Influenza Infection Exerts Prominent Inflammatory and Thrombotic Effects on the Atherosclerotic Plaques of Apolipoprotein E–Deficient Mice

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Background—The role of infection in the development and complications of atherosclerosis has been the focus of much attention. We reported previously that influenza vaccination was associated with reduced risk of recurrent myocardial infarction. Here, we report the effect of influenza A virus on the apolipoprotein E–deficient (apoE−/−) mouse, an animal model of atherosclerosis.

Methods and Results—Twenty-four apoE−/− mice >24 months old were injected with 1 LD50 (lethal dose 50) of influenza A virus. Ten wild-type C57BL/6 infected mice and 11 noninfected age-matched apoE−/− mice served as controls. Multiple aortic sections were studied histologically 3, 5, and 10 days later. The infected mice showed markedly increased intimal cellularity compared with the noninfected apoE−/− mice. No aortic abnormalities were seen in infected wild-type mice. Ten infected apoE−/− mice had a significant subendothelial infiltrate composed of a heterogeneous group of cells that stained positively for smooth muscle cell actin, F4/80 (macrophages), and CD3 (T lymphocytes). One case of subocclusive platelet and fibrin-rich thrombus was seen.

Conclusions—This study shows that influenza infection promotes inflammation, smooth muscle cell proliferation, and fibrin deposition in atherosclerotic plaques. (Circulation. 2003;107:762-768.)

Key Words: influenza • atherosclerosis • inflammation • thrombosis • plaque

In the 1860s, Virchow recognized the role of inflammation in atherosclerosis; however, it was Minick et al1 in 1966, who showed experimental immunologic arterial injury in atherogenesis. Ever since, a growing body of evidence has indicated the central role of inflammation and immune activation in the pathogenesis of atherosclerosis and its thrombotic complications.2 Proposed antigens include oxidized LDL, infectious agents (Chlamydia pneumonia, herpes virus, and cytomegalovirus), and heat-shock protein 60.

The role of infection in atherosclerosis has been suggested since early in the 20th century.3 In the 1970s, Burch et al4 and Fabricant et al5 separately described virus-induced atherosclerosis. Since then, this concept has attracted attention and controversy.6,7 Respiratory infections have been proposed to cause increased cardiovascular mortality in the cold months.8 In a large case-control study, patients with myocardial infarction (MI) had significantly more acute respiratory-tract infections in the 10 days before the index date of MI than the control subjects in the same time period.9 A possible relationship between influenza and MI was first suggested after epidemics of influenza struck Europe and the United States in the early 1900s. In those epidemics, approximately half of the excess mortality was attributed to causes other than influenza, primarily heart disease.10 One prospective study11 and several retrospective studies have suggested that acute respiratory infection might trigger MI.12,13

We reported previously in a case-control study that vaccination against influenza was negatively associated with the development of recurrent MI.14 Another recent study found a significant decrease in the sudden cardiac death rate in people receiving influenza vaccine.15 Recently, Lavallee et al16 showed reduction in brain infarction among patients vaccinated against influenza.

Therefore, we hypothesized that influenza infection may play a role in the complications of atherosclerotic plaque in patients with established atherosclerosis. We chose the apolipoprotein E–deficient (apoE−/−) mouse model because it offers a practical model for studying atherosclerosis. More-
over, the mouse influenza model is an accepted model for studying experimental influenza virus infection.

Methods

Lethal Dose Titration

Because influenza infection has never previously been studied in apoE−/− mice, we first had to determine the median lethal doses (LD50) in these transgenic mice. In a series of studies in which >250 apoE−/− and wild-type mice were used, mice were inoculated with serial dilutions of influenza A/Hong Kong/68 (H3N2) virus intranasally. It was determined that the LD50 of this virus for apoE−/− mice was ~50 median tissue culture infectious doses (TCID50). When the mice were inoculated with this dose of virus, maximum virus titers (ie, 7.3 log10/g lung) occurred on day 4 after virus infection. Moreover, it was established that these animals developed overt pulmonary infection and disease similar to that seen in wild-type mouse strains.

Animal Study

Twenty-four apoE−/− mice of either sex bred in our lab, 2 to 2.5 years old, were fed water and normal mouse chow diet ad libitum. They were not kept in specific pathogen-free conditions. However, the combined load of infection and treatment over their life spans has been similar between the infected and noninfected groups because they have been kept in the same place, under the same conditions, and on the same protocol until they were randomly assigned to the infected and control groups at the beginning of the study. The mice were lightly anesthetized with isoflurane (Abbott) and then inoculated intranasally with 102 TCID50 of the influenza A/HK/68 virus. Ten inoculated C57BL/6 mice and 11 noninfected apoE−/− mice were used as controls. The protocol was approved by the Animal Welfare Committees of the University of Texas–Houston Health Science Center and Baylor College of Medicine.

The mice were weighed at the start of the experiment before infection, on days 3 and 5, and on the day they were killed. Heart rate and O2 saturation were measured simultaneously by pulse oximetry machine (Surigvet, Sims BCI, Inc). The animals were killed with air saturated with CO2. The mice were scheduled for euthanization at days 3, 5, and 10. However, mice that died before these dates were autopsied on the day of death.

Lung Virus Quantification

Pulmonary virus titers in 6 mice were determined on days 3 (2 mice) and 5 (4 mice) after virus inoculation. At this time, the mice were killed with CO2, and their lungs were removed and individually homogenized. Each homogenate was serially diluted (0.5 log10 dilutions) with minimal essential medium without FCS but containing 2 μg/mL Worthington trypsin (Trypsin-TPCK, Millipore Corp). The diluted lung suspensions were transferred to 96-well tissue culture plates (Falcon 3077) containing monolayers of Madin-Darby canine kidney (MDCK) tissue cells (American Type Culture Collection; catalog No. ATCC CCL 34). After incubation for 5 days at 37°C, 0.05 mL of a 0.5% suspension of chicken erythrocytes was added to each well. Wells exhibiting hemagglutination were considered positive for the presence of virus but not from any of the control animals not inoculated with virus. The viral titer of the animals harvested on day 3 after inoculation was greater than 7.3 log10 TCID50/g of lung, and that of those harvested on day 5 after inoculation ranged between 6.8 and 7.3 log10 TCID50/g of lung. These data suggest that the attack rate in the virus-inoculated mice was 100%.

Pathological Findings

Of the 24 mice experimentally inoculated with influenza A/HK/68 virus, 13 died of infection between days 3 and 10, and 11 were killed at days 3 (n=2), 5 (n=4), and 10 (n=5) after inoculation. All the inoculated animals lost substantial weight (30.6±5.6 g at baseline versus 24.1±3.3 g at the latest weight before death or euthanasia, P<0.001). Ten infected mice (40%, 6 killed and 4 fatalities) showed striking subendothelial infiltrates between the necrotic core and the endothelium composed of round, ovoid, and spindle cells (Figure 1, A and B). However, despite severe infection and similar weight loss, the intimal infiltration was not found in the wild-type infected C57 mice (Figure 1, E and F). Intimal infiltration was also absent in the noninfected apoE−/− mice (Figure 1, C and D).

The infiltrates were found in 1 or more sections obtained from different areas (root, arch, descending, and abdominal levels). The infiltration was located superficially and closer to the lumen than the core. Most specimens lacked a well-demarcated fibrous cap, a common phenomenon in apoE−/− mice. The deeper cells of the infiltrate were often foamy. In some specimens, an unusual histopathologic picture was seen with a fibrous “cap” deeper in the plaque than the cellular infiltrate, as seen in Figure 2.

When present, the inflammatory and smooth muscle cell infiltrate involved only portions of the atherosclerotic plaque. The nonatherosclerotic intima was never infiltrated (Figure 1A, white arrows).
Most infected animals had clusters of platelets and in some cases, fibrin strands overlying the plaques and the infiltrates, when present. The thin cap typical of these mice was difficult to delineate in areas in which large inflammatory and smooth muscle cell infiltration and platelet clusters were present. One unequivocal case of premortem subocclusive platelet-rich and fibrin-rich thrombus with early organization was observed (Figure 3). Neither the subendothelial infiltrate nor the platelet clusters were observed in the noninfected mice.

The infiltrating cells stained positively for several antibodies, including F4/80 and Kp-1 (macrophages), CD3 (T lymphocytes), and smooth muscle cell actin (Figure 4). The relative proportions of these infiltrates varied between the experiments (see Discussion).

Next, we performed a quantitative assessment of the average cellularity per HPF of each infected mouse that survived >3 days after the infection (n = 20) and compared it with the 3 control groups: (1) age-matched apoE<sup>-/-</sup> noninfected mice; (2) wild-type infected mice; and (3) wild-type noninfected mice. As illustrated in Figure 5, the infected apoE<sup>-/-</sup> mice had 105±40 cells per HPF and the noninfected, 53±9 cells per HPF. Wild-type (C57BL/6) mice infected with the influenza virus had no infiltration in the subendothelial intima at the light microscopic level, nor did the noninfected normal C57BL/6 mice. The difference was statistically significant across all groups (ANOVA, P<0.0001). However, probability values were not significant between the infected and noninfected wild-type (C57BL/6) mice.

**Discussion**

The present study was designed to determine whether influenza A infection induces histopathologic changes in the atherosclerotic lesions of aged apoE<sup>-/-</sup> mice.

**Figure 1.** A, Abdominal aorta with eccentric plaque encompassing ~75% of circumference. There is a cellular infiltrate in subendothelial region over most of involved plaque, particularly concentrated at 5 and 8 o'clock positions. Remainder of plaque is markedly hypocellular. Clusters of platelets are seen close to infiltrate, and center of lumen is occupied primarily by red blood cells. White arrows point to a wall area free of atherosclerosis and with no inflammatory and smooth muscle cell infiltrate (H&E, magnification ×10). B, A higher magnification shows a significant portion of deeper cells to be foamy and mostly oval nuclei to be interspersed with cholesterol clefts. Clusters of platelet-rich thrombi are seen in lumen (H&E, magnification ×40). Mice died 10 days after infection. C, Representative section of noninfected apoE<sup>-/-</sup> (K/O) mice with severe nonconcentric atherosclerosis. There is no well-developed fibrous cap (H&E, magnification ×10). D, Higher magnification highlights paucicellular plaque and lack of fibrous cap. E, Aortic section of infected C57BL mouse killed 10 days after inoculation. Note normal intima (H&E, magnification ×10). F, Higher magnification shows lack of cellularity between endothelium and internal elastic lamina.

**Figure 2.** A, Multilayered intimal infiltrate composed of ovoid and spindle cells with some plump endothelial cells. Clusters of platelets overlie infiltrate (H&E, magnification ×10). B, Cluster of platelets in close contact with cellular infiltrate that is present on surface. Fibrous “cap” is deeper in plaque than cellular infiltrate (H&E, magnification ×40). Mice died 10 days after infection.
We observed that influenza infection in aged (>2 years old) apoE<sup>−/−</sup> mice induced significant infiltration of inflammatory and smooth muscle cells, superficial platelet aggregation, and occasional subocclusive fibrin thrombus. We also found that the nonatherosclerotic (spared) segments of the apoE<sup>−/−</sup> mouse aorta were not affected by influenza infection. To the best of our knowledge, pathological changes like those observed in relation to influenza in the present study have not been reported previously in human or animal atherosclerotic plaques. The fact that only the atherosclerotic plaques were affected and the nonatherosclerotic segments were spared excludes panarteritis. Also, none of the known forms of aortitis seen in aortic wall infections or connective tissue disorders resemble the pathology described in this article.8

Thus, although the numbers are small, the response is clearly uniform and not idiosyncratic.

**Plaque Complications in the apoE<sup>−/−</sup> Mouse**

The apoE<sup>−/−</sup> mouse is a good model of plaque development. However, plaque complications (rupture, intraplaque hemorrhage) are extremely uncommon. Murine plaques are paucicellular and lack cap inflammation, a key element in the development of the vulnerable plaque. Recently, plaque rupture was seen in the innominate artery<sup>19</sup> and was attributed to loss of...
smooth muscle cells. Calara et al reported spontaneous plaque ruptures with superimposed luminal thrombus. Rosenfeld et al reported intraplaque hemorrhage, fibrotic conversion of necrotic cores, and loss of the fibrous cap in the innominate artery. Of note, to the best of our knowledge, no report has implicated cap inflammation as a factor in the plaque complication. The present report shows heavy plaque inflammation and smooth muscle proliferation after influenza infection in the apoE−/− mouse model. The difference in cell counts would have been even more dramatic had we limited the counting to the inner quadrants with infiltration only. Counting the entire circumference “dilutes” the observed effect, because most infiltrates were not circumferential. However, we counted the whole circumference of the artery and still found significant differences in cell numbers. The reason for not counting the entire intima is based on the fact that vulnerable plaques are defined by, among other parameters, cap inflammation. We therefore believe that our count analysis is more relevant to the clinical condition than the counting of the entire plaque.

Supporting Evidence

Although several epidemiological studies have focused on the relationship between influenza infection and cardiovascular
apoE is known to have an immunomodulatory effect in RANTES, monocyte chemotactic protein-1, monocyte chemotactic protein-3, IFN-γ inducible protein 10, etc. The critical role of the above-mentioned cytokines in the development of atherosclerosis and its link to thrombosis have been described by others. Key among the latter is the stimulation of interferon-γ production by natural killer cells and T cells. The cytokines, alone or in combination, lead to exaggerated attraction and adhesion of monocytes to the vessel wall, smooth muscle cell proliferation, and rapid progression of atherosclerosis. Also, release of other T cell–activating and proliferating cytokines such as IL-15 and IL-2 may be involved. Houkamp et al recently showed that IL-15 in atherosclerotic plaques can serve as an alternative pathway for T-cell activation.

Another potential mechanism relates to possible influenza virus–induced deposition of immune complexes in the plaque. The immune complex deposits peak in the lungs 10 days after inoculation and have also been detected in the heart and kidneys. If they were shown to react against epitopes in oxidized LDL or other factor specifically found in atherosclerotic lesions, it could explain the restriction of the inflammatory reaction to the plaque. The apoE−/− mouse is a model of lipoprotein oxidation, and oxidized LDL has been shown to be chemotactic for monocytes and T lymphocytes. Finally, we cannot rule out the possibility that the virus could be present in the plaque. This hypothesis seems unlikely, because only a few studies were able to locate the virus outside the respiratory tract. Moreover, in our preliminary studies to date, we have been unable to find evidence of influenza virus in the plaques of infected apoE−/− mice examined by reverse-transcription polymerase chain reaction (data in preparation).

Other possibilities or contributing factors might include thrombotic diathesis mediated for example by hyperfibrinogenemia and hemoconcentration, endothelial apoptosis, endothelial dysfunction, oxidative stress, exaggerated lipid abnormality, and some other remote possibilities discussed by Naghavi et al.

**Limitations**

A limitation of the present study is that only one dose (LD50) was used. Whether other doses also induce the same response is unknown. However, the LD50 is accepted as the standard method for studying the immunologic response of influenza A virus in mice and is widely used for testing flu vaccine. The emphasis in this study was on the histological findings in the short-term period after virus administration. Important issues (more thorough investigation of the possible presence of the virus in the plaque, evaluation of the cytokines involved, longer-term studies to evaluate the effect of the influenza infection on the development of atherosclerotic plaques in younger mice, possible presence of immune complex in the lesions, immunogenetic factors, etc) are being pursued in our laboratory.

**Need for Future Studies**

In the present study, we examined the short-term effect of influenza infection on aged apoE−/− mice with a severe degree of atherosclerosis. What would have been the most likely outcome had the animals been allowed to survive? Plaque rupture (or erosion) and subsequent healing is recognized to be a major cause of rapid plaque progression. Therefore, it is likely that the influenza infection could increase the plaque burden. The degree of inflammation in the plaque also raises the possibility that a late effect of the
infection might be the development of aortic (or coronary) aneurysm. In young animals, influenza could accelerate the progression of the disease through several of the mechanisms previously discussed.

Implications

Influenza is a significant public health burden. At present, public health policy regarding vaccination is directed primarily toward the elderly and individuals with known cardiopulmonary disease. Unfortunately, sudden death is the first manifestation of coronary artery disease in approximately half of the victims, and broader primary prevention is therefore of paramount importance. This article raises the possibility that primary prevention against coronary artery disease could include vaccination against the influenza virus.

Conclusions

In summary, we show that infection with influenza A virus at doses of 1 LD₅₀ induces significant inflammatory and thrombotic effects in aged apoE⁻/⁻ mice between 7 and 10 days after infection.

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References

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