Cytomegalovirus Infection Accelerates Inflammation in Vascular Tissue Overexpressing Monocyte Chemoattractant Protein-1

M. Kent Froberg, Alice Adams, Nicole Seacotte, Jan Parker-Thornburg, Pappachan Kolattukudy

Abstract—Cardiovascular disease is the leading cause of mortality in the United States. Atherosclerosis is responsible for most of this pathology and is an inflammatory disease with multiple cytokines and adhesion molecules expressed during atherogenesis. Cytomegalovirus (CMV), monocytes, and monocyte chemoattractant protein-1 (MCP-1) have all been implicated in human atherogenesis. A transgenic mouse overexpressing MCP-1 in the myocardium and pulmonary arteries develops myocarditis and pulmonary vascular inflammation. We infected MCP-1 transgenic mice with a sublethal dose of murine cytomegalovirus (MCMV) to look for evidence of accelerated inflammation in vascular tissues overexpressing MCP-1 to determine if MCMV could interact with monocytes and MCP-1 in a manner similar to what may occur in atherogenesis. MCMV infection of MCP-1 transgenic mice caused ascites, myocarditis, and pulmonary artery inflammation, which was not present in mock-infected MCP-1 or MCMV-infected wild-type mice. Inflammatory infiltrates in these tissues consisted of macrophages and T lymphocytes similar to the infiltrates seen in atherosclerosis. Virus presence in inflamed tissues was demonstrated by infecting transgenic mice with MCMV recombinant virus containing the gene sequence for the enhanced green fluorescent protein (EGFP). Human CMV could be involved in atherogenesis in a similar manner by interacting with monocytes and MCP-1 specifically expressed in vascular walls. (Circ Res. 2001;89:1224-1230.)

Key Words: cytomegalovirus ■ atherosclerosis ■ monocyte chemoattractant protein-1 ■ monocytes
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receptors resulting in uptake of modified LDL and further formation of atheromatous lesions in apoE when compared with apoE knockouts alone.22

for MCP-1, develop markedly reduced atheromatous lesions efficient for both apoE and CCR2, the primary in vivo receptor for MCP-1, with monocyte migration, which was blocked by antibodies to MCP-1.19 The significance of MCP-1 activity to atherogenesis has been demonstrated in a number of animal models of atherosclerosis. LDL receptor–deficient mice develop severe atherosclerosis, but when double null mice are produced that are LDL $^{-/-}$×MCP-1 $^{-/-}$, the area of atheromatous plaques is reduced by over 80%.20 MCP-1 overexpression accelerates atherogenesis in apoE $^{-/-}$ mice.21 Mice deficient for both apoE and CCR2, the primary in vivo receptor for MCP-1, develop markedly reduced atheromatous lesions when compared with apoE knockouts alone.22

A herpesvirus known as Marek’s disease virus (MDV) was found to cause the formation of atherosclerotic lesions in the aortas of chickens and led to accumulation of cholesterol and cholesterol esters in the aortic wall of MDV-infected chickens.23–25 Subsequent studies in humans have suggested a role for CMV in atherogenesis. CMV nucleic acids have been identified in atheromatous plaques by in situ DNA hybridization and PCR.26,27 In one study, 90% of advanced atherosclerotic lesions (Grade III) obtained at surgery were positive for CMV nucleic acids, whereas 50% of Grade I lesions obtained at autopsy from age- and sex-matched patients were positive, suggesting CMV may play a role in atherogenesis.27

CMV seropositivity is a strong risk factor for restenosis following coronary atherectomy28–30 and transplant-associated atherosclerosis in heart transplant patients.31,32 CMV infection has been implicated in atherogenesis in animal models of atherosclerosis,33 and CMV accelerates formation of atheromatous lesions in apoE $^{-/-}$ mice.34 In vitro CMV infection of endothelial cells causes increased expression of ICAM-1 and VCAM-1 resulting in increased adhesion of leukocytes to endothelial cells.35,36 Viral infection activates macrophages in vitro leading to increased expression of cytokines.37,38 Both macrophages and T lymphocytes within atherosclerotic lesions show increased cytokine and HLA-DR expression.1,39 Macrophages increase expression of scavenger receptors resulting in uptake of modified LDL and further oxidative injury.40 CMV infection increases accumulation of cholesterol and cholesterol esters within infected smooth muscles cells in vitro.41

CMV infection could activate monocytes/macrophages drawn to sites of endothelial injury by MCP-1 secretion and accelerate or exacerbate atherogenesis. Alternatively, virions could infect and activate endothelial cells leading to increased MCP-1 expression and initiation of atherogenesis. To investigate the relationship of CMV, monocytes, and MCP-1 in vivo, we infected MCP-1 transgenic mice with murine cytomegalovirus (MCMV) to determine if this accelerated or exacerbated inflammation specifically in vascular tissues overexpressing MCP-1.

Materials and Methods

Mouse Injections

Eighteen male and 5 female 2-month-old MCP-1 transgenic mice were obtained from the Ohio State University Transgenic Animal Facility courtesy of Dr Pappachan Kolattukudy. Mice were maintained in separate cages and given food and water ad libitum. Mice homozygous for MCP-1 expression were produced by breeding heterozygotes as previously described.15 Age-matched mice of the same genetic strain (FVB/N) that were not transgenic for MCP-1 were purchased (Harlan, Indianapolis, Ind). Transgenic mice were given either 10$^5$ plaque-forming units (PFU) of live Smith strain MCMV (n=9) (VR-1399, American Type Tissue Culture, Rockville, Md) or mock infection (n=6) by intraperitoneal injection (IP). Virus was cultured in SC-1 cells (CRL-1404, ATTC), a cell line of fetal mouse embryo origin, in minimal essential media (MEM) with Earle’s salts and 1-glutamine (Gibco, Rockville, Md) or mock infection (n=6) by intraperitoneal injection (IP). Virus was cultured in SC-1 cells (CRL-1404, ATTC), a cell line of fetal mouse embryo origin, in minimal essential media (MEM) with Earle’s salts and 1-glutamine (Gibco, Rockville, Md) supplemented with 5% calf serum (Sigma Diagnostics, St Louis, Mo), 100 IU/mL penicillin, and 100 $\mu$g/mL streptomycin. For virus production, confluent layers of SC-1 were infected with MCMV at a multiplicity of 0.1, and the virus-containing supernatants harvested 4 to 5 days later. Confluent monolayers of SC-1 cells in individual wells of 24-well titer plates were used to quantitate virus by plaque assay for subsequent mouse injection.42 MCMV-treated mice received an equivalent amount of culture media without virus in 1 mL of phosphate buffered saline. Mock-infected mice were given an IP injection of an equivalent amount of culture media without virus in 1 mL of phosphate buffered saline to ensure that culture media alone did not accelerate inflammation.

Recombinant cytomegalovirus containing the gene sequence for the enhanced green fluorescent protein (MCMV/EGFP, kindly provided by Dr Edward Mocarski of Standford University) was used to determine if virus was present within inflammatory tissues in areas of MCP-1 overexpression.43 Viral culture and injections were performed similar to that for the wild-type virus. Eight male 2-month-old homozygous MCP-1 transgenic and 4 nontransgenic mice were given 10$^4$ PFU of MCMV/EGFP and euthanized 7 days after infection. Tissues were fixed in formalin, stained briefly with hematoxylin, and examined under a Nikon Eclipse E600 microscope equipped with a Y-FL fluorescent package (both Nikon Corp) for evidence of green fluorescence.

**TABLE 1. Study Design and Results**

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>Treatment</th>
<th>n</th>
<th>Time Euthanized After Injection</th>
<th>Myocarditis</th>
<th>Pulmonary Artery Infiltrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic</td>
<td>MCMV 5</td>
<td>5</td>
<td>1 week</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Transgenic</td>
<td>Mock 3</td>
<td>3</td>
<td>1 week</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wild-type</td>
<td>MCMV 8</td>
<td>8</td>
<td>1 week</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Mock 7</td>
<td>7</td>
<td>1 week</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Transgenic</td>
<td>MCMV 4</td>
<td>4</td>
<td>60 days</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Transgenic</td>
<td>Mock 3</td>
<td>3</td>
<td>60 days</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wild-type</td>
<td>MCMV 8</td>
<td>8</td>
<td>60 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Mock 2</td>
<td>2</td>
<td>60 days</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

+ indicates present; 0, absent. Wild-type and transgenic mice are of the same strain (FVB/N).
Pathology
Mice were euthanized by CO2 inhalation at 1 week or 2 months after infection (aged 9 or 16 weeks) and examined for evidence of gross or microscopic pathology (Table 1). Total body weight as well as the weight of heart, spleen, and liver were recorded for each mouse. Mean organ weights were compared between MCMV- and mock-infected mice directly and by determining mean organ to mean total body weight ratios for the spleen, liver, and heart to quantify any change in organ size due to MCMV infection. Ratios were used to reduce weight differences due to variation in body weight. Mean organ weights were also compared between MCMV-infected transgenic and wild-type mice. Samples of tissue from heart, spleen, liver, and lung were placed in 10% buffered formalin or frozen at −20°C for routine histological and immunohistochemical studies.

Immunohistochemistry
Sections of spleen, liver, heart, and lung (6 μm) obtained at necropsy from each mouse were stained with antibodies for MCP-1, CD-3, CD-20, and F4/80 using the avidin-biotin peroxidase complex method and standard methods.44 MCP-1 was detected using the goat anti-mouse JE/MCP-1 primary antibody at a dilution of 1:500 (R&D Systems, Minneapolis, Minn). For T and B lymphocyte identification, we used rabbit anti-mouse CD3 and mouse anti-human CD20, respectively (both Scy Tec, Logan, Utah), using prediluted antibody. Monocytes/macrophages were identified using the rat anti-mouse F4/80 primary antibody (Serotec, Raleigh, NC) diluted at 1:50 following trypsin pretreatment of frozen tissue sections. For negative controls, the primary antibody was replaced with an unrelated antibody.

Viral Assays
Presence of MCMV in specific mouse organs was semiquantified by viral assay to ensure MCMV infection was similar in transgenic and wild-type mice. Equivalent homogenized samples of spleen, liver, heart, and lung were placed in 10% buffered formalin or frozen at −20°C for routine histological and immunohistochemical studies.

Statistical Analysis
Comparison of mean organ, mean total body, and mean organ/body weight ratios were compared for MCMV versus mock-infected mice for groups euthanized at 1 week and 2 months, respectively. Results were analyzed using the Student’s t test and a level of significance of P=0.05 on StatView software (Abacus Conceptus).

Results
All 9 MCMV-infected transgenic mice developed ascites by 6 days after infection, whereas none of 6 mock-infected transgenic mice or 16 MCMV-infected wild-type mice developed ascites. MCMV-infected transgenic mice euthanized at 1 week had significantly higher mean total body weight (P=0.0219), mean liver (P=0.0044), mean spleen (P=0.0087), and mean spleen to total body weight (P=0.0141) compared with mock-infected transgenic mice (raw data for mice euthanized 1 week after treatment are shown in Table 2). Mean heart weight was not different between MCMV- and mock-infected groups (P=0.2773), and only mean spleen weight was significantly different between MCMV- and mock-infected mice euthanized at 2 months after infection (P=0.0273) (raw data not shown). Mean total body and organ weights were not significantly different between MCMV-infected transgenic and wild-type mice. Mean heart weight was not significantly different between MCMV and mock-infected transgenic mice at 1 week (P=0.2773) or 2 months (P=0.8864) after infection, or between MCMV-infected transgenic and wild-type mice at 1 week after infection (P=0.2398).

Sections of hearts from MCMV-infected transgenic mice showed scattered mononuclear inflammatory infiltrates within the ventricles consisting of a mixture of monocytes/macrophages and T lymphocytes by immunohistochemistry, whereas mock-infected transgenic mice and MCMV-infected wild-type mice showed no significant infiltrates in sections of hearts or pulmonary arteries (Figures 1A through 1D). In MCMV-infected transgenics, infiltrates were more intense within the atria with areas of myonecrosis and some cells having the appearance of foamy macrophages (Figure 1G). JE/MCP-1 immunoreactivity (IR) was present in ventricular and atrial myocardium and in the walls of pulmonary arteries of all transgenic, but not wild-type mice (Figures 1E and 1F). MCMV transgenic mice also had perivascular pulmonary artery infiltrates at 1 week and 2 months after infection (Figure 2A through 2C). Somewhat denser appearing infiltrates were seen in MCMV transgenic mice euthanized at 2 months after infection. In addition, MCMV-infected transgenic mice euthanized at 2 months after infection had subendothelial mononuclear pulmonary artery infiltrates resembling fatty streaks (Figures 1F, 1H, and 2A). Some subendothelial inflammatory cells within the lungs also showed JE/MCP-1 IR (Figure 2A). By immunohistochemistry, inflammatory cells were an admixture of F4/80 positive macrophages and CD3 positive T lymphocytes (Figure 2B and 2C). Only very rare CD20 positive B lymphocytes were identified. Mock-infected transgenic mice had no ventricular and very sparse atrial infiltrates with rare pulmonary perivascular infiltrates at 1 week. Myocardial and pulmonary inflammatory infiltrates in mock-infected transgenic mice were similar to those seen in MCMV-infected transgenic mice at 2 months, but were less dense and had fewer arteries showing subendothelial infiltrates. Spleens of MCMV-infected transgenic and wild-type mice showed follicular hyperplasia, whereas livers were free of inflammatory infiltrates but showed cell swelling in both transgenic and wild-type MCMV-infected groups. Most MCMV-infected transgenic and wild-type mice showed mild patchy pneumonitis.

<table>
<thead>
<tr>
<th>Mice</th>
<th>n</th>
<th>Treatment</th>
<th>Body</th>
<th>Heart</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>5</td>
<td>MCMV</td>
<td>30.55 (1.69)</td>
<td>0.1382 (0.003)</td>
<td>1.808 (0.072)</td>
<td>0.2898 (0.037)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>3</td>
<td>Mock</td>
<td>23.62 (0.140)</td>
<td>0.1283 (0.009)</td>
<td>1.172 (0.022)</td>
<td>0.1027 (0.006)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>4</td>
<td>MCMV</td>
<td>32.03 (0.780)</td>
<td>0.1490 (0.012)</td>
<td>1.635 (0.095)</td>
<td>0.2480 (0.025)</td>
</tr>
</tbody>
</table>

Weights are in grams (raw data are not shown for mice euthanized at 2 months after treatment); standard errors are shown in parentheses.
Viral assays of spleen, liver, and salivary gland tissues from MCMV-infected transgenic and wild-type mice were similar with low titers of virus in liver and spleen and higher titers in salivary glands. Virus was detectable in splenic tissue 6 to 8 days after infection at total organ burdens of 10^2 to 10^3 PFU with slightly smaller burdens in the liver. Similar levels of virus were seen in MCMV-infected transgenic and wild-type mice euthanized at 2 months after infection with 10^3 to 10^4 PFU in salivary gland tissue. Levels in spleen and liver were markedly decreased at 2 months after infection. Green fluorescence of mononuclear inflammatory cells was present in the spleen, heart, pulmonary arteries, and cells lining hepatic sinusoids of MCMV/EGFP-infected MCP-1 transgenic mice euthanized 1 week after infection (Figure 2D through F). Green fluorescent cells were not observed in the hearts or pulmonary arteries of MCMV/EGFP-infected wild-type mice.

Discussion

MCMV infection accelerated inflammation in vascular tissues overexpressing MCP-1 by 1 week after infection, demonstrating that MCMV infection could activate monocytes and T lymphocytes in tissues with high MCP-1 expression leading to edema and necrosis. Kolattukudy et al. reported...
myocardial infiltrates of monocytes in 9-month-old mice heterozygous for MCP-1 without myonecrosis or cytokine expression by monocytes. Our mice were homozygous for MCP-1 expression, and most mock-infected transgenic mice showed no myocarditis, no myonecrosis and little perivascular pulmonary artery inflammation at 1 week after infection, indicating that MCP-1 overexpression alone was not responsible for the inflammation. Periartrial and subendothelial mononuclear inflammatory infiltrates from lungs of MCMV-infected transgenic mice also showed MCP-1 IR suggesting MCP-1 overexpression may be more responsible for the inflammation. Periarterial and pulmonary artery inflammation at 2 months when compared with mock-infected transgenic mice, although the contrast between treatment and control groups was not as striking as at 1 week after infection. This suggests that MCP-1 overexpression may be more responsible for the infiltrates as the mice get older.

It is well established that monocytes/macrophages are key cells in atherogenesis. Adhesion molecules and chemokines expressed by injured endothelial cells can bind ligands on CMV-infected monocytes acting as virus vectors and facilitate transmigration into the intima to become foam cells. Guetta et al45 has shown that in cocultures of endothelial cells and smooth muscle cells, infected monocytes were able to transmit HCMV to ECs and SMCs. They also demonstrated that CMV major immediate-early promoter (MIEP) expression was increased in CMV-infected monocytes when exposed to ECs, SMCs, or oxidized LDL. Waldman et al46 demonstrated bidirectional transmission of CMV between ECs and peripheral blood mononuclear cells. Thus, CMV-infected monocytes could interact with ECs and SMCs in a complex manner to deliver CMV to sites of endothelial injury and facilitate atherogenesis. CMV antigens and nucleic acids have been identified within human vessel walls and within atherosclerotic lesions.26,27,47 Viral infection has been shown to activate inflammatory cells enhancing cytokine production and lead to SMC proliferation.1,37 CMV reactivation within vessel walls could also contribute to atherogenesis. Restenosis following angioplasty is associated with CMV infection or reactivation and may involve virus inactivation of p53 with subsequent exuberant smooth muscle proliferation, a hallmark of this lesion.28–30,48 Speir et al48 showed a high concordance between p53 immunoreactivity in restenosis lesions and presence of CMV nucleic acids by PCR.

CMV infection of monocytes can increase cytokine expression, which in turn may lead to increased expression of adhesion molecules and MCP-1 by ECs, thus enhancing monocyte adhesion and migration. Iwamoto et al58 demonstrated in vitro that monocytes/macrophages transfected with the HCMV IE gene increased IL-1β production by ~30-fold. IL-1β treatment has also been shown to induce MCP-1 expression in endothelial cells.38 Other sources of endothelial injury, such as hypertension or oxidized LDL, have been shown to enhance MCP-1 expression, which could secondarily enhance delivery of CMV-infected monocytes to areas of vascular injury and influence atherogenesis. In this MCP-1 transgenic mouse model, we demonstrated accelerated inflammation and tissue injury in vascular tissues overexpressing MCP-1 following MCMV infection. By immunohistochemistry, the infiltrates consisted of monocytes/macrophages and T lymphocytes similar to what is seen in atherosclerosis.

The course of MCMV infection appeared similar in MCP-1 transgenic and wild-type mice because in the acute phase similar levels of virus were present in the spleen and liver by viral assay. By 2 months after infection, only the salivary glands contained relatively high levels of virus by plaque assay in both transgenic and wild-type mice consistent with a chronic phase of virus infection. This suggests that MCP-1 overexpression did not alter the distribution or viability of MCMV in different host tissues but acted to attract MCMV activated monocytes and T lymphocytes to tissues overexpressing MCP-1 leading to pathology. This was supported by studies of mouse tissues following infection with the MCMV/EGFP virus which demonstrated virus-infected mononuclear cells within tissues showing MCP-1 overexpression. HCMV could be involved in atherogenesis in a similar manner, ie, HCMV-infected monocytes could be trafficked to sites of arterial injury with high MCP-1 expression and transmit virus to endothelial and smooth muscle cells leading to an escalating cycle of cell proliferation with increased expression of proinflammatory elements. Primary endothelial injury could be initiated by CMV infection or any of the putative etiologic agents of atherogenesis. The relatively small number of fluorescent cells found in these sites of inflammation is not unlike human CMV pneumonitis in which few cytomegalic cells are seen.

The differences in mean spleen and liver weights between MCMV- and mock-infected transgenic mice may have been due to MCMV infection of those organs or, in part, to passive congestion secondary to myocarditis. Although the spleen had prominent nodular hyperplasia with germinal centers, no significant inflammatory infiltrates were seen with the liver of MCMV-infected transgenic mice. The ascites would account for the difference in mean total body weights between MCMV and mock-infected groups. Only mean splenic weights were significantly different between MCMV- and mock-infected transgenics at 2 months, suggesting that chronic MCMV infection was still causing an antigenic stimulus. MCMV infection did not contribute to cardiomegaly because there was no significant difference in mean heart weight between MCMV- and mock-infected transgenic mice or MCMV-infected wild-type mice. Pneumonitis was seen in both MCMV transgenic and wild-type mice and appeared unrelated to MCP-1 overexpression. Pneumonitis has been reported as part of the natural course of MCMV infection in mice.49

MCP-1 and CMV also have a direct relationship. HCMV-infected cells express a G protein–coupled receptor under the control of an open reading frame labeled US28 that binds MCP-1 with high affinity.50,51 Bodaghi et al52 suggest this chemokine sequestration by viral chemoreceptors may be a mechanism to escape host immune responses. Streblow et al50 have shown that US28 mediates smooth muscle cell migration in vitro. Smooth muscle cell migration to the intima is an
important facet of atherogenesis and is a dominant component of restenosis atherosclerosis. A G protein–coupled receptor homolog called M33 is found in murine CMV, but its relationship to MCP-1 has not been defined. Davis-Poynter et al have demonstrated that M33 null virus grows normally in cell culture, but produces an attenuated infection in mice suggesting M33 may be important to pathogenesis. If the M33 receptor also binds MCP-1, it is possible this receptor is expressed on infected monocytes and binds MCP-1 in transgenic mice, thereby delivering the virus to sites of MCP-1 overexpression and contributing to the inflammatory process.

Moderate ethanol consumption has a protective effect on cardiovascular disease and recent in vitro and laboratory animal studies suggest that this effect may in part be due to downregulation of MCP-1 expression. Thus, therapeutic mechanisms could be used in the treatment or prevention of atherosclerosis aimed at blocking MCP-1 activity.

The acceleration of inflammation in vascular tissue overexpressing MCP-1 demonstrates MCMV, monocytes, and MCP-1 may interact to cause focal inflammation and necrosis similar to what may occur in atherogenesis. This model may be useful in studying the mechanism by which CMV could contribute to atherogenesis, even though the MCP-1 expression has a different distribution than human atherosclerosis. If CMV is determined to play a role in atherogenesis, antiviral therapies may be used to treat or prevent de novo or restenosis atherosclerosis.

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References


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