Active Cytomegalovirus Infection of Arterial Smooth Muscle Cells in Immunocompromised Rats

A Clue to Herpesvirus-Associated Atherogenesis?

M.C.J. Persoons, M.J.A.P. Daemen, J.H. Bruning, C.A. Bruggeman

Abstract

The susceptibility of medial and neointimal arterial smooth muscle cells (SMCs) to acute cytomegalovirus (CMV) infection was investigated in immunocompetent and immunocompromised rats. The left common carotid artery of all the rats was injured by balloon catheterization. On days 14 and 17 after injury, rats were either intravenously infected with a rat CMV (RCMV) or mock-infected. Active RCMV infection was shown in the neointima of the injured arteries of immunosuppressed rats, characterized by specific cytopathological changes in hematoxylin-eosin–stained sections and by the presence of early viral antigens and the presence of the virus at different stages of maturation at the electron-microscopic level. Viral genome was shown as well by use of in situ hybridization procedures. However, medial cells were hardly ever infected, and RCMV did not infect neointimal parts that were recovered by endothelial cells. No infection was seen in control right carotid arteries or in the injured arteries of immunocompetent rats. Acute intimal RCMV infection was accompanied by infiltration of inflammatory cells, predominantly mononuclear cells that stained positive with an ED-1 monoclonal antibody. The majority of infected neointimal cells were SMCs containing smooth muscle actin. No changes were found in medial or neointimal cross-sectional areas of the infected arteries. In the present study, an active RCMV infection of arterial SMCs was established in vivo, and it was concluded that neointimal SMCs are far more susceptible than are medial SMCs and that the absence of endothelium and immunosuppression are necessary conditions for arterial RCMV infection. (Circ Res. 1994;75:214-220.)

Key Words • cytomegalovirus • arterial smooth muscle • neointima

Cytomegalovirus (CMV) is a ubiquitous agent and a common cause of infection in humans. Although the vast majority of infections is asymptomatic, the virus may incite serious, often life-threatening disease in immunocompromised patients.1 Like other herpesviruses, CMV may establish latent or persistent infection and reside lifelong in the infected patient, although the site of latency is not clear.

Much emphasis has been laid on an association of herpesviruses with atherogenesis.2-4 Fabricant and colleagues5-8 described avian herpesvirus–induced atherosclerotic changes in chickens, similar to the lesions found in humans. Since then, CMV, in particular, has been found repeatedly in human arterial tissue, predominantly derived from patients suffering from atherosclerosis. The presence of CMV antigens and DNA has been described in cultures of smooth muscle cells (SMCs) derived from arterial tissue that had been surgically removed from patients with advanced arterial disease. Furthermore, CMV antigens and genome were found in coronary arteries and aortas of trauma victims, predominantly in focal intimal areas showing early or advanced atheromatous changes.4 More recently, CMV nucleic acid sequences, but no viral antigens, were demonstrated in large arteries of patients with and without atherosclerosis.8 With the polymerase chain reaction, CMV genome was found in 90% of arterial specimens obtained from patients with severe atherosclerosis and in 53% of nonatherosclerotic arterial specimens.9

Epidemiologic support for an association between CMV and human atherosclerosis has come from studies on human heart transplants, where a significantly higher rate of accelerated transplant atherosclerosis was found in CMV-infected heart transplant recipients compared with non–CMV-infected recipients.10-12 In accordance with these findings in patients, accelerated atherosclerotic alterations were recently described in aortic allografts of rats that had been infected with a rat-specific CMV (RCMV).13,14 Although a correlation between CMV and atherosclerosis has been suggested, the actual role of the virus in atherogenesis remains to be established. Since CMV is found in arterial SMCs and proliferating intimal SMCs are thought to be part of the early atherosclerotic plaque,15 it is tempting to consider that (intimal) arterial SMCs play a role in virus-induced atherogenic changes. However, although CMV infection and replication have been demonstrated in cultured arterial SMCs,16,17 no active CMV infection of arterial SMCs has been found in the above-mentioned in vivo studies. Therefore, it has been hypothesized that the arterial wall may be a place for CMV latency from which the virus can reactivate, starting a productive infection. In the present study, the susceptibility of medial and neointimal SMCs to acute infection with RCMV18 was
investigated in immunocompetent and immunocompromised rats in vivo. The characteristics of an active arterial SMC infection were analyzed, including a possible effect of the virus on intimal or medial cross-sectional area.

Materials and Methods

Virus

The RCMV used in the present study consisted of a pool of homogenated salivary glands of acutely infected laboratory rats. Experimental rats were intravenously infected with 10^7 plaque-forming units (PFU) of salivary gland–derived RCMV diluted in 1 mL Eagle’s minimal essential medium and containing 2% newborn calf serum.

Animals

The experiments were performed, according to institutional guidelines, with 16- to 18-week-old male specific pathogen-free Wistar-Kyoto rats bred at the Department of Experimental Animal Service at the University of Limburg, Maastricht, The Netherlands. During the experiments, the rats were given standard rat chow and tap water at libitum.

Experimental Design

The left common carotid artery of all rats was balloon-injured, as previously described. In short, after anesthetizing the animals with pentobarbital (60 mg/kg body wt IP), a 2F balloon embolectomy catheter (Baxter) was introduced through the left external carotid artery and advanced into the aortic arch. Subsequently, the inflated catheter was withdrawn through the common carotid artery. This procedure was repeated three times. Then the catheter was removed, the external carotid artery was ligated, and the wound was closed. The right carotid artery was left untraumatized and served as an internal control. Rats were divided into four groups (A through D, n = 7 or 8 per group). Thirteen days after balloon injury, when medial SMC proliferation had returned to control levels but intimal SMC proliferation was submaximal, rats of groups A and B were immunosuppressed by total-body x-irradiation of 5 Gy. One and 4 days later, animals of groups A and C were intravenously injected with 10^7 PFU RCMV; rats of groups B and D were mock-infected with a salivary gland homogenate derived from noninfected rats. It has been proved that during these first few days after x-irradiation, the animals were immunosuppressed sufficiently to give the virus a chance to proliferate and initiate an infection. The choice to administer RCMV 14 days after balloon injury was based on previous experience with the restimulation model (Reference 23; and E.M. van Klee, personal communication), where it was shown that neonatal cells are very sensitive to stimuli like angiotensin and do respond in a manner different from medial SMCs.

By aortic bleeding under ether anesthesia, the animals were killed 2 weeks after the first RCMV administration and were perfused in situ with 0.9% NaCl. One hour before death, all animals intravenously received 0.5% Evans blue in 0.9% NaCl to stain nonendothelialized vascular tissue. Two-millimeter segments were taken from the central Evans blue–retaining part of the balloon-injured left common carotid artery as well as from the proximal part near the aortic arch that was not stained by Evans blue. Comparable segments were taken from the noninjured right carotid artery. Furthermore, tissue biopsies of liver, spleen, lung, and kidney were collected. The salivary glands, preferential sites for CMV, were taken to confirm a generalized RCMV infection.

All tissues were fixed in 3.7% formaldehyde in phosphate-buffered saline, routinely processed, and paraffin-embedded. Cross sections (3 μm) were cut and mounted on chromium alum gelatin–coated, glutaraldehyde-inactivated glass slides for immunohistochemical and in situ hybridization techniques as well as for hematoxylin-eosin and Lawson elastin staining.

Virus Detection

For the detection of RCMV early antigens, immunocytochemical techniques were used as previously described. After blocking of endogenous peroxidase with 0.6% H2O2, deparaffinized tissue sections were preincubated with 2% bovine serum albumin before incubation with a mixture of two mouse monoclonal antibodies (8 and 35) against nuclear and cytoplasmic RCMV antigens. A second incubation was performed with biotinylated affinity-purified sheep anti-mouse IgG antibodies (Amersham, Nederland B.V.), followed by incubation with streptavidin–horseradish peroxidase complex (Amersham, Nederland B.V.). Specific antibody–antigen binding was visualized with diaminobenzidine substrate (DAB). To control for aspecific positive staining, all sections were incubated with an anti-human CMV monoclonal antibody, nonreactive with RCMV, as well as with no monoclonal antibody at all. Antibodies were of IgG1 subclass and used in the same concentration (1:100). Tissue sections were counterstained with hematoxylin and embedded in Entellan (Merck). In situ hybridization procedures to detect viral genome were performed as previously described. Probe DNA consisted of a mixture of DNA fragments (C, D, and E), accounting for 23.7% of the RCMV genome, as had been analyzed in our laboratory. The fragments were labeled with biotin 11-dUTP (Sigma Chemical Co) by use of the primed labeling kit (Boehringer). The specificity of the DNA probe was evaluated in uninfected rat embryonic fibroblasts. Hybridization was carried out overnight at 42°C. Formed hybrids were visualized by use of the BLU-gene TM kit (Bethesda Research Laboratories). To rule out nonspecific hybridization, in situ hybridization procedures were also accomplished without the DNA probe, using the plasmid vector as a control.

For electron microscopy, cross-sectional samples of the carotid arteries were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.2. The arterial samples were postfixed in 1% osmium tetroxide, dehydrated in graded alcohol solutions, and embedded in Epon 812. Ultrathin sections were cut on a diamond knife, using a Reichert Ultracut and mounted on copper grids. The sections were stained with uranyl acetate and lead citrate and observed with a Philips CM10 electron microscope at 80 keV.

Histopathology

Immunohistochemical staining procedures were carried out to mark arterial SMCs by use of HHF 35 (Brunschwig), a monoclonal antibody that recognizes actin isotypes (α and γ) common to all SMCs. After incubation with HHF 35, the sections were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako). Visualization was performed using DAB substrate. Serial sections were stained, either with HHF 35 or monoclonal antibodies 8 and 35 against viral antigens, to determine the SMC origin of RCMV-infected cells.

To recognize infiltrated cells, immunoperoxidase staining of arterial sections was carried out by use of ED-1, a monoclonal antibody reacting with rat macrophages/monocytes and W3/13, a monoclonal antibody that reacts with rat T lymphocytes. After incubation with these monoclonal antibodies, the sections were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako). Visualization was performed with DAB as substrate. To control for specificity of signals, the same procedures were performed with aspecific antibodies and with no monoclonal antibodies.

Morphometric Analysis

Cross-sectional areas of the intima and media of the carotid arteries were measured by using a computer-assisted morphometry system (Quantimet, 570; Leica) on Lawson-stained cross sections. The cross-sectional area of the media was defined as
the area surrounded by the external and internal elastic lamina. The neointimal cross-sectional area was defined as the area surrounded by the internal elastic area and the arterial lumen.

Statistics
Comparisons of the intimal and medial cross-sectional areas between groups were made by a one-way ANOVA. Differences between the individual means were further tested by Fisher’s protected least-squares difference. For comparisons between the intima covered by endothelium and the intima deprived of endothelial cells and between left and right media, Student’s t test was used. Significance was assumed at \( P<.05 \).

Results

Animal Conditions
No apparent clinical signs of illness were observed in any of the animals during the experiments. Starting body weights ranged from 340 to 390 g; at the end of the experiments, the animal weights ranged from 370 to 440 g. Although the infected rats that had been immunocompromised (group A) tended to gain less body weight compared with the animals from the other groups, these differences were not significant (data not shown).

Virus Detection
Intravenous administration of RCMV led to a generalized infection in the rats, as shown by the presence of viral antigens and genome in the salivary glands. The amount of viral DNA or antigens containing cells in these organs was much higher in immunocompromised animals, which is in agreement with previous observations.\(^22\) In spleen, liver, lung, and kidney, no viral nucleic acids nor antigens were found, which is also a usual finding 2 weeks after infection.\(^19\)

In the RCMV-infected animals that had been immunosuppressed (group A), a large number of cells in the intima of the common carotid arteries were positive for RCMV antigens and genome, as shown with immunohistochemical and in situ hybridization procedures (Fig 1A, 1B, and 1C). Enlarged cells showing typical CMV inclusions and normal-shaped SMCs proved to contain viral antigens and DNA. These cells were predominantly found in the neointimal areas bordering the arterial lumen. Infected cells were present in the outermost intimal parts, whereas they were sporadically found in the underlying media. RCMV-positive cells were only present in the neointimal parts that were not covered by endothelial cells. In sections of the proximal parts of the common carotid artery retaining no Evans blue, where an endothelial cell layer overlaid the neointima, no viral antigens nor DNA was ever found (Fig 1D).

Electron microscopy confirmed the presence of RCMV in the carotid intimas of RCMV-infected rats of group A. Massive amounts of viral nucleocapsids in different stages of maturation were observed in the nuclei and cytoplasm of SMCs (Fig 2) and in some inflammatory cells. In the arteries from groups B, C, and D, no signs of viral infection were found, either with immunohistochemical and in situ hybridization techniques or with electron microscopy.

Histopathology
Four weeks after balloon injury, a multilayered neointima had been formed in the left carotid arteries. In
the roentgen-radiated and RCMV-infected rats of group A, neointimal cells showed clear evidence of infection and inflammation. Cells were often swollen and contained cytoplasmic and nuclear inclusions, giving them an owl-eyed appearance characteristic of active CMV infection. Mononuclear and polymorphonuclear inflammatory cells as well as pyknotic cells were seen, predominantly in the innermost layers of the intima (Fig 3A and 3B). No features of infection or inflammation were found in the neointima covered by endothelium nor in any of the arteries derived from rats of group B, C, or D (Fig 3C).

Cells of the media and intima stained positive with HHF 35, indicating their SMC origin. Most of the infected cells in the neointima of rats from group A also stained positive with the HHF 35 antibody (Fig 4A and 4B).

Cells staining with the macrophage/monocyte marker ED-1 were present as well, predominantly in the innermost intimal layer (Fig 4C). However, they were seen in smaller amounts and did not stain with the anti-RCMV monoclonal antibodies. In heavily infected arteries, large amounts of ED-1–stained cells were present in the adventitia, whereas the media and the outermost intimal parts only sporadically contained cells staining with ED-1.

Cells staining positive with the T-cell marker W3/13 were also found in the carotid intima of animals from group A. However, only few cells had the appearance of true lymphocytes. It is known that W3/13 can stain...
neutrophil granulocytes,\textsuperscript{28} and we found that W3/13 predominantly stains polymorphonuclear cells throughout the infected intima but especially near the lumen. W3/13-positive or ED-1-positive cells were rarely seen in noninfected arteries.

**Morphometric Analysis**

The medial cross-sectional area of the noninjured right carotid artery was not significantly different for the four experimental groups (Table). Although the medial cross-sectional area of the injured left carotid artery tended to be increased compared with the medial cross-sectional area of the right carotid artery, this apparent difference was not statistically significant. No effects of radiation or virus infection were found on the medial cross-sectional area of the left carotid artery.

The neointimal cross-sectional area of the carotid artery segments covered with endothelium was significantly smaller than the neointimal cross-sectional area of the segments that were not covered by endothelial cells. Neither roentgen radiation (group B) nor virus infection (group C) nor the combination of both (group A) changed the neointimal cross-sectional area compared with the control condition (group D).

**Discussion**

Although CMV nucleic acids have been shown in arterial SMCs of atherosclerotic patients,\textsuperscript{6,7} a productive infection of arterial SMCs in vivo has never been described before. We investigated the susceptibility of medial and neointimal SMCs in immunocompetent rats as well as in rats that had been immunocompromised by total-body roentgen radiation. Acute RCMV infection was realized in the latter ones and was almost exclusively localized in the innermost neointima of the in-

---

### Table: Neointimal and Medial Cross-sectional Area of Rat Cytomegalovirus–Infected and Noninfected Carotid Arteries After Balloon Injury

<table>
<thead>
<tr>
<th></th>
<th>Cross-sectional Area, mm(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A (n=7)</td>
</tr>
<tr>
<td><strong>Left carotid artery</strong></td>
<td></td>
</tr>
<tr>
<td>Neointima without ECs</td>
<td>0.30±0.16</td>
</tr>
<tr>
<td>Neointima with ECs</td>
<td>0.13±0.08</td>
</tr>
<tr>
<td>Media</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td><strong>Right carotid artery</strong></td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td>0.13±0.03</td>
</tr>
</tbody>
</table>

Group A indicates rat cytomegalovirus (RCMV) infection and roentgen radiation; group B, only roentgen radiation; group C, only RCMV infection; group D, neither RCMV infection nor roentgen radiation; and ECs, endothelial cells. Values are mean±SD.
jured carotid artery. Acute, so-called productive, infection was proved by the presence of typical nuclear CMV inclusions and the presence of early viral antigens in the cytoplasm and nucleus of infected cells. Moreover, at the electron-microscopic level the virus was visualized at different stages of maturation and replication. To complete the demonstration of RCMV presence, viral nucleic acids were shown as well. The majority of infected neointimal cells were SMCs, as can be concluded from the electron-microscopic images and the positivity of these cells for smooth muscle actin; ED-1–positive and W3/13-positive cells did not contain RCMV early antigens, and endothelial cells were absent in the infected areas.

In our model, immunosuppression proved to be a prerequisite for arterial RCMV infection, which is in agreement with other rat studies in which disseminated RCMV infection was only seen in animals with disturbed immunity.22 In rats with an intact immune system, RCMV DNA and antigens were found only in the salivary glands, which are known to be preferential sites for CMV; in other organs, RCMV could be detected at very low levels and in the acute phase only.29 The enhancing effect of immunosuppression on viral replication is a characteristic feature of CMV and other herpesviruses, and it is commonly known that in humans CMV may cause serious problems in neonates and patients with disturbed immunity, whereas in most healthy adults the infection remains asymptomatic.1

We found neointimal cells of the injured arteries to be highly susceptible to RCMV infection, whereas the virus only sporadically infected a medial SMC in these arteries and was never found in control arteries. The absence of RCMV infection in noninjured arteries is compatible with previous findings from our laboratory, which showed that during a disseminated RCMV infection, the virus was hardly ever found in the large arteries (authors’ unpublished data).

The presence of endothelium seems to be a barrier for acute CMV infection of neointimal SMCs, since neointimal infection was only seen in the intimal parts that were not covered by endothelial cells. In the area of the common carotid artery near the aortic arch, where the neointima was overlaid by an endothelial cell layer, no virus was detected. It is very likely that endothelial cells had been present during the entire postinfection period, since regrowth of endothelial cells from the aortic arch to the most proximal part of the denuded common carotid artery has been described within 2 weeks after balloon catheterization.20 In vitro, endothelial cells have been frequently described to be slightly permissive to CMV infection,20,31 which supports the hypothesis that endothelium forms a possible mechanical barrier to CMV infection of underlying SMCs in vivo.

However, this possibility does not explain why only neointimal and not medial SMCs were infected. Earlier studies showed that arterial SMCs in vitro are highly permissive to CMV infection16,17 and susceptibility of SMCs to herpesvirus infection may be determined by the SMC phenotype17 or may be developmentally regulated.32 Although there are no in vitro data of the susceptibility of neointimal SMCs to viral infection, intimal and medial SMCs have distinct properties both in vitro and in vivo,23,33,34 and the susceptibility to CMV infection may be one of these properties.

It may also be possible that the susceptibility of neointimal cells to RCMV infection is linked to the position of these cells in the cell cycle, since endothelial cells reduce SMC proliferation in culture35,36 and neointimal SMC proliferation has been described to cease as soon as endothelium recovered the neointima.20,21 This might explain the absence of RCMV infection in the neointima when endothelium is present. Moreover, the amount of DNA synthesis in the third and fourth week after balloon injury is higher in the neointimal SMCs than in the underlying media,20 and this may explain the higher susceptibility of the neointimal SMCs to virus infection compared with medial SMCs.

Intimal SMC migration and proliferation are commonly described features of early atherosclerotic changes and are described phenomena after arterial catheterizations in the clinic, although recent data may question this observation.37 Therefore, the high susceptibility to CMV infection of injured arterial areas is intriguing in the context of a possible viral contribution to atherogenesis. In this context too, we studied the presence of inflammatory cells in RCMV-infected arteries. After RCMV infection, mononuclear inflammatory cells and, to a lesser extent, polymorphonuclear cells and T lymphocytes infiltrated into the infected intimal areas. This is interesting because macrophages, in particular, are found in large amounts in atherosclerotic lesions.38–41 These cells synthesize and release a wide range of biologically potent molecules, which are all potential contributors to a local atherogenic process and may influence SMC proliferation. Mitogenic factors may also be released from infected SMCs.

Our observation that the neointimal cross-sectional area was not affected by virus infection may suggest that SMC DNA synthesis was not increased in the infected intima. This is in contrast with our recent findings that RCMV infection enhances SMC proliferation and neointimal thickness in rat aortic allografts.13,14 However, no viral antigens or nucleic acids were found in the allograft neointima, nor were there other signs of active RCMV infection. RCMV-infected cells could only sporadically be detected in the adventitia.14 On the contrary, in the present study a stimulatory effect of RCMV on neointimal thickness may be obscured by an increased degradation of SMCs in the course of intimal RCMV infection. To analyze SMC proliferation, preliminary studies were performed with bromodeoxyuridine labeling, a thymidine analogue that is incorporated into newly synthesized DNA. However, these were hard to interpret, since the compound was incorporated into the SMCs as well as into viral DNA and the cellular infiltrate (data not shown). Comparable results were obtained with an anti–Ki-67 antibody (Immunotech).

In conclusion, for the first time, acute CMV infection of arterial SMCs in vivo is described. In immunocompromised rats, RCMV infection was established in the intima of injured arteries when endothelial cells were absent. The observation that disturbed immunity is a necessity for active RCMV infection of arterial SMCs supports the thought that if the arterial SMC is indeed a potential site for latent CMV infection, the virus may reactivate, eg, during immunosuppression or local injury, initiating an acute infection as described here. The model described in the present study should provide a
basis for further study of the short- and long-term effects of CMV infection on the neointima and the proliferation of SMCs.

Acknowledgments

The authors would like to thank M.C.A. Stuart, C.M. Eerdmans Thijssen, and M.M. Hubbscho for their excellent technical assistance and F.S. Stals for critically reading the manuscript.

References


13. Brunning JH, Persoons MCI, Lemstrom KB, Stals FS, De Clercq E, Bruggeman CA. Enhancement of transplantation associated arteriosclerosis by CMV, which can be prevented by antiviral therapy in the form of HPMPC. Transpl Int. 1994;7(suppl 1):S367-S370.


Active cytomegalovirus infection of arterial smooth muscle cells in immunocompromised rats. A clue to herpesvirus-associated atherogenesis?

M C Persoons, M J Daemen, J H Bruning and C A Bruggeman

Circ Res. 1994;75:214-220
doi: 10.1161/01.RES.75.2.214

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/75/2/214

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/