Adult human heart microvascular endothelial cells are permissive for non-lytic infection by human cytomegalovirus

Doris Ricotta, Giulio Alessandri, Caterina Pollara, Simona Fiorentini, Flavia Favilli, Maria Tosetti, Alberto Mantovani, Manuela Grassi, Emirena Garrafa, Livio Dei Cas, Claudio Muneretto, Arnaldo Caruso

Abstract

Objective: Human cytomegalovirus (CMV) infection has been linked to chronic heart disease. The mechanism of CMV dissemination to the heart remains unknown. CMV antigens and nucleic acid sequences have been detected in endothelial cells (ECs) in vivo, and ECs are fully permissive hosts to CMV replication in vitro. This report examines the characteristics of CMV replication in primary cultures of human heart microvascular ECs (HHMECs).

Methods: Capillary ECs were isolated from heart tissue biopsies of six patients at the time of heart surgery. HHMECs were infected with CMV and viral antigens were detected by immunofluorescence assay using monoclonal antibodies as specific reagents. Cytokine and chemokine release in the supernatant of sham- and CMV-infected cells was quantitated by ELISA. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to analyse expression of mRNA for adhesion molecules.

Results: CMV was found to productively infect HHMECs without cytolytic effects. Infected cultures released high levels of pro-inflammatory chemokines and enhanced their adhesion molecule expression.

Conclusions: Our data provide new insights into the mechanism of CMV dissemination to the heart, signalling the need for further investigation of the pathogenetic role of this virus in cardiac disorders.

Keywords: Infection/inflammation; Cell culture/isolation; Cytokines; Endothelial receptors; Endothelial function

1. Introduction

Cytomegalovirus is a widely distributed β-herpesvirus, as evidenced by the fact that most adults throughout the world are seropositive for the virus [1]. Being a member of the herpes group, CMV shares the tendency to establish latency/persistence in infected individuals, a state that is retained for life [2]. CMV reactivation often follows either HIV infection, with a related decline in the immune function, or pharmacologic immunosuppression in patients undergoing bone marrow, kidney or heart transplant [3–6]. With the exception of a mononucleosis-like syndrome, it is generally believed that CMV does not cause disease in immunocompetent individuals. However, this view may be incorrect because an increasing body of evidence suggests that CMV infection is linked to the development of vascular disease [7,8], including atherosclerosis [9]. More recently, CMV infection has been implicated in chronic inflammatory heart muscle disease, since CMV antigens and nucleic acid sequences have been detected in endomyocardial biopsies of patients with active myocarditis and dilated cardiomyopathy [10–13]. Inflammation is a characteristic of CMV-infected tissues, given that the presence of this virus is frequently accompanied by lymphocytic and monocytic infiltration. The endothelium forms an anatomical and functional barrier between circulating immunocompetent cells and underlying tissues and interacts...
dynamically with both in immunomodulation [14]. Indeed, the expression of endothelial cell adhesion molecules for leukocytes and the production by endothelial cells (ECs) of chemoattractive molecules known as chemokines are assumed to be an important component of the inflammatory process [15,16]. ECs are fully permissive hosts to AD 169 CMV in vitro [17,18], and serve as natural hosts to CMV infection in vivo [19,20], possibly forming a site of latency and persistence [21]. It is also well established that the expression of adhesion molecules on ECs and the production of chemokines by these cells are up-modulated by CMV infection [21,22]. In the light of all such findings, we decided it would be useful to investigate capacity of CMV to infect human heart microvascular ECs (HHMECs) and alter their biological activity. For this purpose, we isolated and purified HHMECs using a technique we have developed [23] and looked at the characteristics of CMV replication in these cells. It is important to perform CMV infection on HHMECs because ECs exhibit phenotypic and functional differences depending on their origin (adult versus foetal), anatomic localization, and vessel size (large versus capillary vessel) [24,25], which results in different abilities to support CMV replication [17,18]. In the present study we show that CMV infects HHMECs — thus producing non-lytic infection and a continuous release of virus for the lifespan of the culture — and influences the expression of adhesion molecules and chemokine production. This evidence supports the argument that CMV can productively infect HHMECs in vivo, initiating a cascade of events that fosters the development of inflammatory processes.

2. Methods

Heart tissue biopsies (auricles) were obtained at the time of heart surgery from six immunocompetent patients suffering from aortic insufficiency and aortic aneurysm (average age 58 years, range 45–79 years). The present study conforms with the principles outlined in the Declaration of Helsinki (Cardiovasc Res 1997;35:2–4).

2.1. Isolation of HHMECs

Isolation and purification of capillary endothelial cells were performed as recently described [23]. Briefly, heart tissue was finely minced with scissors and then digested by incubation for 2 h at 37°C in M199 medium (Gibco, Paisley, UK) containing 0.25% collagenase D (Boehringer, Mannheim, Germany), 0.25% dispase (Boehringer) and 0.25% bovine serum albumin (BSA). The cell suspension was passed through a 50-μm pore size filter (Consul™, Turin, Italy) to discard all macroaggregates, and resuspended in EBM medium (Clonetics, San Diego, CA) supplemented with 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine (Gibco), 100 μg/ml heparin (Park-Davis, Milan, Italy), 1 μg/ml hydrocortisone (Clonetics), 10 ng/ml human epidermal growth factor (EGF, Clonetics), and 10 μg/ml bovine brain extract (BBE, Clonetics) (EBM complete medium). Cells were then seeded into 25-cm² flasks coated with 1 μg/cm² collagen type I (collagen S bovine skin, Boehringer). After 7–10 days of culture, HHMECs were separated from other contaminating cells (mostly fibroblasts) using paramagnetic beads covalently bound with CD31 monoclonal antibody (mAb) (Dako, Carpenteria, CA). This procedure was repeated twice to obtain high-purity HHMEC cultures. The primary cultures, grown in 24-well plates, usually reached confluency after 10–14 days and were then transferred to collagen type I-coated T25 flasks. This was the first in vitro passage. The endothelial origin of HHMECs was assessed by their staining for CD31 and for von Willebrand factor (vWF) antigen expression, using CD31 and vWF mAbs (Dako, Milan, Italy) as specific reagents and confirmed by their staining for CD45 and CD14 markers, as assessed by flow cytometry using specific mAbs as reagents (Leukogate, Becton Dickinson, San Jose, CA). All experiments were performed using HHMECs at the fourth to fifth in vitro passage.

2.2. Viral infection of cell monolayers

CMV AD 169, a CMV strain known to productively infect ECs in vitro [17,18], was obtained from the American Type Culture Collection (Rockville, MD). Virus stock was generated by serial propagation through multiple passages in human pulmonary embryonic diploid fibroblasts. Virus in culture supernatant was titrated as already described [26]. Then 1-ml aliquots of the viral stock (8×10⁶ p.f.u./ml) were stored at −80°C until use. Inactivation of some aliquots of the viral stock was achieved by UV irradiation, leaving supernatants for 4 h in an open sterile Petri dish at a distance of 4 cm from a 30-W UV lamp (Philips, Eindhoven, The Netherlands). The UV-treated CMV was not capable of inducing any cytopathic effect on human pulmonary embryonic diploid fibroblasts. HHMECs were seeded at 2×10⁵ cells into collagen type I-coated 25-cm² flasks or at 2×10⁶ cells/well into 24-well plates containing collagenated glass coverslips.

Infection with CMV was performed in heparin-free medium on a 70% confluent HHMEC monolayer. Cells were washed three times in PBS and infected with CMV, diluted at an multiplicity of infection (MOI) of 10, for 2 h at 37°C in 5% CO₂. Mock HHMEC infection was performed with the same procedure but using UV inactivated viral inoculum. Simultaneous infection of human pulmonary embryonic diploid fibroblasts, used as a positive control, ensured that CMV infectivity occurred within the same time frame in each experiment performed.
2.3. PCR for CMV DNA

CMV-infected or mock-infected (using UV inactivated CMV) HHMECs were split every 6 days and re-fed with fresh medium. Cells were detached at 8 and 20 days post infection, washed with PBS and pelleted by centrifugation at 800×g for 10 min at room temperature. DNA was extracted and purified from pelleted cells by Nuclisens (Organon Tecnika, Boxtel, The Netherlands) following the manufacturer’s instructions. The CMV immediate early (IE) 1 gene was amplified with primer IE1 sense 5’- agacctgcatgcatcctc-3’ and primer IE1 antisense 5’- ggtgccgcaacagttgacatc-3’, given a 250-bp fragment purchased from Sorin Biomedica (Saluggia, Italy). Polymerase chain reaction (PCR) was performed in a total volume of 50 μl containing 10 mmol/l Tris–HCl, pH 8.3, 50 mmol/l KCl, 2.5 mmol/l MgCl₂, 1 mol/l dNTPs, 0.01% gelatin (all purchased from Boehringer), and 2 U of Taq DNA polymerase (AmpliTaq Gold, Perkin-Elmer, Norwalk, CT). Amplification was performed for the first cycle at 94°C for 10 min, at 94°C for 4 min, at 55°C for 1 min and at 72°C for 1 min. The following 49 cycles were carried out as follows: at 94°C for 1 min, at 55°C for 2 min and at 72°C for 1 min. A final elongation step was carried out for 7 min at 72°C. Then 10 μl of the amplified product were analysed by electrophoresis with 3% agarose gel stained with ethidium bromide.

2.4. Detection of viral antigens

Cells cultured into 24-well plates containing collagen type I-coated glass cover slips were fixed for 20 min at room temperature using 100% ice cold methanol. Monolayers were incubated for 1 h at 37°C with one of the following FITC-conjugated antibodies to CMV, immediately (IE) p72 and CMV lower tegument phosphoprotein UL83/pp65 (both purchased from Argene-Biosoft, Varilhes, France), and CMV late antigen UL55/gB, from Virostat (Portland, ME). Monolayers were also double stained for UL55/gB and CD31, using a phycoerythrin (PE)-conjugated anti-CD31 mAb (Becton-Dickinson, San Jose, CA). The glass cover slips were washed three times with PBS and mounted onto slides using gallocate in 50% glycerol, 0.1 M Tris–HCl, pH 8.0. Immunofluorescence was monitored using an upright Leitz fluorescence microscope or, for double immunofluorescence, a confocal laser scanning microscope MCR-1024 (BioRad, Hemel Hempstead, UK) fitted with a 30-mW krypton–argon ion laser and a Zeiss Axiovert 100 microscope. A slowfade antifade (S.D.) of the three replicate samples was less than 10% of the mean in all experiments.

2.5. Extracellular virus titration

Supernatants from infected and mock-infected cells were removed at 2, 3, 4, 8 and 20 days post infection and, following clarification by centrifuging at 1200×g for 10 min, stored as 1-ml aliquots at ~80°C until use. The released virus was titrated on human pulmonary embryonic diploid fibroblast monolayers as above.

2.6. Reverse transcriptase–polymerase chain reaction (RT–PCR) for the analysis of adhesion molecules

Total RNA extraction and RT–PCR analysis were performed as previously described [27]. Briefly, total RNA was purified by RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer’s instructions, with liquid nitrogen to prevent RNA degradation. cDNA synthesis was performed with 1 μg of RNA in a total volume of 20 μl, containing 10 mmol/l Tris–HCl, pH 8.3, 50 mmol/l KCl, 5 mmol/l MgCl₂, 1 mol/l dNTPs (Boehringer), 20 U of ribonuclease inhibitor (Ambion, Austin, TX), 2.5 mmol/l of oligo(dT) (Perkin Elmer), and 50 U of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer). The reaction mixture was incubated at 42°C for 40 min, heated to 94°C for 5 min to inactivate reverse transcriptase, and cooled on ice for 5 min. A 10-μl aliquot of the cDNA thus obtained was amplified in a 20-μl reaction solution containing 50 mmol/l KCl, 10 mmol/l Tris–HCl, pH 8.3, 1.5 mmol/l MgCl₂, 0.2 mmol/l each dNTP, 200 mmol/l of each primer, and 1 U of Taq DNA polymerase (Boehringer). The primers for β-actin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin (Genset, Paris, France) and cycling conditions were as previously described [28]. A sample (15 μl) of each PCR reaction was electrophoresed through 1.5% agarose gel and visualized with ethidium bromide. β-Actin PCR product was used as an external standard and the mRNA expression of each molecule was evaluated by comparison between the PCR product and β-actin PCR product.

2.7. Cytokine and chemokine production analysis

Cell-free culture supernatants from CMV-infected HHMECs and mock-infected HHMECs were harvested at different days post infection and cryopreserved at ~70°C in 100-μl aliquots before testing. Each aliquot was thawed once and discarded after being assayed for the presence of IL-6 and IL-8 with two separate ELISA kits (Biosource International, Camarillo, CA), and for the presence of proinflammatory chemokine monocyte chemoattractant protein (MCP)-1 by ELISA (Biosource International). Each sample was run in triplicate. Standard deviation (S.D.) of the three replicate samples was less than 10% of the mean in all experiments.

3. Results

3.1. Morphologic and phenotypic features of HHMECs

Successful HHMEC cultures were obtained from 50% of
the samples processed. Cultured cells were found to express specific EC markers. They showed an intense granular perinuclear immunofluorescence staining for vWF (Fig. 1a) and the presence of CD31 antigens on the cell surface. CD31 was expressed at a higher density along cell borders when the HHMEC monolayer reached confluency (Fig. 1b). The endothelial cell origin was further demonstrated by the ability of cells to form capillary-like tubular structures when plated on matrigel (data not shown).

HHMECs were morphologically examined by light microscopy (Fig. 2). Subconfluent cultures exhibited a distinctive morphology, with a prominent nucleus and two nucleoli per cell. HHMECs maintained a uniform morphology and a doubling time of 32–48 h even after five to seven in vitro passages, forming a typical homogeneous monolayer of elongated cells in close contact. After eight to ten passages HHMEC cultures developed a morphologic variability due to the appearance of several giant cells and a decline in vWF positivity, which signalled an early aging of cultures. Aging of cultures was not found to influence CD31 surface expression (data not shown).

3.2. CMV productively infects HHMECs

We investigated the ability of CMV to infect primary cultures of HHMECs in vitro. Subconfluent HHMEC monolayers were infected or not with CMV and the occurrence of viral infection was assessed by immuno-
fluorescence assay. HHMECs were fixed at various intervals after infection and examined for the presence of CMV p72, pp65 and gB antigens. Fig. 3 shows that HHMECs were highly susceptible to CMV infection, as demonstrated, respectively, by the presence of CMV p72 (a) and pp65 (b) antigens in the nuclei of infected cells. An examination of p72 expression in HHMECs revealed the presence of this antigen between 3 and 4 days post infection. A peak of p72 expression (mean percentage value of 63.7%, range 35–78.6%) was usually observed at 5 days post infection. Expression of CMV pp65 was delayed, as compared to that of p72, with the first antigen appearing between 5 and 6 days post infection and a peak of expression usually at 7 days post infection. The mean percentage value of pp65-positive cells was 56.6%, within a range of 32.4–68.7%. The frequency of CMV antigen detection in HHMECs did not differ from that observed in human pulmonary embryonic diploid fibroblasts but the kinetics of viral antigen expression differed between HHMECs and human pulmonary embryonic diploid fibroblasts. In fact, the first appearance of p72 in infected human pulmonary embryonic diploid fibroblasts occurred at 1 day post infection and peaked at 3 days post infection, whereas pp65 expression usually occurred at 3 days post infection and peaked at 5–6 days post infection. To confirm that HHMECs are fully permissive hosts for CMV replication, the expression of the CMV late antigen gB, a virion envelope-associated glycoprotein, was investigated within the CD31-positive ECs by double staining immunofluorescence assay. An examination of gB expression revealed the presence of antigen in intracellular vacuoles and at the plasma membrane of CD31-positive HHMECs (Fig. 3c).

Despite expression of the three CMV specific antigens, HHMECs infected at an MOI of 10 exhibited no cytopathic effect, even at 20 days post infection. Since HHMECs expressed virion-associated protein, the release of infectious virus in the supernatant was evaluated. As

![Figure 3](image-url)
shown in Table 1, extracellular virus was recovered at 4 days post infection and reached a plateau at 8 days post infection, which persisted up to 20 days post infection. Viral production by HHMECs averaged more than 100-fold less than that by human pulmonary embryonic diploid fibroblasts until day 8. However, by day 5 many of the human pulmonary embryonic diploid fibroblasts were dying, whereas the HHMECs were still confluent and had to be split several times until physiological senescence of culture.

### 3.3. Effects of CMV infection of HHMECs on the production of IL-6, IL-8 and MCP-1

The effects of CMV infection on the production of pro-inflammatory cytokine IL-6 and inflammatory chemokines IL-8 and MCP-1 by HHMECs was monitored at various stages after infection. Since the viral stock obtained from human pulmonary embryonic diploid fibroblasts may contain inducers of this pro-inflammatory cytokine and chemokines, we used UV inactivated CMV to mock-infect HHMECs as a negative control. Inactivated CMV induced no cytopathic effect on HHMECs and no viral DNA was detected in cell extracts even at 20 days post infection — unlike viable CMV, whose DNA was detected at both 8 and 20 days post infection (data not shown). Supernatants from three different CMV-infected or mock-infected HHMEC cultures were harvested at various times, and fresh medium was added after each harvest. The level of cytokine and chemokine production in each supernatant was measured by immunoassay. Production of IL-6, IL-8 and MCP-1 was consistently up-regulated by CMV infection as early as 48–96 h after CMV infection (data not shown), with peaks of IL-6, IL-8, and MCP-1 production at 6–8 days post infection. As representatively shown in Table 2, peak titers of IL-6, IL-8 and MCP-1 released in the supernatant of CMV-infected cells were more than 2.0-fold higher than those released by mock-infected cells.

### 3.4. Expression of adhesion molecules on CMV-infected HHMECs

Total RNA was extracted from three different CMV-infected HHMECs at the time they expressed the viral protein p72, as assessed by immunofluorescence assay. This usually occurred at 3–4 days post infection. On the same days, total RNA was extracted from mock-infected HHMECs. Using reverse transcription followed by cDNA amplification, we established that despite variation in the constitutive level of adhesion molecules across different batches of HHMECs, the cells usually exhibited a modest expression of ICAM-1 and VCAM-1 mRNAs. E-selectin mRNA was not detectable in any of the HHMEC cultures analysed. As representatively shown in Fig. 4, CMV infection consistently increased the expression of ICAM-1 mRNA in HHMECs. Semi-quantification of PCR product revealed that, in three different HHMEC cultures, the mean value of the ICAM-1:β-actin mRNA ratio was 4.3-fold higher in CMV-infected cultures, as compared to mock-infected ones. In two out of the three different HHMEC cultures, CMV infection also induced an increased level of VCAM-1 mRNA expression, with the average VCAM-1:β-actin mRNA ratio 4.8-fold higher than in mock-infected cultures. In HHMEC cultures showing increased levels of VCAM-1 mRNA expression, CMV was also able to induce de novo expression of E-selectin mRNA (Fig. 4).

![Fig. 4. Detection of ICAM-1, VCAM-1, E-selectin and β-actin mRNA expression by RT–PCR. Total RNA was extracted from uninfected (a) or CMV-infected (b) HHMECs. The results on expression of VCAM-1 and E-selectin are representative of two out of three independent experiments performed with HHMECs from heart biopsies of different patients.](image-url)
4. Discussion

Several studies emphasize the role of CMV in the development of heart disease, as viral antigens and nucleic acid sequences have been detected in ECs of myocardial biopsies obtained from patients suffering from active myocarditis as well as dilated cardiomyopathy [10–13]. Limited evidence on the ability of CMV to productively infect HHMECs has hampered our understanding of the underlying mechanism that links CMV infection to heart disease.

The present study sought to establish whether CMV can infect heart microvessels and produce, either per se or through an inflammatory process, vascular alterations leading to heart disease. For this purpose we isolated and purified ECs from heart microvessels and investigated the features of CMV replication in such cells.

Our investigation shows that CMV AD 169 productively infects HHMECs obtained from different donors: this results in non-lytic infection of cells and in continuous release of infectious extracellular virus for the culture’s lifespan. Our data on non-lytic CMV replication in HHMECs differ from that of strong lytic effect induced by AD 169 in brain microvascular ECs [17], which confirms the need for further research on virus replication in biologically relevant cell types. Productive infection in the absence of cytolytic effects is a pre-condition for establishing persistence. Since in vitro CMV infection of HHMECs is non-cytopathic and HHMECs are infected in vivo, our evidence suggests that the microvascular endothelium may function as a CMV reservoir in vivo.

CMV is known to inhibit the proliferative activity of human fibroblasts by arresting G1/S transition [29,30]. On the other hand, we consistently observed that the number of CMV-infected and mock-infected HHMECs remained comparable as far as 20 days post infection. This inability of CMV to block HHMEC proliferation may be a form of co-existence between virus and cell. If the complexity of CMV influence on the cell cycle is uncovered, the mechanism whereby HHMECs elude blocks in the cell cycle may also be understood.

Despite its non-cytolytic effect on HHMECs, CMV infection induced cells to secrete high amounts of chemoattractants, such as the CXC chemokine IL-8 and the CC chemokine MCP-1. Both chemokines are associated with a number of human inflammatory diseases [31] and are known to promote cellular migration, activation and proliferation through binding of their respective G protein-coupled receptors on target cells [32]. In particular, IL-8 is known to attract mostly neutrophils, whereas MCP-1 is considered a major chemoattractant for monocytes and lymphocytes [15]. Moreover, MCP-1 is a major chemoattractant of CMV-infected vascular smooth muscle cells (VSMC), which according to recent studies may play a critical role in the development of vasculopathy prompted by vessel narrowing [33]. Both chemokines have also been shown to play an important role in inducing transendothelial migration and allowing firm adhesion of leukocytes to ECs through adhesion molecules [34].

We also established that CMV increased the expression of adhesion molecules on HHMECs and it is known that the expression of adhesion molecules for leukocytes on ECs is an important factor in the inflammatory process. In particular, CMV infection was found to constantly increase the expression of ICAM-1 and — in some HHMEC cultures — of VCAM-1 together with the de novo expression of E-selectin. Both ICAM-1 and VCAM-1 are known to be responsible for leukocyte–EC interaction and leukocyte extravasation [35,36] whereas E-selectin is known to mediate the adhesion of neutrophils, monocytes and T-lymphocytes to ECs [37]. These findings are suggestive of high rates of transendothelial leukocyte migration and accumulation in the sites of CMV replication. Moreover, enhanced secretion of the inflammatory cytokine IL-6 by CMV-infected HHMECs probably amplifies the inflammatory process and transendothelial leukocyte migration [38].

Inflammatory processes and local cellular immune response are likely to damage ECs, which support CMV replication. There is evidence, however, that the virus takes advantage of these events: Sodeberg-Naucler et al. [39], have proved that the inflammatory process is capable of inducing CMV reactivation from latency. In addition, CMV is known to be primarily disseminated by cell-to-cell contact, so that any process assisting cell adhesion to CMV-infected ECs could also favour viral dissemination. Finally, neutrophils and monocytes attracted by α and β chemokines can be infected by virus after contact with infected ECs and/or during transendothelial migration [40,41] and become themselves carriers of infectious virus, thus playing a role in virus dissemination.

This paper points to the importance of CMV in HHMEC biology. Though CMV is not capable per se of inducing cytolysis and disruption, it increases the release of inflammatory chemokines and the expression of adhesion molecules that are likely to favour local accumulation of leukocytes and the generation of inflammatory processes. Inflammatory events coupled with VSMC infiltration may predispose to vascular alterations and consequently to heart disease. Our data provide new insights into the mechanism of CMV dissemination to the heart, signalling the need for further investigation of the pathogenetic role of this virus in cardiac disorders. The considerable heterogeneity of myocardial ECs — which is more marked among microvascular and large vessel ECs, reflecting EC specialisation into different functions — calls for extra research into the ability of CMV to replicate in ECs from different regions of the heart. This line of enquiry should be able to account for the mechanisms of CMV replication, persistence and trafficking in such a vital organ.
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