D-4F, an Apolipoprotein A-I Mimetic Peptide, Inhibits the Inflammatory Response Induced by Influenza A Infection of Human Type II Pneumocytes

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Background—Evidence suggests that apolipoprotein A-I (apoA-I) and HDL play important roles in modulating inflammation. We previously reported that an apoA-I mimetic peptide, D-4F, reduced inflammatory responses to influenza virus in mice. To further define the antiinflammatory activity of D-4F, a human alveolar type II cell line, A549, was used.

Methods and Results—Cells were either uninfected or infected with influenza A in the presence or absence of D-4F. Cells treated with D-4F were more viable, and virus-induced cytokine production was suppressed by D-4F. Caspases associated with cytokine production were activated after infection but suppressed by D-4F treatment. Infected A549 cells showed dramatic increases in cellular phospholipid secretion into the media. When infected cells were incubated with D-4F, secretion of parent nonoxidized, noninflammatory phospholipids was unaltered, but production of proinflammatory oxidized phospholipids was inhibited.

Conclusions—Type II pneumocytes respond to influenza A infection by activating caspases and secreting cytokines and cellular phospholipids into the extracellular environment, including oxidized phospholipids that evoke inflammatory responses. D-4F treatment inhibited these events. Our results suggest that apoA-I and apoA-I mimetic peptides such as D-4F are antiinflammatory agents that may have therapeutic potential. (Circulation. 2004;110:3252-3258.)

Key Words: apolipoproteins ■ atherosclerosis ■ infection ■ lipoproteins ■ epithelium

We previously reported that after influenza infection in mice, HDL lost its antiinflammatory properties.1 As a result, the ability of HDL to protect against LDL oxidation in vitro was lost. In subsequent studies using LDL receptor–null mice fed a Western diet and then infected with influenza virus, 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, D-4F.2 Moreover, D-4F treatment increased plasma HDL cholesterol and paraoxonase activity, reduced lung viral titers by 50%, and inhibited increases in LDL cholesterol and levels of interleukin-6 (IL-6) compared with controls after influenza infection.

To investigate in more detail the mechanism(s) by which D-4F suppresses the inflammatory processes after influenza infection, for the present studies we chose an in vitro model system employing a human type II pneumocyte cell line, A549. After influenza infection, A549 cells treated with D-4F were more viable at all time points studied, and virus-induced IL-6 production was suppressed at picomolar concentrations of D-4F. The marked increases in the activities of caspases and interferon (INF)-α/β observed after viral infection were also reduced with D-4F treatment. In addition, infecting A549 cells with influenza A virus resulted in dramatic increases in cellular phospholipid secretion into the media including nonoxidized, noninflammatory arachidonic acid–containing phospholipids, as well as cell-derived oxidized phospholipids that induce monocyte chemoattractant-1 production. However, when infected cells were incubated with D-4F, secretion of parent nonoxidized, noninflammatory phospholipids was not altered, but production of proinflammatory oxidized phospholipids was inhibited. Cellular viral titers increased dramatically 48 hours after infection, but treatment of cells with D-4F reduced titers by 50%. These results in a human pneumocyte cell line, taken together with our previous in vivo findings in mice, demonstrate the therapeutic potential of apoA-I mimetics and suggest that these peptides may be exerting their effects at the earliest steps in the sequence of events after viral infection.
Methods

Cell Culture and Experimental Preparation

Human type II pneumocytes (A549) were purchased from ATCC. Cells were propagated in medium containing Ham’s F12K in 10% FBS with 2 mmol/L l-glutamine adjusted with sodium bicarbonate. In preparation for experiments, type II pneumocytes were plated at a concentration of 1×10^5 cells per well in 12-well plates and grown to 85% confluence before the experiment began. Before the experiment, cells were infected with influenza A virus strain WSN/33 at a multiplicity of infection (MOI) of 0.1 in a volume of 100 μL for 1 hour with gentle agitation. Cells were washed 3 times in PBS and then switched to Ham’s F12K in 2% FBS with 2 mmol/L l-glutamine adjusted with sodium bicarbonate (experimental medium) for the duration of the experiment.

Peptides

D-4F was synthesized as described. A peptide containing the same number of D-amino acids as in D-4F but in a scrambled sequence that does not bind lipids, scD-4F, was used as a control in some of the studies. Sixteen hours before the experiment, peptides were preincubated with cells in experimental medium. At the end of this time, the cells were washed 3 times before viral infection. After viral infection, peptides were coincubated with cells in experimental medium for the course of the experiment.

Other Procedures

Analysis of phospholipids was performed as described previously.5 Caspase activity was measured as described.6,7 Plaque assays were used to determine cell viral titers.8 The concentrations of IL-6 and IFN-α/β were measured by ELISA (Biosource). Lipoprotein cholesterol concentrations were determined with the use of a cholesterol-20 kit (Sigma). Statistical significance was determined with the use of model I ANOVA, and significance was defined as P<0.05.

Results

Production and Release of Oxidized Phospholipids From A549 Cells Are Inhibited by D-4F

Stocker and colleagues reported rates of superoxide anion radical generation by lung cells to be 70-fold higher than in cells from control animals after infection of mice with influenza virus. Oxidized phospholipids derived from HDL and LDL may be part of a system of nonspecific innate immunity.10,11 Our laboratory identified several oxidized phospholipids present in mildly modified/oxidized LDL that are able to induce genes and proteins necessary for the cellular response seen in fatty streaks.11 These oxidized phospholipids are designated 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphorylcholine (POVPC), 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphorylcholine (PGPC), and 1-palmityl-2-(5,6-epoxyprostane E2)-sn-glycero-3-phosphorylcholine (PEIPC). In experiments shown in Figures 1 and 2, we asked whether influenza infection would result in the enhanced production of any or all of these oxidized phospholipids and, if so, whether this response would be altered by D-4F treatment. It was found that uninfected control cells had constitutive levels of all 3 oxidized phospholipids in addition to the parent, nonoxidized phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) (Figure 1). Treatment of uninfected cells with D-4F had no effect on either cellular content (Figure 1) or release into the media (Figure 2) of POVPC, PGPC, or PEIPC. Infected cells showed marked increases in both cellular content and the release into the media of POVPC, PGPC, and PEIPC as well as the parent, nonoxidized phospholipid PAPC (Figures 1 and 2, respectively). D-4F treatment reduced both the viral-induced production and release of POVPC, PGPC, and PEIPC but not of PAPC.

D-4F Inhibits the Viral Induction of IFNs

IFNs are considered the body’s first line of antiviral defense. They are cytokines secreted in response to virus infection and block viral replication at many levels. The global antiviral state of the cell involves cross-talk between IFN signaling and pathways regulating apoptosis, inflammation, and cellular stress-response pathways.12,13 We postulated that D-4F may alter responses to influenza infection by modulating the cellular IFN system. In Figure 3, infected or uninfected A549 cells were treated with or without D-4F for 8, 24, or 48 hours, and the activities of IFN-α and IFN-β were determined by ELISA. No IFN activity was observed in uninfected cells, but by 24 hours after infection, IFN activity could be seen for both α- (top) and β-isoforms (bottom). This response was more marked at 48 hours after infection. D-4F treatment suppressed both IFN-α and IFN-β activities at 24 and 48 hours after infection.

D-4F Treatment Inhibits Caspase Activation and Cytokine Production After Viral Infection

The results from Figure 3 suggest that D-4F may be exerting its effects at another of the earliest stages of infection/inflammation. Influenza A virus can control posttranslational events involved in the production of cytokines. Many cytokines are expressed as inactive proforms and after proteolytic cleavage by caspase enzymes become biologically active. The apoptosis induced by influenza virus can be characterized by activation of caspase cascades.14 Multiple apoptotic signals may first activate initiator caspases such as caspase-8 and -9, which is followed by activation of downstream activator caspase-3, -8, or -6. During apoptosis, caspase-3 has multiple substrates, including structural and regulatory proteins, and has been considered the major caspase regulating apoptosis. Recent studies have shown that caspase-3 and -8 are highly activated during influenza A virus infection. One could speculate, from these observations as well as from our previous studies,1,2 that D-4F inhibits the cytokine cascade via effects on caspase activation. Therefore, we conducted the experiments shown in Figures 4 and 5 to test whether D-4F treatment alters caspase-dependent production of IL-6. In Figure 4, A549 cells were either not infected or infected with influenza virus in the absence or presence of D-4F for 8 (top), 24 (middle), or 48 hours (bottom). At the end of these time points, caspase-3, -8, and -9 activities were measured. Eight hours after infection (Figure 4, top), caspase-8 activity was the same in all treatment groups. However, in infected cells, caspase-3 was activated and caspase-9 activity increased 4-fold. D-4F treatment suppressed activities of caspase-3 and -9. By 24 hours after infection, D-4F treatment had suppressed caspase-8 activity in both uninfected and infected cells (Figure 4, middle). Caspase-3 activation, observed only in infected cells, was again suppressed by D-4F treatment. D-4F treatment also inhibited caspase-9 activity in uninfected cells at 24 hours. At 48 hours after infection (Figure 4,
bottom), caspase-9 activity was low in all cells. In infected cells, however, caspase-8 activity had doubled and caspase-3 activity was enhanced 60-fold, and D-4F treatment of cells suppressed both caspase-8 and caspase-3 activities.

Influenza A virus–infected epithelial cells respond to infection by producing proinflammatory and other immunoregulatory cytokines. Because cytokine maturation and activation depend on proteolytic cleavage by caspase enzymes to become biologically active, we reasoned that the marked inhibition of caspase-3 activity in A549 cells by D-4F would affect the production/release of IL-6 and may explain previous in vivo observations.1,2 The experiment in Figure 5 demonstrated that compared with uninfected A549 cells, viral infection resulted in a dramatic increase in IL-6 concentrations in tissue culture media. However, if cells were incubated with as little as 25 ng/mL of D-4F, concentrations of IL-6 in the media were reduced.

**D-4F Inhibits Viral Replication in A549 Cells**

Productive influenza A virus infection in epithelial cells destroys host cell pre-mRNAs, inhibits translation of cellular mRNAs, and kills host cells by either cytolytic or apoptotic mechanisms.16 Accumulation of viral proteins and mRNAs resulting from viral replication can serve as apoptotic signals activating initiator caspases such as caspase-8 and caspase-9.14 On the basis of results from Figures 4 and 5 showing that D-4F treatment inhibited caspase activation and IL-6 production in A549 cells, we asked whether D-4F acts on an even earlier upstream event, that of influenza virus replication. In Figure 6, A549 cells either were uninfected or were infected for 4, 8, 24, or 48 hours in the absence of peptides or in the presence of D-4F or a scrambled peptide to D-4F, scD-4F, which does not bind lipid. As Figure 6 shows, viral replication was barely detectable at 8 hours, but at 24 hours viral replication was marked. At 48 hours after infection, viral replication had increased 4-fold. D-4F treatment reduced viral titers by 50% at 48 hours after infection, but a scrambled peptide unable to bind lipids had no significant effect in reducing viral replication.

**Discussion**

Compelling evidence has accumulated suggesting that certain infectious agents contribute to the course of atherosclerosis. In influenza infection, reactive oxygen species are released...
into extracellular spaces by inflammatory and airway epithelial cells. The source of the reactive oxygen species may be the epithelial cells of the lungs themselves and specifically type II pneumocytes. Reactive oxygen species can act as molecular triggers of various inflammatory processes. They can directly attack biological membranes and stimulate arachidonic acid metabolism with increased production of prostaglandins and thromboxanes. In the present study we observed in a human type II pneumocyte cell line that influenza infection resulted in the formation and release into the media of 3 oxidized phospholipids derived from oxidation of arachidonic acid–containing phospholipids: POVPC, PGPC, and PEIPC (Figures 1 and 2). Treatment of these cells with D-4F, however, suppressed the increased formation and release of these oxidized phospholipids (Figures 1 and 2). Treatment of these cells with D-4F, however, suppressed the increased formation and release of these oxidized phospholipids (Figures 1 and 2). ApoA-I mimetic peptides, such as D-4F, by virtue of their ability to avidly bind lipids, may be important in binding and deactivating lipid oxidation products, reducing their cellular and media concentrations (Figures 1 and 2). Interestingly, greater amounts of parent nonoxidized phospholipid, PAPC, were also observed after influenza infection. D-4F had no effect on PAPC formation (Figures 1 and 2). In the respiratory tract, type II pneumocytes are responsible for surfactant production, which may increase on exposure to virus and could explain the increase in the cellular content of PAPC (Figure 1). It may also be that during influenza infection there is a generalized loss of cellular lipid or “blebbing” of cell membranes, as a part of the process for viral clearance, contributing to the increase in culture media PAPC observed after influenza infection (Figure 2). Preserving these cellular defense phenomena is consistent with an antiinflammatory effect of D-4F.

Infection-induced increases in circulating cytokines may constitute one mechanism by which infection contributes to atherogenesis. IFN-α/β is a major antiviral cytokine system, which also has antiproliferative and immunomodulatory functions. In our study we observed marked increases in IFN-α/β, particularly at 48 hours after infection (Figure 3). Although one might have predicted that a protective effect of D-4F on influenza virus infection would have included an enhancement of the IFN-α/β system, we observed a reduction rather than a stimulation of IFN-α/β in cells treated with D4-F. From the results in Figure 3, it would seem likely that A549 cells had already mounted a response to the virus, and D-4F treatment acted to suppress the initial response to infection, thus blunting the resulting IFN response.
Upregulation of caspase activity is a hallmark of influenza A virus infection. Virus-activated caspases are involved in the maturation of many inflammatory cytokines. In our study caspase-9 but not caspase-8 activity was upregulated at 8 hours after viral infection (Figure 4, top). Caspase-3 was activated in infected cells at this time as well. By 24 hours after infection, levels of caspase activity were unchanged (Figure 4, middle); however, by 48 hours after viral infection, whereas caspase-9 activity had diminished, caspase-8 activity had doubled, concomitant with a 60-fold increase in caspase-3 activity (Figure 4, bottom). These results are consistent with a “2-step” approach whereby the activation of initiator caspase-8 and -9 cleaves the proform of effector caspase-3 to its active form. D-4F treatment was effective in inhibiting this process, possibly through initially inhibiting caspase-9 activity and then by inhibition of caspase-8 activity. As we had also observed in vivo,1,2 IL-6 levels were markedly increased by influenza infection in A549 cells (Figure 5). Because cytokine regulation is highly dependent on caspase activity, one would predict that an inhibition of caspase activation would have significant effects on cytokine production. Indeed, D-4F treatment of cells, which suppressed caspase activation, also inhibited the IL-6 secretion into the media of infected A549 cells (Figure 5). It is possible that the inhibition of cytokines previously seen in mice...
resulted in reductions in lung viral titers,2 we asked whether we had previously observed in mice that D-4F treatment to activate downstream effectors such as caspases. Because lytic cleavage of viral glycoproteins, which in part can serve caspase activation by D-4F.

Oxidative stress has been more marked; however, treatment of cells with D-4F was able to suppress the viral titers by 50%. Oxidative stress has been implicated in the pathogenesis of a number of viral diseases.9,23 The observation that a scrambled peptide to D-4F that is unable to bind lipids was ineffective at inhibiting viral titers (Figure 6) suggests that the ability to bind lipids, and particularly their oxidation products, may be important in suppressing viral replication. Thus, sequestration by D-4F of the free radicals generated after influenza infection may retard the progression of viral replication, which in turn could affect the activation and induction of the inflammatory cytokine cascade. Our studies demonstrate that apoA-I mimetic peptides, in addition to improving the inflammatory properties of HDL and reducing atherosclerotic lesions in mice,2,4 also possess antiviral activity. Moreover, apoA-I mimetic peptides may hypothetically reduce the risk of cardiovascular events in humans by increasing the resistance to influenza infections.

Acknowledgments
This work was supported in part by UPS grants HL 30568 and HL 34343 and by the Laubisch, Castera, and M.K. Gray Funds at UCLA.

Disclosure
Dr Navab, Anantharamiaiah, and Fogelman are principals in Bruin Pharma, a start-up biotech company.

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Circulation. published online November 8, 2004;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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