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Murine Cytomegalovirus Infection Increases Aortic Expression of Proatherosclerotic Genes

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Background—The possible etiologic role of infection in cardiovascular disease is still debated. Having previously demonstrated that murine cytomegalovirus (MCMV) infection of apolipoprotein (apo) E−/− mice increases atherosclerotic lesion size, we determined if MCMV infection produces proatherogenic changes in aortic gene expression. Additionally, in cholesterol-fed C57BL/6J mice, we examined the effects of MCMV infection on aortic lesion area.

Methods and Results—C57BL/6J apoE−/− and wild-type C57BL/6J mice were infected with MCMV. At various time points, aortas were collected and pooled. Total RNA was extracted and hybridized to Affymetrix murine chips or analyzed for specific gene expression using TaqMan reverse transcription–polymerase chain reaction. Data from infected and uninfected mice were compared. A separate group of cholesterol-fed C57BL/6J mice were infected with MCMV, and lesion area in the aortic sinus was assessed using oil red O staining. Acute MCMV infection altered aortic expression of atherogenic genes in young apoE−/− and C57BL/6J mice—specifically, monocyte chemoattractant protein-1, monokine induced by interferon-γ, and interferon-γ inducible protein 10. Acute infection in adult 9-month-old apoE−/− mice with well-established lesions increased aortic expression of monocyte chemoattractant protein-1. Atherosclerotic lesion area in cholesterol-fed C57BL/6J mice was increased after infection with MCMV.

Conclusions—MCMV infection significantly increases atherosclerotic lesion area and aortic expression of atherogenic genes. These infection-induced effects indicate mechanisms by which cytomegalovirus may contribute to atherosclerotic disease initiation and progression and to the precipitation of clinical events. These results additionally add to data compatible with the concept that infection does play an important role in atherosclerotic disease. (Circulation. 2004;109:893-897.)

Key Words: atherosclerosis • infection • genes

Numerous studies suggest that various infectious agents are associated with an increased risk of cardiovascular disease (CVD), including cytomegalovirus, Chlamydia pneumoniae, Helicobacter pylori, hepatitis A, and herpes simplex virus.1–8 In contrast, there exists a considerable body of literature suggesting that infection is not associated with CVD.9–12 Most recently, negative findings of large prospective randomized antibiotic trials have raised additional questions as to the possible role of infections in CVD.13 However, other reports in smaller populations have suggested that antibiotics may be beneficial in preventing adverse cardiac events.14,15

The present investigation additionally examines the concept that infectious agents contribute to atherosclerotic disease initiation and progression through the inflammatory/immune responses elicited in the host. To this end we hypothesized that cytomegalovirus infection of mice prone to develop atherosclerosis upregulates in the aorta various genes involved in immune or inflammatory responses and that some of these gene products are known to play an important role in atherogenesis. If confirmed, we thought that such findings would indicate mechanisms by which infectious agents and the immune or inflammatory responses they elicit contribute to cardiovascular disease. To test this hypothesis as comprehensively as possible, we used Affymetrix microarrays to determine whether MCMV infection of 2-week-old C57BL/6J apolipoprotein (apo) E−/− mice leads to proatherosclerotic changes in gene expression compared with uninfected controls over multiple time points. Additionally, we examined the effects of MCMV infection in cholesterol-fed wild-type C57BL/6J mice by quantifying the lesion area in the aortic sinus after MCMV infection. Using TaqMan real-time polymerase chain reaction (PCR), we assessed expression changes of several candidate genes identified in the apoE−/− gene chip study after acute MCMV infection in both C57BL/6J and 9-month-old apoE−/− mice.
Methods

Viral Preparation
The Smith strain of MCMV (ATCC) was used for inoculation of mice. The virus and uninfected SC-1 cell (mouse embryonic fibroblasts, ATCC) supernatant was prepared as previously described. 16

Experimental Animals
C57BL/6J apoE−/− and C57BL/6J mice (breeders obtained from the Jackson Laboratory, Bar Harbor, Maine) were bred in house. All animals were housed in microisolator cages and were given free access to sterile food and water. Mice were injected intraperitoneally with either 30,000 pfu of MCMV or SC-1 cell supernatant control at either 2 weeks or 9 months of age. For the cytokine studies, mice were euthanized (n=10) at 2, 4, 8, 12, and 14 weeks after infection. For the various gene expression studies, mice were euthanized at 1, 4, or 14 weeks after infection and were collected using RNA later (Ambion) and stored at −80°C. RNA from a total of 60 aortas (30 per group) was used for the 1-week time point. RNA from 40 aortas (20 per group) was used for the 4-week time point, and RNA from 32 aortas (16 per group) was used for the 16-week time point. For the TaqMan experiments, RNA from 8 to 10 mice per group was used. Male and female animals were used in equal numbers. For the aortic lesion analysis, male C57BL/6J mice (bred in house) were infected as described above and weaned to a high cholesterol diet (containing 1.25% cholesterol and 0.5% sodium cholate; Harlan Teklad). At 16 weeks of age, hearts were collected and fixed in 10% buffered formalin solution for analysis. This protocol was approved by the Institutional Animal Care and Use Committee at MedStar Research Institute.

Isolation of Splenocytes
Spleens were removed from infected and noninfected animals, and splenocytes were isolated. Briefly, spleens were passed through a sterile nylon mesh filter and erythrocytes were lysed using ACK lysing buffer. Splenocytes were cultured at a concentration of 106 cells/mL in 96-well plates. MCMV antigen (1:40 dilution of heat-inactivated 107 pfu stock), PHA (Sigma), or medium alone was used as a stimulus. Splenocytes were cultured at 37°C in 5% CO2 for 18 hours. Supernatants were collected and stored at −80°C until testing.

Quantification of Interferon-γ Secreted by Splenocytes
Concentrations of interferon (IFN) γ in cell culture supernatants were measured using a commercially available mouse IFNγ ELISA kit (BioSource International). Assays were done in duplicate according to the manufacturer’s instructions.

MCMV Serology
The presence of anti-MCMV antibody in mouse serum was detected using a mouse anti-MCMV IgG ELISA kit (Charles River Laboratories).

Quantitative Analysis of Atherosclerotic Lesions
Lesion analysis was performed according to the method of Paigen et al. 17 Frozen sections of the aortic sinus were cut (10 μm) using a cryostat, and every other slice was collected. Sections were stained using oil red O and hematoxylin. Five sections per mouse were analyzed for lesions by a technician who was blinded throughout the study. Area analysis was completed using Image Pro Plus software (Media Cybernetics, Inc).

Cholesterol Levels
Serum samples, collected at time of euthanasia at 16 weeks of age, were individually evaluated for total cholesterol using Cholesterol-SL-ASSay, a 4-aminoantipyrine-based enzymatic assay (Diagnostic Chemical Limited).

RNA Extraction
Aortas from MCMV-infected mice and mock-infected mice were pooled, and total RNA was extracted using TRIzol reagent (Invitrogen) as previously described. 16 The pooled samples from each group were divided, and 2 in vitro transcription reactions were run for each group (infected and noninfected).

Microarrays
The duplicate cRNA samples were hybridized to separate Affymetrix murine U74A version 2 GeneChips for 16 hours. The chips were washed and stained on the Affymetrix Fluidics Station 400 using instructions and reagents recommended by Affymetrix. Fluorescence was read using the Hewlett-Packard G2500A Gene Array Scanner.

Data Analysis
Scanned raw data were processed with Affymetrix GeneChip version 5.0 software. The average intensity value for each probe set, which directly correlates with mRNA abundance, was calculated as an average of fluorescence differences for each perfectly matched versus single-nucleotide mismatched probe. Data sets on each GeneChip were normalized by scaling total chip fluorescence intensities to a common value of 800 before comparison. All data were imported into GeneSpring 5.1 software (Silicon Genetics) for additional analysis. The fold changes of each gene were calculated based on the normalized values and represented as relative to uninfected controls at each time point. Selected candidate genes had to be present on both replicate chips, with at least a 2-fold change in expression level compared with the controls.

TaqMan PCR
Intron-spanning monocyte chemoattractant protein (MCP)-1-specific and monokine induced by IFNγ (MIG)-specific oligonucleotide primers and internal TaqMan probes were designed using Primer Express (Applied Biosystems). Sequences are shown below and were confirmed to be sequence-specific by BLAST search. Probes were fluorescently labeled with TAMARA and FAM. Interferon-γ inducible protein-10 primers and probes were purchased from Applied Biosystems Inc. MCP-1 primers were as follows: S (5’-GAGCATCCACGTTGCTGCT3’), AS (5’TGTTGAAATGGATAGCACGAGT3’), and probe (5’-AGCCAGATGCAGTGGTTTGGATGCA3’). MIP primers were as follows: S (5’-CATCT-TCGAGAGCAGCAGTGG3’), AS (5’TGGATGAGATCGTGTCC-TG3’), and probe (5’-ACCCTAGTGAAGGGCAGAGATC-CTTCC3’).

Total RNA in multiple dilutions (100 and 200 ng) was reverse transcribed using random hexamer primers and Multiscribe Reverse Transcriptase (Applied Biosystems). Quantitative PCR was performed in duplicate. cDNA equivalent of 100 ng of total RNA per tube containing TaqMan PCR Universal Master Mix (Applied Biosystems), 100 nmol/L of probe, and 200 nmol/L of each primer were used. As a control for RNA integrity and for assay normalization, 18S rRNA was amplified using TaqMan rRNA control reagents kit (Applied Biosystems). Cycling conditions for TaqMan PCR were 2 minutes at 50°C and 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Relative quantitation of gene expression was determined using the methods described in User Bulletin No. 2, ABI Prism 7700 Sequence Detection System. Negative controls were included for each reaction to ensure the absence of contamination.

Statistics
Data are given as mean±SEM. Comparisons between 2 experimental groups were made by Student’s t test (2-tailed). Values of P≤0.05 were considered statistically significant.

Results
IFNγ Secreted by Splenocytes From ApoE−/− Mice
Levels of IFNγ were measured in supernatant from splenocytes isolated from MCMV-infected and noninfected ani-
mals. All samples produced IFNγ when stimulated with PHA (data not shown). The splenocytes isolated from MCMV-infected mice produced IFNγ after incubation with MCMV antigen, whereas the splenocytes isolated from uninfected mice did not produce IFNγ under identical conditions (Figure 1).

**MCMV Serology**

All mice that were infected with MCMV tested positively for anti-MCMV IgG by a commercially available MCMV ELISA kit. The mice that were sham infected did not test positively for MCMV IgG (data not shown).

**Cholesterol Levels of ApoE−/− Mice at 16 Weeks After Infection**

There was no difference in total cholesterol levels between MCMV-infected and uninfected mice (780±29 versus 783±15 mg/dL).

**Lesion Analysis**

C57BL/6J mice were infected with MCMV at 2 weeks of age and placed on a high cholesterol diet on weaning. At 16 weeks of age, aortic lesion area was assessed and found to be 4.5-fold higher in the MCMV-infected animals than in the sham-infected mice (2316±701 μm² versus 513±103 μm², P=0.01). Lesion analysis data are shown in Figure 2. Representative aortic sections from an MCMV-infected and sham-infected mouse are shown in Figure 3.

**Microarrays**

Total RNA was extracted from the aortas of apoE−/− mice to assess any alterations in gene expression 1, 4, and 16 weeks after infection with MCMV at 2 weeks of age. As expected, infection did produce changes in gene expression in young apoE−/− mice. Specifically, MCP-1, IP-10, and MIG were found to be upregulated at 1 week after infection (Table). These observed infection-induced changes in gene expression were no longer apparent at 4 and 16 weeks after infection. It is important to note that roughly 100 genes and expressed sequence tags were differentially expressed in this model. We chose to focus on these 3 important proatherosclerotic genes in the hopes of providing new and compelling data indicating mechanisms by which infectious agents and the immune or inflammatory responses they elicit contribute to cardiovascular disease.

**TaqMan PCR**

TaqMan PCR was used to confirm the results of the microarray study and, specifically, the increases in expression level for MCP-1, IP-10, and MIG at 1 week after infection in the apoE−/− animals. MCP-1, IP-10, and MIG mRNA levels were elevated 9.5-fold, 10.2-fold, and 7.5-fold, respectively (Table).

In a separate study designed to examine the effects of MCMV infection on wild-type C57BL/6J mice, aortic RNA samples isolated 1 week after infection with MCMV at 2 weeks of age were analyzed using TaqMan for MCP-1, IP-10, and MIG gene expression. Infected C57BL/6J mice had a 15±3.01-fold increase in MCP-1 expression, 92±14.50-fold increase in IP-10 expression, and 38±8.15-fold increase in MIG expression compared with the uninfected C57BL/6J controls.

To determine the effects of MCMV infection in older apoE−/− animals with established lesions, aortas were isolated from adult 9-month apoE−/− mice 1 week after MCMV infection and TaqMan PCR was used to measure MCP-1, IP-10, and MIG expression levels. In these older animals, MCP-1 expression was elevated 3.5±0.47-fold after viral infection. Although IP-10 and MIG expression increased by

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**Figure 1.** IFNγ production in C57BL/6J apoE−/− mice after splenocyte stimulation with MCMV antigen. White bars represent splenocytes taken from uninfected mice. Black bars represent splenocytes taken from MCMV-infected mice. The time points represent weeks after MCMV infection.

**Figure 2.** Aortic lesion area in C57BL/6J wild-type mice on a high cholesterol diet. Animals infected with MCMV had a significantly larger lesion area than the uninfected controls (2316±701 versus 513±103, P=0.01).

**Figure 3.** Aortic sections from 16-week cholesterol-fed C57BL/6J mice. A, Sham infected; B, MCMV infected. Sections were stained with oil red O and hematoxylin. Original magnification, ×100.
MCP-1 in plaque instability. Blocking of MCP-1 in apoE-/- mice with preexisting atherosclerotic lesions was shown not only to limit the progression of aortic root lesions but also to alter the lesion composition to a more stable phenotype containing fewer macrophages and lymphocytes, less lipid, and more smooth muscle cells and collagen. MCP-1 has also been linked to plaque instability in a recent patient study. Specifically, elevated baseline plasma levels of MCP-1 were associated with an increased risk of death or myocardial infarction in a cohort of patients with acute coronary syndromes. In all of our animal models—2-week-old apoE-/- and C57BL/6J and 9-month-old apoE-/- mice—aortic MCP-1 expression is upregulated during acute infection with MCMV. We believe that this finding is significant, because it suggests a potential mechanism by which acute infection with cytomegalovirus can contribute to the initiation and progression of atherosclerotic disease and to plaque instability.

In this study, we demonstrated that MCMV infection of 2-week-old C57BL/6J mice weaned to a high cholesterol diet resulted in an increased aortic lesion area at 16 weeks of age. In the gene expression component of the study, we collected the aortas from mice at 3 weeks of age (1 week after infection) before weaning. In this group of young, normocholesterolemic animals, MCMV infection resulted in an increase in MCP-1, IP-10, and MIG expression. Similarly, in a group of young apoE-/- mice (at 3 weeks of age) with elevated serum cholesterol levels but before gross evidence of atherosclerotic lesions, MCMV infection increased MCP-1, IP-10, and MIG gene expression. These findings suggest that acute infection with MCMV in a young animal can contribute to the initiation of lesion development, even in the absence of high cholesterol.

Furthermore, we tested the effects of MCMV infection on gene expression in a group of older, 9-month apoE-/- mice with established disease. One week after infection, MCP-1 expression was elevated in the aortas of these animals, although IP-10 and MIG levels remained unchanged compared with controls. This finding highlights the possibility that acute infection with cytomegalovirus in a situation of established atherosclerotic disease can cause changes in aortic gene expression compatible with the phenotype of less-stable plaques.

In addition to macrophages, T lymphocytes are also present in atherosclerotic lesions. Several lymphocyte-specific chemokines, including IP-10, MIG, and IFN-inducible T cell α chemoattractant, have been localized in atherosclerotic lesions. Of these 3 T cell chemoattractants known to play a critical role in the accumulation of lymphocytes in plaques, we found 2, IP-10 and MIG, to be highly upregulated in the mouse aorta after MCMV infection. Each of the chemokines we studied is induced by IFNγ. We therefore assessed the effects of acute MCMV infection on IFNγ expression. We found that incubating splenocytes obtained from MCMV-infected mice with MCMV antigen increased IFNγ release. We and others have previously shown that MCMV infection leads to an increase in serum levels of IFNγ. Additionally, we have shown in a series of in vitro studies that serum from MCMV-infected mice has the capacity to induce MCP-1 expression in vascular endothelial cells and that these serum-induced changes in MCP-1 expression are attributable, at least in part, to IFNγ.

The role of IFNγ in a murine model of atherosclerosis has been demonstrated in a study by Gupta et al in which apoE-/- animals were crossed with IFNγ receptor-/- animals, resulting in a 60% reduction in lesion area. Additional
studies describing the administration of exogenous IFNγ to apoE−/− animals28 and the crossing of IFNγ−/− mice with LDL receptor−/− mice29 additionally support the proatherosclerotic role of IFNγ. The chemokines, IP-10 and MIG, are stimulated by IFNγ. IP-10, a chemotractant for T cells and monocytes, is secreted by endothelial cells, monocytes, and fibroblasts and promotes T cell adherence to endothelial cells.30 MIG is closely related to IP-10 and is a chemotractant for T cells. Although both of these chemokines were upregulated after MCMV infection in young mice, the older apoE−/− mice, with established lesions, did not exhibit a significant increase in IP-10 or MIG after acute infection.

The elevations in MCP-1, IP-10, and MIG expression were only found after acute infection and were no longer detectable at 4 or 14 weeks after infection. This suggests to us the possibility of the “hit and run” phenomenon described in other diseases,31–33 in which infection produces early transient effects that can have a long-term impact on lesion development. This concept appears valid given our demonstration that infection in these mice does indeed result in larger lesions at 16 weeks of age.5,16

Taken together, these findings suggest that viral infection could initiate or accelerate atherosclerotic disease progression by causing an upregulation in the expression of specific proatherosclerotic chemokines, leading to an increase in the number of monocytes and T lymphocytes present in the aortic wall. Additionally, the upregulation of MCP-1 in older apoE−/− mice with established lesions indicates a mechanism by which acute MCMV infection could lead to a decrease in plaque stability and thus lead to the precipitation of acute coronary events.

References


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