Enhanced Cytomegalovirus Infection in Atherosclerotic Human Blood Vessels

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Human cytomegalovirus (CMV) is a possible co-factor in atherogenesis and vascular occlusion, but its ability to actively infect medium and large blood vessels is unclear. A vascular explant model was adapted to investigate CMV infection in human coronary artery, internal mammary artery (IMA), and saphenous vein (SV). Vascular explants were inoculated with CMV Towne or low-passage clinical isolate and examined *in situ* for CMV cytopathic effect and immediate-early and early antigens, as indicators of active infection. At 5 to 7 days after inoculation, we found that CMV Towne actively infected eight of eight different atherosclerotic blood vessel explants (coronary artery, $n = 4$; SV and IMA grafts, $n = 4$), whereas it only infected 2 of 14 nonatherosclerotic blood vessel explants (SV, $n = 10$; IMA, $n = 4$) ($P = 0.001$). The CMV clinical isolate actively infected none of six sets of nonatherosclerotic SV explants at 5 to 7 days after inoculation. The active CMV infections involved adventitial and, less frequently, intimal cells. A small subset of infected cells in atherosclerotic tissue expresses the endothelial cell marker CD31. Smooth muscle cells residing in both atherosclerotic and non-atherosclerotic blood vessels were free of active CMV infections even after all vascular tissue layers were exposed to the virus. In contrast, active CMV Towne infection was evident at 2 days after inoculation in smooth muscle cells and endothelial cells previously isolated from the SV tissues. We conclude that active CMV infection is enhanced in atherosclerotic blood vessels compared to atherosclerosis-free vascular equivalents, and this viral activity is restricted to subpopulations of intimal and adventitial cells. (*Am J Pathol* 2004, 164:589–600)

Experimental findings in animals and isolated human vascular cells, as well as some epidemiological studies in

humans, support the hypothesis that human cytomegalovirus (CMV) may be a co-factor in atherosclerosis,^{1–4} arterial restenosis,⁵ acute arterial occlusion,^{6–8} and post-transplant coronary artery (CA) disease.^{9,10} CMV might contribute to vascular disease by direct invasion of the blood vessel wall or by acting from distant sites through host-inflammatory response or perturbation of lipid metabolism.^{11–13} Several investigators have reported that CMV nucleic acid is often present in walls of atherosclerotic arteries,^{14–19} implicating a role for CMV in directly initiating or advancing this disease. However, very little is known about the virus's ability to replicate within atherosclerosis-prone blood vessels, despite long-standing awareness of CMV's proclivity for replicating in and damaging small blood vessels and capillaries of persons with CMV disease.²⁰ Notably, rat CMV replicates in mechanically injured carotid arteries but not in healthy contralateral arteries of acutely infected rats, implying that pre-existing vascular injury or inflammation can render large arteries conducive to viral replication.²¹

Endothelial cells (ECs) and smooth muscle cells (SMCs) that are isolated from human arteries and subsequently inoculated with CMV are able to support viral replication.^{22,23} The CMV replicative process disrupts cell-cycle control^{16,24} and increases amounts or activities of procoagulant proteins,²⁵ reactive oxygen species,^{26–28} leukocyte adhesion molecules,^{29–32} cholesterol uptake and esterification,³³ cell motility,³⁴ and proinflammatory cytokines.^{5,35–37} Thus, findings in isolated ECs and SMCs suggest multiple mechanisms by which CMV might promote atherogenesis and its complications.

Human CMV has uniquely evolved with its host, has little genetic similarity to animal CMV counterparts, and only replicates in humans.³⁸ Given these considerations, the present study makes use of a blood vessel explant model in which to investigate CMV's ability to actively replicate in atherosclerosis-free and atherosclerotic human vascular tissues. Novel findings have emerged from this tissue explant model to provide important insights

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into CMV's biology in humans that are relevant to the virus's potential role in the genesis, progression, and complications of atherosclerotic vascular disease.

Materials and Methods

Cells and Virus

ECs and SMCs were isolated from saphenous veins (SVs) collected at the time of bypass surgery using previously described methods.^{39,40} The identities of ECs and SMCs were confirmed by uptake of acetylated low-density lipoprotein and positive immunostaining for α -smooth muscle actin, respectively. ECs and SMCs were grown in Clonetics endothelial growth medium-2 and smooth muscle growth medium-2 (Clonetics, San Diego, CA), respectively, containing 20% fetal bovine serum (Harlan Bioproducts for Science, Inc., Indianapolis, IN). Confluent cells at passage 3 to 7 were trypsinized and placed in six-well plates for experiments. Primary human foreskin fibroblasts (HFFs) were grown in Eagle's minimal essential medium supplemented with 10% newborn bovine serum and penicillin/streptomycin (100 U/100 μ g per ml) as described previously.⁴¹

Human CMV Towne and a recombinant CMV Towne expressing green fluorescent protein (GFP) were propagated on HFFs, as reported previously.⁴¹ The recombinant CMV exhibits normal growth kinetics⁴² and expresses GFP in the early phase of infection in HFFs.⁴³ The clinical CMV isolate came from a gastric ulcer of a patient with a heart transplant. This virus was grown on HFFs (passage number, 3 to 5) and on human umbilical vein ECs (passage number, 2 to 3). Cell-free CMV was collected by filtration of the infected cell medium or extract through a 0.45- μ m filter (Nalgene; NalgeNunc International, Rochester, NY). The filtrate was supplemented with 20% fetal bovine serum before inoculation onto vascular cells and tissue explants. Negative control inoculum consisted of the same medium except for omission of virus. For preparation of extract, infected cells were scraped into growth medium, twice freeze (dry ice)-thawed, and sonicated for 4 minutes. Plaque-forming units (PFU) per ml of CMV inoculum was determined using methods published previously.⁴¹

Organ Culture

The human blood vessel culture method was adapted from previously published studies.^{44–46} The University of Iowa Institutional Review Board approved these studies. Unused SVs and internal mammary artery (IMA) were obtained at the time of CA bypass surgery. Atherosclerotic SV and IMA grafts were obtained either at the time of repeat CA bypass grafting or from explanted hearts at time of cardiac transplantation. Atherosclerotic CAs were obtained from explanted hearts of patients undergoing cardiac transplantation. It was not possible to obtain both atherosclerotic and atherosclerosis-free blood vessels from the same patient. Blood vessels were transported and rinsed in RPMI 1620 containing HEPES 20 mmol/L,

penicillin/streptomycin/gentamicin (100 IU/100 μ g/50 μ g per ml), and L-glutamine 4 ml/L, and then cross-sectioned into 3- to 8-mm lengths. Some cross-sections were opened longitudinally, and some of these were pinned onto gauze (NuGauze; Johnson and Johnson Gateway, Inc., Piscataway, NJ) on top of a 2- to 3-mm layer of Sylgard resin (DowCorning, Midland, MI) in 100-mm tissue culture dishes (Becton Dickinson, Mountain View, CA), while the other tissues were cultured free-floating in 24-well tissue culture plates (Becton Dickinson) to continuously expose all surfaces to the medium. The two methods of vessel culture (pinning onto gauze *versus* free-floating) produced equivalent experimental outcomes. The vascular explants were cultured in RPMI 1620 containing NaHCO₃ 2 g/L, penicillin/streptomycin (100 IU/100 μ g per ml), L-glutamine 4 ml/L, and 30% fetal bovine serum and incubated at 37°C in 5% CO₂. Growth medium was changed daily. In some experiments, the endothelium was gently denuded using a cotton swab after pinning tissues onto gauze.

Infections

Isolated ECs and SMCs were incubated for 1 to 2 hours at 37°C in 5% CO₂ with CMV Towne expressing GFP at a multiplicity of infection of 10 and 1 PFU/ml, respectively. After removal of the viral inoculum, the infected cells were washed once with 1 $\times$ phosphate-buffered saline (PBS) and growth medium was applied. In some experiments, the infected and uninfected cells were fixed in 7% buffered formalin (Fisher Scientific, Pittsburgh, PA) and processed for analysis by fluorescence imaging and immunohistochemistry.

Vascular explants were incubated with and without CMV for 2 hours at various times after culture at 37°C in 5% CO₂. At the indicated times, vascular tissues were processed by standard formalin fixation and paraffin-embedding, sectioned (6 μ m thickness), and mounted on glass slides for staining with hematoxylin and eosin (H&E) and, in some experiments, Verhoeff-van Gieson's stain for elastin.

Detection of Viral and Cellular Proteins

In isolated ECs and SMCs, expression of CMV GFP and immediate-early (IE)/early antigens was analyzed by immunofluorescence imaging. Formaldehyde-fixed cells were treated with 0.1% trypsin for 20 minutes at 37°C and blocked with serum-free blocking solution (DAKO, Carpinteria, CA) for 30 minutes at 4°C. Pooled murine monoclonal anti-CMV DDG9 (p72) and CCH2 (p52) antibodies (DAKO) were applied at 1:50 dilution (in 1% fetal bovine serum in PBS) overnight at 4°C and secondary goat anti-mouse antibody conjugated to Alexa-568 (Molecular Probes, Eugene, OR) was applied for 30 minutes. Cells mounted in Vecta-Stain mounting media (Vector, Burlingame, CA) were imaged at 488 nm and 568 nm to detect fluorescence of GFP and CMV IE/early proteins, respectively. Controls done in parallel include CMV-

infected HFFs, uninfected ECs and SMCs, and infected cells in which primary antibody was substituted with normal mouse serum (1:1000 dilution).

Immunohistochemical assay of CMV IE (p72)/early (p52) antigen in vascular tissues involved an immunoperoxidase reaction for pooled murine monoclonal anti-CMV DDG9 (p72) and CCH2 (p52) antibodies (DAKO), using the avidin-biotin-peroxidase complex method. Endogenous peroxidase activity was quenched using 3 to 6% hydrogen peroxide before proteolytic digestion with either 0.1% trypsin for 20 minutes or 0.05% proteinase K for 5 minutes at 37°C. Avidin and biotin blocking reagents (Vector) were each applied for 15 minutes. Primary antibody DDG9/CCH2 was applied at 1:25 to 1:50 dilution. Power block reagent (Biogenex, San Ramon, CA) was applied for 10 minutes on tissues having high levels of background staining. LASB Plus (DAKO) and DAKO DAB Plus were applied according to the manufacturer's directions, followed by counterstaining with 10% Harris hematoxylin without acid. As a control, adjacent tissue sections were reacted with normal mouse serum (1:1000 dilution) instead of primary antibody DDG9/CCH2. The Vectastain ABC kit (Vector) was used initially for immunohistochemical assay followed by counterstaining with nuclear fast red. Negative and positive controls consisted of vascular explants not incubated with CMV and tissues (jejunum, lung, or placenta) of persons with CMV disease, respectively.

Immunohistochemical assays to identify lineage of CMV-infected cells were performed on adjacent sections of formaldehyde-fixed, paraffin-embedded atherosclerotic CAs, using previously described methods.⁴⁷ Tissue sections were digested with 0.05% Proteinase K for 5 minutes before addition of primary antibodies to CMV p52 (CCH2, DAKO) at 1:20 dilution or CD68 (PG-M1, DAKO) at 1:100 dilution. Heat-induced antigen retrieval was used for detection of CD31 (pressure cooker) and CD45 (microwave, twice for 5 minutes each); the antibodies to CD31 (JC170A, DAKO) and CD45 (DAKO) were used at 1:20 dilution and 1:4000 dilution, respectively. Antibody to α -smooth muscle actin (Sigma Chemical Co., St. Louis, MO) was used at 1:100 dilution, without previous epitope retrieval. Primary antibody binding was detected using avidin-biotin technique and 3',3'-diaminobenzidine-tetrahydrochloride dihydrate as the chromogen. The secondary antibody was polyvalent and reactive with both polyclonal (rabbit) and monoclonal (mouse IgG and IgM and rat IgG) primary antibodies. Sections of CMV-infected placenta and of multitissue control blocks were used as positive and negative controls, respectively.

Proliferating cells were identified with a murine monoclonal antibody to proliferating cell nuclear antigen (DAKO) applied at 1:1500 dilution. Deparaffinized tissue was incubated in 3% hydrogen peroxide for 5 minutes before antigen retrieval by heating in microwave (twice for 4 minutes) in citrate buffer. The Envision kit (DAKO) was subsequently used according to the manufacturer's directions, followed by counterstaining with Harris hematoxylin.

Results

Active CMV Infection Is Greatly Restricted in SV Explants Unlike the ECs and SMCs Isolated from this Tissue

We examined CMV's ability to actively infect human SVs, which have propensity to develop atherosclerosis when used in CA bypass grafting. SV remnants obtained at time of harvesting for CA bypass surgery that had negligible histological findings of atherosclerosis were subjected to study in a blood vessel explant culture model.⁴⁴⁻⁴⁶ The SV explants retain viability for up to 10 days of culture, increase in tissue thickness as early as day 4, and exhibit neointimal proliferation (Figure 1, A and B), which are characteristic features of this model.⁴⁴⁻⁴⁶ As reported previously, most neointimal cells contain α -smooth muscle actin (not shown), confirming their identity as SMCs.⁴⁶ Moreover, many SMCs in the neointima and media express proliferating cell nuclear antigen (Figure 1C), implying an active state of SMC proliferation. Cells residing at the luminal surface (Figure 1D) and in adventitia and vasa vasorum (Figure 1E) also express proliferating cell nuclear antigen.

SV explants at days 1 to 2 of culture were incubated without and with CMV Towne (10^7 PFU/ml) or clinical isolate previously grown in fibroblasts or ECs (10^4 PFU/ml). Tissues were examined at 2 to 7 days after inoculation for CMV cytopathic effect (CPE) and immediate-early (IE)/early antigen expression, both indicators of activate infection, by H&E staining and immunohistochemical assay, respectively. Active infection was absent in all SV explants not receiving CMV. Remarkably, CMV CPE and IE/early antigens were only detected in 2 of 16 sets of CMV-inoculated SVs from 12 different donors, while they were invariably detected in positive control tissues. SVs from 2 of 10 different donors were susceptible to active infection with CMV Towne. As shown for one of these SVs, actively infected cells appear in patches in adventitia, but are not observed in intima and media despite exposing their cut margins to viral inoculum (Figure 2A). The actively infected adventitial cells are randomly scattered or concentrated around vasa vasorum (Figure 2, B and C). They did not express the CD31 EC marker or SMC-specific actin (not shown). Actively infected cells in SV of the other donor were distributed likewise in adventitia, but were also rarely evident at the luminal surface (not shown). Notably, the experimental outcome was unchanged by varying the timing (0 to 5 days after culture) or duration (2 to 24 hours) of exposure to CMV or by mechanical removal of endothelium at 2 to 24 hours before viral inoculation (not shown).

The active infection indicators were not detected at 5 to 7 days after inoculation in six of six sets of SV explants from four donors that were inoculated with low-passage clinical CMV isolate grown in either fibroblasts or ECs. A representative experiment shown in Figure 2, D and E, reveals SV explant in direct contact with clinical CMV isolate in cell lysate, yet absence of CMV IE/early antigen in all tissue components up to 7 days after inoculation. In

Figure 1. Viability of human SV explants sustained in culture. **A and B:** Histological examination of extent of neointima on day of procurement (**A**) and at 5 days in culture (**B**) assessed by Verhoeff-van Gieson stain for elastic fibers of fixed tissues. **C–E:** Detection of cell proliferation by immunohistochemical assay for proliferating cell nuclear antigen in neointima and media (**C**), along luminal surface (**D**), and in adventitia and vasa vasorum (**E**) of SV explants at 6 days in culture. **C to E** are counterstained with hematoxylin. **White arrows** point to internal elastic lamina; A, L, M, and N denotes adventitia, lumen, media, and neointima, respectively. **A to E** are representative sections of multiple SVs. Original magnifications: $\times 100$ (**A, B**); $\times 200$ (**D, E**); $\times 400$ (**C**).

SV from one donor, active infection was observed with CMV Towne at 10^7 PFU/ml, but not with clinical CMV isolate at 10^4 PFU/ml (not shown). Whether this one discrepancy is the result of difference in inoculum titer, viral tropism, or other experimental variables is unknown.

The apparent resistance of SV ECs and SMCs *in situ* to active CMV infection was unexpected given published reports of susceptibility of isolated arterial and pulmonary vein ECs and SMCs to active CMV infection in culture. We examined the possibility of ECs and SMCs from SVs differing from cellular equivalents of other blood vessel types with regard to CMV infection. ECs and SMCs were isolated from SVs of two different donors and inoculated in culture with CMV Towne expressing GFP, a marker of viral early gene expression.⁴³ Nearly all SMCs, and most ECs, exhibited viral GFP fluorescence as early as 2 days after inoculation (Figure 3, A and B), whereas the uninfected SMCs and ECs did not fluoresce (not shown). All SMCs (Figure 3; C to E) and ECs (not shown) producing GFP fluorescence also expressed CMV IE/early antigen, as detected by immunohistochemistry. Although these experiments were performed in subconfluent proliferating

cells, CMV IE/early protein expression was not diminished in confluent SV SMC and EC cultures, implying that infectivity is not dependent on cell-cycling activity (not shown). These findings are consistent with those produced in SMCs and ECs isolated from other blood vessel types.^{22,24,28,48} Thus, active CMV infection is minimal to negligible in SV explants despite inoculation with high multiplicity of infectious viral particles, unlike findings in SMCs and ECs isolated from this tissue. Level of infectivity did not correlate with activity of cellular proliferation.

Active CMV Infection in Atherosclerotic Blood Vessels

Given the frequent presence of CMV nucleic acid in atherosclerotic blood vessels,^{14–19} we determined whether CMV would actively infect atherosclerotic SV bypass grafts. Like native SV, the diseased SV bypass grafts of three donors were cultured, inoculated with and without CMV Towne (10^7 PFU/ml), and examined for CMV IE/early antigen and CPE at 0 to 5 days after inoculation.

Figure 2. Active CMV infection is greatly restricted in human SV explants. **A–C:** Immunohistochemical assay for CMV IE/early antigen in SV explant at 7 days after inoculation with CMV Towne (10^7 PFU). **A:** CMV⁺ cells are present in patchy distribution in adventitia (arrows), but are absent in or at cut-margin of luminal endothelium, neointima, and media. **B:** Adventitial CMV⁺ cells are randomly scattered or located around vasa vasorum. On high-power view (asterisk), many CMV⁺ cells exhibit cytomegaly and nuclear inclusions (**C**), which is also evident on H&E staining (not shown). **A** to **C** are representative of multiple sections of quadruplicate SV explants, with 38 ± 9 CMV⁺ adventitial cells per section. Immunohistochemical assay for CMV early antigen only (not shown) yielded 36 ± 11 CMV⁺ adventitial cells per section. **D** and **E:** Immunohistochemical assay for CMV IE/early antigen in SV explant at 7 days after inoculation with clinical CMV isolate in fibroblast lysate (10^{4-5} PFU). CMV antigen is absent in luminal endothelium, neointima, and media (**D**), but present in lysate debris (arrowhead) at adventitial surface (**E**). White arrow points to vasa vasorum. Parallel studies of clinical CMV isolate prepared as cell-free virus or in EC lysate (10^4 PFU) also revealed absence of active CMV infection at 7 days after inoculation (not shown). **A** to **E** are counterstained with hematoxylin. Original magnifications: $\times 100$ (**A**, **B**, **D**, **E**); $\times 200$ (**C**).

The atherosclerosis was advanced, with extensive fibroatheromatous plaques and foci of calcifications that persisted in explant culture (Figure 4A). After inoculation with virus, CMV CPE was observed by H&E staining in patches of adventitial cells at 5 days after inoculation (Figure 4B). CMV IE/early antigen was also detected in the adventitial cells (Figure 4C) and was less frequently observed in intimal cells (not shown). Many of the cells expressing viral antigen also had distinctive morphological changes of CMV CPE (Figure 4D). Inoculation of atherosclerotic SV bypass graft explants of the other two donors with CMV Towne produced similar findings. Importantly, parallel analyses uniformly revealed absence of CMV IE/early antigen or CPE in atherosclerotic SV bypass graft explants that had not been inoculated with CMV.

Susceptibilities to active CMV infection were also examined in atherosclerosis-free native IMA from four donors and in an atherosclerotic IMA bypass graft removed at time of cardiac transplantation. Like SVs, they remained viable in explant culture and developed neointimal proliferation. Moreover, neither CMV IE/early antigen nor CPE was detected in these IMA explants at 5 days after inoculation with CMV Towne (10^7 PFU/ml) (not

shown). In contrast, explants of a severely atherosclerotic IMA bypass graft contained many cells expressing CMV IE/early antigen at 5 days after inoculation with CMV Towne (10^7 PFU/ml) (Figure 4E). The CMV antigen-expressing cells were located in adventitia and near the intimal surface, and many of them exhibited CMV CPE. IMA explants not receiving CMV were devoid of findings of CMV IE/early antigen and CPE, regardless of whether atherosclerosis was present or absent (not shown).

The applicability of these findings was determined in four native CAs from three hearts with advanced atherosclerosis. The explants were examined for presence of viral IE/early antigen and CPE at 0 to 7 days after inoculation with and without CMV Towne. In all explants receiving CMV, viral IE/early antigen and CPE were detected at 5 to 7 days after inoculation. These findings are exemplified in left anterior descending and right CAs containing fibroatheromata (Figure 5; A to D). Again, patchy involvement of the vessels was observed, with CMV⁺ cells located in adventitia and in proximity to or at the luminal surface. The actively infected adventitial cells are located at the outer surface, scattered in distribution, or focused around vasa vasorum.

Figure 3. CMV actively infects ECs and SMCs derived from human SVs. Isolated ECs (**A**) and SMCs (**B**) were incubated with GFP-expressing CMV Towne at 10 and 1 PFU/cell, respectively, and examined at 2 days after inoculation by fluorescence confocal microscopy. In separate experiments, infected SMCs (1 PFU/cell) were examined for expression of GFP (green fluorescence, **C**), CMV IE/early antigen (red fluorescence, **D**), or both GFP and CMV IE/early protein (yellow fluorescence, **E**). **A** to **E** represent results obtained in triplicate; uninfected ECs and SMCs did not emit GFP- or CMV antigen-specific fluorescence.

In severely atherosclerotic CA of another heart, an embedded intracoronary stent was removed to expose deep tissue layers to viral inoculum. Although CMV IE/early antigen and CPE were evident in cells in adventitia and in proximity to the luminal surface at 5 days after inoculation (Figure 5, E and F), they were not detected in the exposed muscular media. In yet another donor's atherosclerotic CA that was splayed open before inoculation with virus, the indicators of active CMV infection were absent at exposed tissue margins of the muscular media at 7 days after inoculation, but were frequently present in adventitia (not shown). Importantly, parallel studies of atherosclerotic CA explants incubated without virus did not reveal findings of CMV IE/early antigen or CPE in any of the four different CAs of three hearts. Thus, atherosclerotic CAs and bypass grafts commonly contain subpopulations of intimal and adventitial cells that are susceptible to active CMV infection.

Active CMV Infection in CD31⁺ Cells

The types of cells supporting active CMV infection were investigated by immunohistochemical assay for cell type-specific markers in an atherosclerotic CA explant inoculated with CMV Towne. As shown in Figure 6, both CMV early antigen and CPE are evident in cells in close proximity to the vessel lumen at 5 days after inoculation (Figure 6; A-C). The CMV⁺ cells did not express the common leukocyte antigen CD45 (Figure 6D) or the CD68 macrophage marker (not shown), although cells expressing these surface markers were detected in the tissue. In

contrast, a small subset of CMV-infected intimal cells express CD31 (Figure 6, E and F), an EC marker, and resemble ECs with regard to their location and morphological appearance. Such cells also reside in the lining of vasa vasorum (not shown). Notably, CMV early antigen and CPE are not observed in cells marking for SMC actin (Figure 6, G and H), suggesting that the infected cells are not SMCs or myofibroblasts. This result did not differ when medial SMCs are placed in direct contact with viral inoculum (Figure 6G) or when immunohistochemical analysis of CMV IE antigen is applied for detection of incipient viral replication (not shown). Thus, some CD31⁺ cells in atherosclerotic blood vessels support active CMV infection, whereas SMCs do not permit this infection.

Discussion

Human CMV is implicated as a co-factor in atherogenesis and vascular occlusion.^{2,3,11-13} We found that CMV Towne actively infected eight of eight different atherosclerotic blood vessel (SV/IMA grafts and CA) explants at 5 to 7 days after inoculation, whereas it only infected 2 of 14 nonatherosclerotic blood vessel (SV and IMA) explants (Fisher's exact test, $P = 0.001$) (Table 1). The CMV clinical isolate, grown previously in either fibroblasts or ECs, actively infected none of six sets of nonatherosclerotic SV explants at 5 to 7 days after inoculation. These findings suggest that atherosclerotic blood vessels are more conducive to active CMV infection compared to vascular counterparts without atherosclerosis. The predilection for atherosclerotic vascular explants accords with previously published reports indicating high frequency of CMV nucleic acids in atherosclerotic blood vessels, but not in atherosclerosis-free vascular equivalents, of persons who have acquired CMV *in vivo*.¹⁴⁻¹⁹

In atherosclerotic CA and bypass graft explants, we repeatedly found that active CMV infection presents as patchy involvement of intima and adventitia, suggesting that infectivity varies within a single vessel. A small subset of actively infected intimal and adventitial cells expresses CD31 antigen, an EC marker. These cells are likely of EC lineage based on their location at the luminal surface and in lining of vasa vasorum, as well as on the additional finding (not shown) of faint staining for CD34 by immunohistochemistry assay. This inference is also consistent with CMV's potential tropism for ECs⁴⁹ and infrequent reports of CMV inclusions in ECs lining the lumens of medium- and large-sized blood vessels of persons suffering from CMV disease.⁵⁰⁻⁵² Previous studies in isolated ECs have shown that CMV precludes cell proliferation²⁴ and elevates levels of procoagulant proteins,²⁵ leukocyte adhesion molecules,²⁹⁻³² and proinflammatory cytokines.³⁵⁻³⁷ Our finding that CMV may target ECs in atherosclerotic vascular tissue supports the hypothesis that virus-EC interactions might have proatherogenic and thrombotic effects that advance arterial disease and its complications.

The majority of actively infected intimal and adventitial cells are of an unknown cell type. They do not express markers of SMCs, myofibroblasts, lymphocytes, and

Figure 4. Active CMV infection in atherosclerotic SV and IMA bypass graft explants. **A–C:** Atherosclerotic SV bypass graft at 5 days after inoculation with cell-free CMV Towne (10^7 PFU/ml). H&E staining reveals fibroatheroma and calcification (**A**), with high-power view showing CMV CPE in adventitial cells (**B**). **C:** Immunohistochemical assay for CMV IE/early antigen shows patch of CMV⁺ cells in adventitia. **White arrow** points to calcification; **black arrows** point to CMV⁺ cells. **A to C** are representative of multiple sections of duplicate atherosclerotic SV explants. **D and E:** Atherosclerotic SV bypass graft of another donor at 7 days after inoculation with CMV Towne (10^7 PFU/ml). Immunohistochemical assay for CMV IE/early antigen reveals presence of CMV⁺ adventitial cells (**D**), with high-power view showing CMV⁺ cell lining lumen of vasa vasorum (**E**). **Arrowhead** points to adventitial surface. **D and E** are representative of multiple sections of quadruplicate atherosclerotic SV explants; 49 ± 19 CMV⁺ adventitial cells per section. **F:** Atherosclerotic IMA bypass graft explant at 5 days after inoculation with CMV Towne (10^7 PFU/ml). Immunohistochemical assay for CMV IE/early antigen reveals patch of CMV⁺ adventitial cells, 22 ± 6 CMV⁺ cells per section. **Arrowhead** points to adventitial surface. **F** is representative of multiple sections of this explant. A denotes adventitia. **C and F** are counterstained with fast red; **D and E** are counterstained with hematoxylin. Original magnifications: $\times 100$ (**A**); $\times 200$ (**C, D, F**), $\times 400$ (**B, E**).

macrophages. Their scattered distribution and focus around vasa vasorum in adventitia is a striking characteristic that is also evident in the exceptional cases of actively infected explants of native SVs. This observation has important implications because of growing awareness of the adventitia's role in development of atherosclerosis, in which direct adventitial injury or disease can produce neointimal lesions.⁵³ The aberrant increase in cellular infiltration and activation in adventitia of atherosclerotic arteries^{53,54} might furnish permissive targets for CMV infection that could consequently worsen vascular disease. Notably, reactive oxygen species and inflammatory cytokines in the atherosclerotic milieu^{55–58} enhance the CMV replicative cycle;^{27,28} also, the CMV major IE promoter, unlike the Rous sarcoma virus promoter, is much more active in atherosclerotic blood vessels as compared to normal vessels.⁵⁹

The inability to actively infect SMCs *in situ* was surprising, given the abundance of SMCs in the tissue and rapid activation of the CMV life cycle in isolated SMCs (Figure 3), regardless of whether they originate from neointima or

media (not shown). CMV antigens were not even expressed by tissue-resident SMCs in direct contact with viral inoculum at incision margins or after removing potential anatomical barriers, ie, endothelium or neointima (Figures 2 and 6). The discrepant outcomes in vascular explant and isolated cell cultures are unlikely to result from differences in SMC mitotic activity, because SMCs proliferate similarly in both experimental conditions (Figure 1).⁴⁶ Extracellular matrix of elastin, collagen fibers, and basement membrane regulate SMC function.⁶⁰ Although growth of isolated SMCs on a matrix of collagen or fibronectin did not alter their susceptibility to CMV infection, another physiological matrix component(s) might produce a difference. Pre-exposing isolated SMCs to heparin or growing them in fresh or conditioned medium used for culturing vascular explants also did not diminish CMV infectivity, indicating that resistance of tissue-resident SMCs to active infection is not a result of these experimental variables. Lastly, our findings are consistent with those reported by Yoshikazu and colleagues,⁶¹ who rarely found CMV nucleic acid *in situ* in SMCs of athero-

Figure 5. Active CMV infection in atherosclerotic CA explants after inoculation with cell-free CMV Towne (10^7 PFU/ml). **A–C:** Left anterior descending CA explant at 7 days after inoculation. H&E staining of fibroatheroma and calcification (asterisk; **A**). Immunohistochemical assay of CMV IE/early antigen in cell at luminal surface (**B**) and in adventitia cells (**C**). Arrow in **B** points to CMV⁺ cell; arrowhead in **C** points to vasa vasorum; 2 ± 1 and 133 ± 23 CMV⁺ intimal and adventitial cells per section, respectively. **D:** Right CA explant with fibroatheroma examined by immunohistochemical assay for CMV IE/early antigen at 7 days after inoculation. Arrows point to CMV⁺ cells located near or at the luminal surface. CMV⁺ intimal and adventitial cells per section, 2 ± 1 and 201 ± 47 , respectively. **E** and **F:** Atherosclerotic CA explant of another donor in which an intra-CA stent was removed before culture was subjected to immunohistochemical assay for CMV IE/early antigen in adventitia at 5 days after inoculation. Tissue contains 79 ± 12 CMV⁺ cells per section. **E** and **F** are representative of multiple sections of the vessel. A, L, M, and N denote adventitia, lumen, media, and neointima, respectively. **B** to **E** are counterstained with hematoxylin. Original magnifications: $\times 200$ (**A**, **C**); $\times 400$ (**B**, **D**, **E**).

matous abdominal aortic aneurysms with inflammation despite frequent detection of virus in other cell types, including ECs, primarily located in intima and adventitia. The basis for the difference in CMV's interaction with tissue-resident and isolated cells is the subject of future studies.

We can only speculate as to the reason for the greatly restricted CMV replicative activity up to 7 days after inoculation in explants of atherosclerosis-free SV and IMA. The findings could simply result from absence of cellular targets or receptor(s). Other possibilities include blockade by an extracellular matrix component(s), defective viral translocation to the nucleus, or delay or silencing of viral antigen expression despite viral entry. Furthermore, the possibility of minimally active or quiescent CMV infection taking place in these tissues cannot be excluded by our study. Only monocytes and monocyte-dendritic cell precursors have been shown, thus far, to support CMV latency.^{62–65} Whether these or other cell types in vascular explants are silently infected with CMV remains to be determined.

CMV Towne actively infects isolated SVs (Figure 3) and arterial ECs in culture.^{24,48} Therefore, we reasoned that this virus is appropriate for examining CMV's interaction with vascular tissue. Because CMV Towne replicates poorly in cultured human umbilical ECs,^{23,66–68} a low-passage clinical CMV isolate was also included in our studies. CMV interstrain variability has not been reported to affect viral replication in isolated vascular SMCs. Because the vast majority of published studies have used cell-free CMV to characterize the virus's interaction with

vascular cells in culture, this study was likewise designed to investigate potential outcomes conferred by infectious CMV particles free in medium or in cell lysate. Transfer of CMV to vascular tissue by cell-cell contact could plausibly yield different results, given that clinical CMV isolates are efficiently transmitted to isolated human umbilical vein ECs by neutrophils and monocytes/macrophages.^{36,69–71} Low-passage clinical CMV isolates may have greater need for cell-cell contact for transmission to blood vessel tissue than does CMV Towne, as the clinical isolate in cell supernatant or lysate appears to be less effective than Towne in actively infecting vascular explants (Figure 2). This hypothesis can be feasibly tested in the vascular explant model. Lastly, we only routinely examined vascular explants for up to 5 to 7 days after inoculation because beyond 10 days of culture there was progressive loss of tissue viability. The frequency and distribution of cells actively infected with CMV Towne did not appreciably differ when vascular explants were examined at 14 days after inoculation (not shown). Although it is possible that the actively infected cell profile might change after 14 days after inoculation,⁷² such a deviation in outcome would not detract from our primary conclusions.

In summary, we report that CMV actively infects atherosclerotic human blood vessels with greater efficiency than nonatherosclerotic vascular equivalents. This infection is limited to cells in intima and adventitia, and some of the infected cells express CD31 antigen, an EC marker. ECs and, in particular, SMCs residing in human blood vessels are relatively resistant to active CMV infec-

Figure 6. Identification of cell types in atherosclerotic CA supporting active CMV infection. Removal of intra-CA stent from atherosclerotic CA exposed underlying SMCs in media to viral inoculum. Adjacent sections of intimal aspect of CA explant at 5 days after inoculation with cell-free CMV Towne (10^7 PFU/ml) were examined by immunohistochemical assay for CMV early antigen (**A–C**), CD45 (**D**), CD31 (**E** and **F**), and α -SMC actin (**G** and **H**). The **black arrows** point to cells with CMV CPE. The SMC layer in contact with viral inoculum is evident in **C** and **G**. Immunohistochemical assay for each antigen was repeated once or twice on multiple sections of the single diseased CA. **A** to **H** are counterstained with hematoxylin. Original magnifications: $\times 40$ (**G**); $\times 100$ (**E**); $\times 200$ (**B–D**, **F**, **H**); $\times 400$ (**A**).

Table 1. Association of Atherosclerosis with Active CMV Towne Infection

Disease status of vessel*	Vessel type [†]	No. of donor vessels [‡]	No. of vessels with active CMV [§]	
Nonatherosclerotic	IMA	4	0	$P = 0.01^{¶a}$ $P = 0.001^{¶b}$
	SV	10	2	
	SV + IMA	14	2	
Atherosclerotic	Graft (IMA,SV)	4	4	
	CA	4	4	
	Graft + CA	8	8	

*Determination of atherosclerosis based on histological findings of H&E staining of tissue sections.

[†]SV, saphenous vein; IMA, internal mammary artery; CA, coronary artery; Graft, SV or IMA bypass graft of CA.

[‡]Number (No.) of different donors' vessels examined in replicate experiments, except for 4 CA examined from 3 different donors.^{||}

[§]Number (No.) of different donors' vessels supporting active CMV. Towne infection in explant culture after challenge with virus.

[¶]Fisher's exact tests comparing groups with a) atherosclerosis vs. those without, and b) atherosclerotic SV and IMA grafts with nonatherosclerotic SV and IMA.

tion as compared to SMCs and ECs isolated from this tissue. Although these findings do not resolve whether CMV contributes to atherosclerosis, they add importantly to our understanding of the nature of CMV's interaction with atherosclerosis-prone human blood vessels.

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