Herpesvirus Infection Accelerates Atherosclerosis in the Apolipoprotein E–Deficient Mouse

Dagmar G. Alber, PhD; Kenneth L. Powell, PhD; Patrick Vallance, FRCP; David A. Goodwin, BSc; Cairistine Grahame-Clarke, MRCP

Background—Human herpesviruses have been implicated but not proven to be involved in the etiology of atherosclerosis. To determine whether there is a causal relationship, the effect of herpesvirus infection on the development of atherosclerosis was assessed in the apolipoprotein E–deficient (apoE−/−) mouse.

Methods and Results—In the present study, 3- to 4-week-old apoE−/− mice were infected with murine γ-herpesvirus-68 (MHV-68). Atheroma formation was accelerated over a 24-week period in infected apoE−/− mice compared with control uninfected apoE−/− mice. Acceleration of atherosclerosis was reduced by antiviral drug administration. Histological analysis of the atheromatous plaques showed no difference between lesions of infected and control mice. Viral mRNA was present in the aortas of infected mice before lesion development on day 5 after infection. This suggests that the virus may initiate endothelial injury, which is believed to be an early event in the development of atherosclerosis. Therefore, the virus may play a direct role in atherosclerosis rather than be an “innocent bystander.”

Conclusions—These data demonstrate that a γ-herpesvirus can accelerate atherosclerosis in the apoE−/− mouse. This study provides the first report of a murine model in which to study the causative role of herpesvirus infection in the development of atherosclerosis. (Circulation. 2000;102:779-785.)

Key Words: infection ■ atherosclerosis ■ viruses ■ apolipoproteins ■ pathology

The classic risk factors for atherosclerosis, namely, cigarette smoking, hypercholesterolemia, hypertension, and diabetes, account for only 50% of its incidence.1 Infectious agents have been suggested as additional risk factors:2–5;2 strong candidates are Chlamydia pneumoniae6–7 and herpesviruses. Herpesviruses have been proposed as potential initiators of arterial injury,8–10 endothelial dysfunction, and local inflammation, which might trigger or exacerbate atherosclerosis.11 Current evidence for a role of herpesviruses in vascular disease is conflicting, with data for and against causation.12–14

In the present study, apolipoprotein E (apoE)-deficient (apoE−/−) mice were infected with murine γ-herpesvirus-68 (MHV-68). ApoE−/− animals on a normal diet have high cholesterol levels and spontaneously develop atheroma.15,16 resembling the human disease.17 MHV-68 virus is a naturally occurring mouse pathogen18–21 that causes arteritis in immune-deficient animals.22 It is homologous to both human herpesvirus 8 (HHV-8, also known as Kaposi’s sarcoma herpesvirus) and Epstein-Barr virus.23,24 We show that infection of apoE−/− mice with MHV-68 significantly increases the amount of atherosclerosis in these animals. This suggests a direct correlation between virus infection and atherosclerosis. Possible mechanisms are discussed.
Histology
Aortas were fixed overnight in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (PB, pH 7.4), washed in PB, and postfixed in 1% osmium tetroxide in PB for 90 minutes. Tissue was washed in PB and dehydrated through a graded series of ethanol with a final change in propylene oxide. This procedure was followed by infiltration and embedding in epoxy resin. Sections (1 mm) were cut and stained with toluidine blue.

Quantification of Atherosclerotic Lesions
Aortas were excised and placed in cold PBS. Adventitial fat was carefully removed, and the aorta was opened up longitudinally from the cusps to the iliac bifurcation and divided into 2 strips. These were then placed sequentially in 70% ethanol (5 minutes), oil red O (90 minutes), 70% ethanol (5 minutes), and water (5 minutes) and then mounted en face in glycerol-gelatin mounting medium (Sigma). Images were analyzed by use of the computer program ImageStat 1.0 (http://www.ucl.ac.uk/ccaamrg/imagestat.html). The amount of atheroma was expressed as a percentage of the total area of the aorta.

Serology
An indirect ELISA was used as previously described, with the exception that 96-well plates were coated with purified MHV-68.

Cell-Mediated Immunity
T-cell proliferation assays were carried out by culturing lymphocytes isolated from the spleen or the para-aortic lymph nodes of infected or control apoE−/− mice in the presence of UV-inactivated MHV-68 or influenza antigen (control antigen). Assays were set up as previously described. The stimulation index (SI) was calculated as SI = mean cpm (test)/mean cpm (control).

Detection of Viral Message by RT-PCR
Total RNA was isolated from tissue by using TRIZOL reagent (Sigma). Reverse transcription (RT)–polymerase chain reaction (PCR) reactions were set up according to the manufacturer’s instructions (Perkin-Elmer). For the RT reaction, an anchored oligo(dT) (17-mer) was used. As a negative control and to assess for possible DNA contamination, the RT reaction was set up without the reverse transcriptase enzyme for each sample tested. β-Actin primers (GACATGGAGAAGATCTGGCA and GCTCGAAGTCTAGAGCAACA) were used as a positive control for the PCR reaction (436-bp PCR product). Specific primers against viral genes were designed to correspond with fragments of the genes encoding the major capsid protein (AACGTCAGCTCCTCAGTITG and AGCAGTCACAACATCCCTC) and the DNA binding protein (AGGCTACTACACCAACGTG and TCAGTGACAGGACAGGAGT) of MHV-68 (472 and 387 bp, respectively). PCR conditions were 94°C for 4 minutes and 30 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute. Samples were run on a 2% agarose gel containing ethidium bromide, and bands were made visible by UV transillumination.

Localization of Virus by Immunohistochemistry
Frozen sections (10 μm thick) were cut from aortas of C57BL/6J mice, air-dried, and fixed in ethanol. Slides were washed with
Tris-buffered saline and 0.3% Tween (TBST, pH 7.4). Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 10 minutes. Sections were washed 3 times with TBST and blocked with 10% serum in TBST for 10 minutes, and primary antibody (anti–MHV-68 polyclonal rabbit serum) was added at a 1/250 dilution in TBST. Sections were incubated at room temperature for 1 hour in a humidified chamber and washed 3 times with TBST. Horseradish peroxidase anti-rabbit antibody (1/100 dilution, DAKO) was used as the secondary antibody, and sections were incubated for 30 minutes before they were washed 5 times with TBST. Horseradish peroxidase activity was detected with use of a DAB solution (DAKO). The reaction was stopped, and sections were counterstained with Mayer’s hemalum, dehydrated, and mounted with DPX (BDH). Viral antigen stained brown; nuclei stained purple.

Results

Virus Isolation From Lungs of ApoE−/− Mice Infected With MHV-68

Intranasal infection of apoE−/− mice (n=5) with MHV-68 caused a subclinical infection. Virus titers isolated from the lungs 5 days after infection were similar to those measured in infected C57BL/6J mice, the parental strain, indicating that apoE−/− mice were equally susceptible to MHV-68 infection (data not shown).

Lesion Development in Mice Infected With MHV-68

Typical macroscopically visible atherosclerotic lesions were seen in the aorta 24 weeks after infection. These plaques were yellowish white in appearance and projected into the lumen of the aorta (Figure 1A). To quantify the amount of atheroma, aortas were stained with oil red O (Figure 1B and 1C). The extent of atheroma was considerably greater in the infected animals than in the control animals 24 weeks after infection (Figure 1B and 1C). Aortas taken from C57BL/6J mice infected with MHV-68 for 24 weeks did not stain with oil red O, and no macroscopic lesions were detected (Figure 1D).

Histological analysis of aortas of apoE−/− mice taken 24 weeks after infection showed typical atherosclerotic lesions (Figure 2). These lesions showed thickening of the vessel wall with disruption of the elastic fibers and deposition of cholesterol crystals. The increase in smooth muscle cells (SMCs), the large number of foam cells, and the presence of inflammatory cells were also typical of atherosclerotic lesions in these animals.

Time-Dependent Virus-Accelerated Atherogenesis in ApoE−/− Mice Infected With MHV-68

The increase in atheroma was time dependent in infected and control apoE−/− mice. Compared with the control condition, MHV-68 infection led to a progressive increase in the amount of atheroma in the aorta, and this increase was most significant 24 weeks after infection (Figure 3A).

Effect of Antiviral Treatment of Infected Mice on the Development of Atherogenesis

The induction of atheroma by virus was similar when mice were infected either intranasally (group 1) or intraperitoneally (group 2) and examined 20 weeks after infection (Figure 3B). Compared with infected control mice (group 2, Figure 3B), mice that received antiviral treatment with 4′-S-EtdU (group 3) showed a mean 67% reduction in atherosclerosis.

Possible Mechanisms of Virus-Induced Atherogenesis

Cholesterol Levels in ApoE−/− Mice Infected With MHV-68

To determine whether differences in lipid metabolism might have contributed to the virus-induced accelerated atherosclerosis, we examined cholesterol levels. Total serum cholesterol levels were not significantly different at any time point in any group of mice and were in the range expected for apoE−/− mice (data not shown).

Immune Response of Infected ApoE−/− Mice

Induction of an inflammatory immune response is a potential mechanism in the development of atherosclerosis in mice.27–29 We examined whether mice showed an altered humoral or cell-mediated immune response against MHV-68.
over a period of 24 weeks. Figure 4A shows that there was no significant difference in the antibody response measured by ELISA in serum samples of mice culled at any time after infection. The antibody response in serum samples was significantly lower in infected mice treated with antiviral treatment. Mice were mock-inoculated with PBS (group 4, c) or intraperitoneally (group 2, ip) or infected and treated with 4'-S-EtdU (group 3, inf ip). The end-point ELISA antibody titer was calculated for each serum sample, and data represent mean±SEM values (n=6 to 9). *P<0.02 (by Student t test). C, T-cell proliferative response of lymphocytes isolated from infected or control mice is shown. LN indicates lymph nodes. Data represent mean±SEM values (n=3 to 5). P<0.03 (for infection vs control by Student t test). Mice were culled 20 weeks after infection.

The antibody response in serum samples was significantly lower in infected mice treated with 4'-S-EtdU than in infected control mice (Figure 4B).

Cell-mediated immune response was measured by in vitro T-cell proliferation assays in lymphocytes isolated from the spleen of infected mice. There was no significant difference between the proliferative response of splenocytes isolated from group 1 (infected intranasally, data not shown), group 2 (infected intraperitoneally), and group 3 (infected and treated with 4'-S-EtdU, Figure 4C). Uninfected mice (group 4) did not mount a MHV-68–specific immune response. Interestingly, lymphocytes isolated from the para-aortic lymph nodes of infected mice also proliferated when stimulated in vitro with MHV-68. Lymphocytes did not proliferate in the presence of a control influenza antigen (data not shown). These results suggest that viral antigen may be present in the aorta and that this maintains an MHV-68–specific T-cell response in the para-aortic lymph nodes.

Detection of Viral Message in Aortas of Infected ApoE−/− Mice and in ECs

To establish whether replicating MHV-68 was present in the aorta of infected mice, aortas were harvested 5 days after infection, and total RNA was isolated. Viral mRNA was detected by RT-PCR in the aortas. Two bands corresponding to the mRNA encoding the major capsid protein and the DNA binding protein were detected (Figure 5A). No viral message was detected in whole blood– derived RNA samples at this time point. This demonstrates that the aorta itself was infected with MHV-68. No MHV-68 RNA was detected in aortas from control mice.

To establish whether MHV-68 could directly infect endothelial cells (ECs), a murine endothelial cell line (sEND1) was infected with MHV-68 at a multiplicity of infection of 0.1 for 3 days. Viral mRNA was detected by RT-PCR in infected, but not in mock-infected, sEND1 cells (Figure 5A).

To determine whether MHV-68 could directly infect aortic tissue, an in vitro system was used. Dissected aortas were cultured for 24 hours and infected on day 2 with 1×10^3 PFU of MHV-68 or were mock-infected (control). Aortas were harvested 3 days after infection. Virus replicated within the aorta, as measured by RT-PCR (data not shown). Viral antigen was localized predominantly at the luminal side of the aorta (Figure 5B), which suggests that SMCs and ECs were both infected. No viral antigen was detected in mock-infected aortas (Figure 5C).

Discussion

The data in the present study demonstrate that infection of apoE−/− mice with MHV-68 accelerates atheroma forma-
tion. Previous work in apoE−/− mice showed that repeated inoculation with C pneumoniae causes a slight increase in atheroma formation, and that C pneumoniae is found within plaques.30,31 In the present study, we show that a single infection with MHV-68 is sufficient to markedly enhance atherosclerosis without altering lesion histology. Furthermore, MHV-68 was detected in the aorta before atheroma developed. Antiviral treatment reduced lesion formation. These findings are not consistent with the suggestion that infective organisms are merely “innocent bystanders” in mature atheromatous plaques32 but suggest a direct role for MHV-68 in accelerating atherogenesis.

**Evaluation of the ApoE−/− Mouse Infected With MHV-68 as a Model for Atherosclerosis**

The quantification of atheroma with oil red O is a well-established method to assess atherosclerosis in a murine model. Histological analysis of lesions confirmed a typical appearance of atherosclerosis with no change in cellular composition between infected and control animals. This indicates that the virus accelerates rather than fundamentally changes atheroma. This acceleration was present early in the time course of atherogenesis and became even more marked at 20 to 24 weeks. Whether the amount of atheroma in infected animals increases further remains to be established. Although inhibition of virus-accelerated atheroma formation by antiviral treatment just failed to reach statistical significance, the effect of treatment with the antiviral drug 4′-S-EtdU would support the finding that the acceleration seen in infected animals was due to the virus. 4′-S-EtdU does not prevent the establishment of virus latency,20 which may explain why the effect of virus infection on the development of atherosclerosis was not completely inhibited by drug treatment. It is possible that the antiviral treatment has some other effect on atherogenesis, and further studies would be required to test this hypothesis. Our findings extend and support the early work of Fabricant et al9 but, for the first time, demonstrate that a herpesvirus can induce atherosclerosis in a mammalian model.

**Antibody Response and Serology**

The need for an appropriate mammalian model in the study of the role of herpesvirus infection in atherogenesis is para-
mount, because despite mounting evidence from clinical studies, no conclusive causative link has been demonstrated. Positive serology for human cytomegalovirus has been associated with the presence of atheroma, restenosis, accelerated atheroma, and subsequent graft rejection after cardiac transplantation. Yet, recently, a large study showed no evidence of an association between human cytomegalovirus (HCMV) or HSV serology and systemic inflammation or C-reactive protein, both of which are known to be predictive for cardiovascular risk. In the present study, the serum IgG response to MHV-68 was similar at 5 weeks after infection, when little atheroma was present, or later (at 24 weeks), when a marked increase in atheroma was seen. Furthermore, apoE−/− mice inoculated at 9 to 10 weeks seem to develop less atheroma than those infected at 4 to 5 weeks (authors’ unpublished data, 2000) despite generating similar antibody responses. It may be that the age at which the initial infection is acquired and the frequency of viral reactivation are both crucially important in determining the effect on atherogenesis. If this is the case in humans, then studies based on serology might yield false-negative results.

Possible Mechanisms for Virus-Induced Accelerated Atherogenesis

How does the virus, whether alone or in conjunction with other known risk factors, influence atherogenesis? In the model of atherogenesis examined by Ross, various noxious stimuli induce inflammatory changes within the arterial wall to initiate a fatty streak, which then may develop into mature plaque. Viruses could act at any stage in this process either indirectly or directly.

Indirectly, viruses may act by increasing serum cholesterol levels and thus promoting atherosclerosis. In the present study, we found that the virus had no effect on total cholesterol levels. However, it would be important to examine cholesterol subfractions to determine whether infection alters the lipid profile in more subtle ways. In the Marek’s disease model of atherosclerosis in chickens, changes in lipid metabolism have been detected, but no increase in total cholesterol was seen. Previous studies in the apoE−/− mouse have suggested that the development of atherosclerosis is caused by hyperlipidemia, although other yet-unknown mechanisms could contribute to this process. Infection of C57BL/6J mice, which have normal cholesterol levels, did not cause atherosclerosis; thus, virus infection alone is not sufficient to induce atherosclerosis. It would seem that a high level of total serum cholesterol is a necessary prerequisite for the virus to enhance atheroma.

A direct mechanism by which MHV-68 may accelerate atherogenesis is to target 1 of the 2 main cellular constituents of the plaque, either the EC or the SMC. In the present study, we have demonstrated that (1) MHV-68 can be detected in aortas of infected apoE−/− mice, (2) MHV-68 localizes predominantly to the luminal site of the aorta after infection of cultured aortas in vitro, and (3) MHV-68 can infect sEND1 cells. Human herpesviruses have similar properties. Both HCMV and HSV can infect ECs, initiating cellular responses similar to those in atherogenesis. Furthermore, HCMV behaves differently in aortic ECs (a vessel susceptible to atheroma) than in brain microvascular ECs (in which atheroma is not found). In aortic ECs, HCMV is nonlytic and is released persistently, whereas in small vessel ECs, it causes rapid lysis. The human homologue of MHV-68 is HHV-8, and this is known to transform ECs. Thus, viral infection may alter EC function and thereby promote atherosclerosis.

Weck et al showed that MHV-68 can also infect SMCs. Benditt et al were the first to show that SMCs from a single plaque were monoclonal rather than polyclonal in origin. Thus, a single SMC may proliferate in a manner analogous to tumor development. It is plausible that MHV-68 is enhancing atherosclerosis via SMCs by as-yet-unexplored means.

Clinical Significance

The present study shows that a murine γ-herpesvirus can induce atherogenesis in a murine model of hyperlipidemia and atheroma. We selected MHV-68 because it is a naturally occurring infection in mice and establishes a latent infection. It is unknown whether the results of our experiments relate to general systemic inflammation and atherosclerosis, to herpesviruses as a family and atherosclerosis, or, more specifically, to γ-herpesviruses and atherosclerosis. It will be logical to test whether these findings can be reproduced with an α-herpesvirus (HSV-1) or a β-herpesvirus (MCMV).

The human homologue of MHV-68, HHV-8, infects immunosuppressed individuals and is thought to be the cause of AIDS-related Kaposi’s sarcoma. These data might suggest a possible link between HHV-8 and AIDS-induced atherogenesis, in particular in those patients with high lipid levels. Such a link is eminently testable by epidemiological studies.

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References


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