 (M199) without any additions was added for an additional 8 hours. At end of incubation, supernatants were collected from cocultures, diluted 40-fold, and assayed for monocyte chemotactic activity as described in Methods. Solid bars represent data for control mice that received vehicle, PBS; open bars, mice that received D-4F. Values shown are mean±SD of numbers of monocytes in 36 fields in 12 cocultures for each treatment. *P<0.05 between PBS treatment and D-4F treatment. Results shown are representative of 3 separate experiments.

Figure 5. D-4F treatment prevents HDL from becoming proinflammatory after influenza infection. Forty-nine mice were divided into 7 groups of 7 mice each and inoculated intranasally with 10^5 PFU of influenza A strain WSN/33. After indicated number of days, HDL was isolated as described in Methods. Artery wall cocultures were incubated in absence of additions (no additions, N/A, hatched bar) or were incubated with 250 μg/mL human LDL in absence (LDL alone, hatched bar) or presence of 350 μg/mL mouse HDL for 18 hours. Line on x axis, LDL + HDL, represents cultures receiving both LDL and HDL, and numbers on x axis above this line represent day after inoculation on which HDL was collected. Supernatants were collected, cocultures were subsequently washed, and fresh culture medium 199 (M199) without any additions was added for an additional 8 hours. At end of incubation, supernatants were collected from cocultures, diluted 40-fold, and assayed for monocyte chemotactic activity as described in Methods. Solid bars represent data for control mice that received vehicle, PBS; open bars, mice that received D-4F. Values shown are mean±SD of numbers of monocytes in 36 fields in 12 cocultures for each treatment. *P<0.05 between PBS treatment and D-4F treatment. Results shown are representative of 3 separate experiments.

Figure 6. A, Effect of cytokines on macrophage trafficking. Forty-eight hours before euthanasia, 5 female C57BL/6J mice on a chow diet (BL/6 CHOW), 5 female LDL receptor–null mice on a Western diet (LDL-R−/− WEST), and 5 female LDL receptor–null mice on a Western diet injected with cytokines (LDL-R−/− WEST + CYT) were infused via tail vein with 10^10 macrophages from male donors. At euthanasia, heart was perfused, aortic arch and innominate artery were removed by dissection, and contents of Sry DNA were determined as described in Methods. B, D-4F treatment prevents influenza-induced macrophage trafficking into aortic arch and innominate arteries of mice. Forty-nine mice were divided into 7 groups of 7 mice each and inoculated intranasally with 10^5 PFU of influenza A strain WSN/33. Forty-eight hours after indicated number of days, female mice were infused via tail vein with 10^10 macrophages from male donors. On indicated day, mice were euthanized, hearts perfused, aortic arch and innominate artery were removed by dissection, and contents of Sry DNA determined as described in Methods. Solid bars represent data for control mice that received vehicle, PBS; open bars, mice that received D-4F. Results are mean±SD and are representative of 2 separate experiments. *P<0.05.

D-4F Inhibits Influenza-Induced Macrophage Trafficking Into the Aortic Arch and Innominate Arteries of Mice

Female mice were injected with male macrophages, and the uniquely male gene Sry was used to measure the arterial association of Sry DNA as an indicator of macrophage recruitment into the artery wall. As demonstrated in Figure 6A, and as Patel et al. had shown previously, macrophages were very effective in homing to arteries of hypercholesterolemic mice. Compared with C57BL/6J mice on a chow diet, LDL receptor–null mice on a Western diet showed a 3-fold increase in macrophage recruitment into the aortic arch and innominate artery. Consistent with the findings of Kim et al., injection of tumor necrosis factor-α and IL-6 at 30 minutes after the injection of donor macrophages resulted in a 25-fold increase in macrophage recruitment into the aortic arch and innominate arteries (Figure 6A). Figure 6B demonstrates that influenza infection in control mice resulted in a marked and progressive increase in Sry DNA in the aortic arch and innominate arteries (left), which was not seen in mice treated with D-4F (right).

D-4F Has Antiviral Activity

Based on previous studies in vitro, we measured viral titers in lungs from PBS- or D-4F–treated mice before and at
2, 3, 5, 7, and 9 days after inoculation (Figure 7). As had been seen previously in wild-type C57BL/6J mice on a chow diet, peak viral titers were achieved 3 days after inoculation and then decreased, returning toward baseline by 9 days. In mice treated with D-4F, viral titers were reduced by 50% compared with controls at all time points.

**D-4F Alters the Contact-Mediated Activation of Monocytes by T Cells**

IL-6 is a proinflammatory cytokine and is strongly induced in monocytes after direct contact with stimulated T lymphocytes. IL-10 is a potent inhibitor of monocyte/macrophage function, suppressing production of many proinflammatory cytokines. Hyka and colleagues reported that human serum contained an inhibitory activity for the contact-mediated activation of monocytes by stimulated T cells. This inhibitory activity was found to be caused by apoA-I. To determine whether D-4F might be acting similarly to apoA-I, freshly isolated human monocytes were incubated for 48 hours with activated T cells in the absence or presence of increasing concentrations of D-4F, and the release of IL-6 and IL-10 into the medium was measured (Figure 8). As shown in Figure 8A, direct contact of monocytes with activated T cells resulted in a marked increase in IL-6 released into the tissue culture medium, which was inhibited in the presence of D-4F. In contrast, D-4F had a stimulatory effect on IL-10 release under the same conditions (Figure 8B). Sugiyama and co-workers demonstrated that GM-CSF is a potent endogenous mediator during monocyte/macrophage activation and differentiation. Figure 8C shows that D-4F, in addition to inhibiting IL-6 release, was shown to inhibit the contact-mediated release by monocytes of GM-CSF. No inhibition was observed when a scrambled peptide to D-4F was used at the same concentration (Figure 8C).

**Discussion**

Our results demonstrate for the first time that in mice, influenza infection can lead to a marked increase in arterial macrophage trafficking. Based on previous work by Kim et al., we chose to use the highly sensitive technique of real-time PCR for these studies, coupled with the technique for studying macrophage homing to the artery wall described by Patel et al. Our results indicate that this combination of techniques is highly effective for studying arterial macrophage trafficking (Figure 6). If the observations in this study hold true for humans as they do for mice after influenza infection, they may provide a new strategy for the treatment of atherosclerosis.
infection, our data might provide an explanation for increases in heart attack and stroke after influenza infection. Gurfinkel et al.\(^2\) recently suggested that influenza vaccination might reduce the risk of death and ischemic events in patients suffering from infarction and those recovering from angioplasty during flu season. D-4F had antiviral activity in vivo (Figure 7), as was observed in vitro with other apoA-I mimetic peptides,\(^2,4\) and also resulted in a cytokine profile in vitro that would be predicted to reduce macrophage activation in vivo\(^2,7,20\) (Figure 8). These results demonstrate that D-4F may be acting through multiple pathways. Taken together with previous reports,\(^4,11,15,22–24\) our results suggest that apoA-I and apoA-I mimetic peptides such as D-4F are potent antiinflammatory agents that may have therapeutic potential.

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