

# Endothelial cells in human cytomegalovirus infection: One host cell out of many or a crucial target for virus spread?

Barbara Adler<sup>1</sup>; Christian Sinzger<sup>2</sup>

<sup>1</sup>Max von Pettenkofer Institut für Virologie, Ludwig-Maximilians-Universität München, München, Germany; <sup>2</sup>Institut für medizinische Virologie, Universität Tübingen, Tübingen, Germany

## Summary

Endothelial cells (EC) are assumed to play a central role in the spread of human cytomegalovirus (HCMV) throughout the body. Results from in-situ analyses of infected tissues and data from cell culture systems together strongly suggest that vascular EC can support productive replication of HCMV and thus contribute to its haematogeneous dissemination. By inducing an angiogenic response, HCMV may even promote growth of its own habitat. The particular role of EC is further supported by the fact that entry of HCMV into EC is dependent on a complex

of the envelope glycoproteins gH and gL with a set of proteins (UL128–131A) which is dispensable for HCMV entry into most other cell types. These molecular requirements may also be reflected by cell type-dependent differences in entry routes, i.e. endocytosis versus fusion at the plasma membrane. An animal model with trackable murine CMV is now available to clarify the pathogenetic role of EC during haematogeneous dissemination of this virus.

## Keywords

Human cytomegalovirus, HCMV, endothelial cell, gH/gL complexes

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## Introduction

Human cytomegalovirus (HCMV) is a ubiquitous human herpesvirus that after primary infection persists for the life span of the host. Infections of immunocompetent humans are mainly asymptomatic and the lifelong persistence does usually not result in reactivations causing disease. Yet, in immunocompromised patients like transplant and AIDS patients, HCMV infection and reactivation often results in acute disease and is a major cause of morbidity and mortality. It is also the leading cause of birth defects among congenitally transmitted viral infections. In acute systemic infection, HCMV spreads throughout the organism. Dissemination presumably happens through the haematogeneous route and besides leukocytes, vascular endothelial cells are the candidate host cells to promote this route of virus spread. As endothelial cells are located at the border between the circulation and organ tissues they might also function in spread of virus from the circulation to the organs and thus be directly involved in the development of HCMV-associated organ infections in acute disease. HCMV infected endothelium is also assumed to

play a role in modulation of inflammatory responses during acute infection and in development of vascular diseases like atherosclerosis, arterial restenosis and transplant vascular sclerosis. Recently determinants of HCMV endothelial cell tropism have been identified.

This article depicts the role of the endothelial cell (EC) in the HCMV infection and the genes and mechanisms determining the endothelial cell tropism of the virus. In addition it proposes new approaches to study the role of infected EC in CMV infections.

## The vascular endothelium as a target of HCMV infection

*In vivo*, HCMV shows an exceptionally wide host cell range, including epithelial cells of glands and mucosal tissues, connective tissue cells in various organs, hepatocytes, smooth muscle cells, various leukocyte populations and vascular endothelial cells (1, 2). In acute infection, virtually any organ can be infected (3). The finding of viremia in posttransplant patients (4) and transmission

Correspondence to:  
Barbara Adler  
Max von Pettenkofer-Institut  
Genzentrum, Virologie  
Feodor-Lynen Straße 25  
D-81377 München, Germany  
Tel.: +49 89 218076862, Fax: +49 89 218076899  
E-mail: adlerb@lmb.uni-muenchen.de

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of HCMV by blood transfusion (5) indicate that virus can spread through the haematogeneous route. Infectivity is predominantly found in the polymorphonuclear fraction of whole blood (4) and removal of white blood cells from whole blood prior to transfusion almost completely abolishes the risk of HCMV transmission (5) thus emphasising the role of circulating cells for virus dissemination. Granulocytes themselves are abortively infected but may serve as a vehicle for infectious virus (6, 7). Monocytes become permissive upon differentiation into tissue macrophages and may then release infectious progeny (8–10). Vascular endothelial cells are particular as they have been found productively infected within the circulation (11, 12) strongly suggesting their contribution to viral dissemination.

In immunocompromised patients infected vascular endothelial cells could be detected in capillaries and venules of various organ tissues by immunochemistry (1, 13, 14). They express immediate early, early and late proteins and thus seem to support the complete viral replication cycle. The focal distribution of HCMV infected endothelial cells in vessels strengthens the conclusion that the EC infection *in vivo* is productive (15). Often, the tissue surrounding infected endothelium shows other HCMV infected cell types and additionally infiltrations of inflammatory cells (13, 16). Infected EC are a regular finding in tissues of post-transplant and AIDS patients and congenitally infected newborns. Yet, the degree of endothelial involvement is variable, showing large interindividual differences (13). Infected EC can also detach from the vessel wall and enter the blood stream. They can be detected as circulating cytomegalic infected cells (CCIC) (12, 17). Staining for EC surface markers identified them as endothelial cells. CCIC express late viral antigens and electron microscopical analyses showed viral capsids in the nucleus and cytoplasm (11, 17). Thus, CCIC may be capable of spreading the infection. From *in-vitro* co-culture experiments it is known that HCMV infected neutrophils cannot transfer infection to placental cytotrophoblasts whereas HCMV infected uterine microvascular endothelial cells readily transmit infection to cytotrophoblasts, which led to the hypothesis that also *in vivo* endothelial cells might play a crucial role in intrauterine infections (18).

In contrast to the detection of infected endothelial cells in acute disease, analyses of different vascular tissues of healthy seropositive individuals for HCMV DNA and antigen are controversial. Some experimental data show HCMV DNA in vessel walls of major arteries of healthy sero-positive individuals (19–21), data which were taken as evidence for either a persisting productive infection or a latent infection in vessels. Others could not detect viral DNA, neither in endothelial nor in smooth muscle cells (22–24). Functional diversity of endothelial cells from different anatomical locations has been suggested as one explanation for controversial results, but still there is rather circumstantial evidence than formal proof for HCMV persistence or latency in endothelial cells (25).

The vascular endothelium can be productively infected *in vivo*, and virus very likely spreads from infected endothelial cells by release into the blood stream, contact with adjacent cells or transiently adhering leukocytes or by detachment of infected EC. Yet, it remains an open question whether the endothelial cell is just one more cell type infected by HCMV-infected circulating leukocytes passing by or whether the vascular endothelium plays

a crucial role in promoting virus spread by mediating transfer from the circulation to organs and vice versa.

## Vascular endothelium pathologies associated with HCMV infection

The pathogenetic role of HCMV infected endothelial cells is a matter of speculation. During an acute HCMV infection the detection of HCMV infected EC is associated with mixed-cellular inflammatory infiltrates consisting of granulocytes, macrophages and lymphocytes (13). The infected endothelial cells are characterised by an increased expression of the adhesion molecules ICAM-1 (inter-cellular adhesion molecule-1) (CD54) and VCAM-1 (vascular cell adhesion molecule-1) (CD106) and of the activation marker CD40 (26, 27). Upregulation of activation and adhesion markers like, ICAM-1, VCAM-1, CD40 and ELAM1/E-selectin (CD62A) can also be observed after *in-vitro* infections of EC cultures with HCMV (27–29). In cell culture it could be shown that upregulation of these surface markers is through direct infection and also through paracrine effects on non-infected bystander cells (30). The activation of the endothelial cells results in increased adhesion and activation of leukocytes and platelets (7, 28, 31). Infected EC monolayers show an increased permeability which facilitates migration of monocytes through the cell layer and thereby bilateral infection of EC and monocytes (32, 33). Binding of HCMV to EC induces an angiogenic response in the cells through activation of the phosphatidylinositol 3-kinase and the mitogen-activated protein kinase signalling pathways (34, 35). Whereas attraction of leukocytes by activated endothelial cells is discussed mainly in the context of dissemination of the virus, the angiogenic response is considered a potential mechanism for HCMV associated vascular disease. Dysregulated angiogenesis after viral infection may lead to atherosclerotic plaques in the vessel wall (36, 37). In transplant vascular sclerosis, a hallmark of chronic rejection of heart transplants, there is convincing evidence for a role of HCMV (38–40). Here treatment with ganciclovir, a potent inhibitor of HCMV replication, significantly delays posttransplant vascular disease. A general HCMV aetiology for the development of atherosclerosis could not convincingly been shown (41–43). There is evidence for increased anti-HCMV IgG levels and HCMV DNA in atherosclerosis patients (41), yet it could be shown that *ex vivo* atherosclerotic blood vessels are more susceptible to HCMV infection than non-atherosclerotic vessels (44).

## Infection of endothelial cells in culture

Similar to the *in-vivo* situation, a wide spectrum of primary cell cultures also support the complete HCMV replication cycle *in vitro*, including skin and lung fibroblasts, vascular smooth muscle cells (45), epithelial cells of the retina (46), placental trophoblast cells (47), hepatocytes (48), neuronal and glial brain cells (49), kidney epithelial cells (50), monocyte-derived macrophages (8), monocyte-derived dendritic cells (51), and vascular epithelial cells (52, 53). The first successful infection of endothelial cells *in vitro* was the infection of umbilical vein en-

endothelial cells (HUVEC) (52), which have become the standard endothelial cell culture for HCMV. HCMV has also been successfully used to infect venous, arterial and microvascular endothelial cells from umbilical vein, placenta, intestine and brain (9, 54–57). *In vivo*, endothelial cells are a highly diverse population of distinct cell types (58) and therefore the source of the primary endothelial cell culture may affect the susceptibility and cellular response to HCMV infection (59). It is for example known, that micro- and macrovascular endothelial cells differ in their expression levels of surface molecules like integrins, epidermal growth factor receptor (EGFR) and platelet-derived growth factor- $\alpha$  receptor (PDGF- $\alpha$ R) which are involved in HCMV entry (59, 60). Indeed, infection of HUVEC compared to microvascular cells (56) and microvascular cell lines (Scrivano and Adler, unpublished observation) is reduced. In addition, it has been shown in a comparative analysis of infections of brain microvascular and aortic macrovascular cells that virus release from macrovascular cells is much more efficient than virus release from microvascular cells. The macrovascular cells consequently showed reduced levels of intracellular virus compared to supernatant virus and this efficient removal of intracellular virus prolonged the survival of the aortic cells as compared to the survival of the microvascular cells (25).

Production of infectious virus in endothelial cell culture is accompanied by pathogenic effects like cell rounding and enlargement, formation of inclusion bodies and lysis of infected cells. Thus, the HCMV infection of EC strongly resembles the infection phenotype of fibroblast infection, the standard cell culture system to propagate HCMV *in vitro*. Productive, lytic infection in cell culture supports the concept that *in vivo* HCMV can directly induce lesions in the vascular endothelium.

EC culture systems have also been used to characterise functional changes of endothelial cells after HCMV infection. Here, it was of particular interest to study effects of HCMV infection on interactions of endothelial cells with leukocytes. As described in the previous section activation of HCMV, directly or through paracrine effects of infected neighbouring EC, leads to the up-regulation of activation and adhesion markers and to an increased adhesion of polymorphonuclear cells, monocytes and lymphocytes to the endothelial cells. At contact EC and adhering leukocytes can transfer infection from one cell to the other (7, 61). It is noteworthy, that the mere contact of virions with endothelial cells, in the absence of productive infection, may be sufficient to induce an angiogenic response. This could be shown by infection experiments using non-endotheliotropic HCMV strains and UV-inactivated virus which both elicited comparable angiogenic responses after co-culture with EC (34, 35).

In contrast to cell culture-adapted HCMV strains, recent clinical isolates grow strictly cell-associated (62). They do not release cell-free infectious virus progeny in cell culture supernatants, but show an efficient focal spread in cell monolayers (63). With passage, clinical isolates adapt to the culture conditions by switching from a cell-associated way of spreading to propagation of virus infection through supernatant virus. If passing takes place in fibroblast cultures, this is usually associated with the loss of the capability to infect endothelial cells (64). Most of the HCMV strains used over the last decades to study HCMV biology *in vitro*, like AD169, Towne and Toledo, are

therefore not capable of infecting endothelial cells. Several studies could show that clinical isolates are mixtures of endotheliotropic and non-endotheliotropic virus variants (64–66). The loss of endothelial cell tropism of HCMV isolates passaged on fibroblasts is selection and enrichment of non-endotheliotropic variants (63, 64). Yet, even after prolonged passage on fibroblasts, virus progeny often remains a mixture of genetically different endotheliotropic and non-endotheliotropic viruses and endotheliotropic variants can be reselected by passage on EC (66–69).

## HCMV gH/gL complexes as determinants of endothelial cell tropism

Comparing genomes of endotheliotropic and non-endotheliotropic HCMV strains, obtained by passage on endothelial cells or fibroblasts, respectively, revealed restriction length polymorphisms. This indicated that during passage certain genetic variants are lost or selected for (63, 70). It was soon recognised that the UL/b' boundary in the HCMV genome is deleted, reversed or shows point mutations in genomes of laboratory strains when compared to genomes of fresh isolates (71, 72). Three genes in this region, UL128, UL130 and UL131A, were identified to be consistently mutated in laboratory strains (73, 74) and mutation of one, two or all three genes abolishes virus growth in endothelial cells (74, 75). Clinical isolates all express functional UL128, UL130 and UL131A genes which in contrast to many virion proteins are highly conserved among all isolates tested so far (76).

Cloning of complete herpesvirus genomes as bacterial artificial chromosomes, mutation of these genomes in *E. coli* and subsequent reconstitution of mutant genomes as infectious virus in eukaryotic cells, has started a new era of genetic analysis of the functions of herpesviral genes (77, 78). Since the cloning of the prototypic laboratory strain AD169 (78), many different HCMV strains, endotheliotropic and non-endotheliotropic, have been cloned as bacterial artificial chromosomes, sequenced and successfully used to study single gene functions by mutation of these genes within the viral context (69, 79–81). They have also been important tools to study the role of the UL128, UL130 and UL131A genes in endothelial cell infection. The genome of the HCMV strain AD169 carries a stop mutation in the UL131A reading frame. Repair of this mutation restored the tropism of the non-endotheliotropic AD169 strain for endothelial and epithelial cells (82, 83). Repaired viruses showed an impaired release of virus progeny from fibroblasts and thus a slower virus spread in fibroblast cultures (82, 83). This might explain why non-endotheliotropic HCMV variants with mutations in the UL128–131A locus are selected from clinical isolates during passage in fibroblast culture (64, 73). The UL128, UL130 and UL131A genes share no sequence homology with genes of other herpesviruses. All three proteins have been shown to be constituents of the virion and form a complex with the gH/gL fusion complex in the viral envelope (83–85). Antibodies directed against these proteins block infection of endothelial and epithelial cells, but not infection of fibroblasts (83, 84). The UL128 gene codes for a protein with a CC-chemokine domain and like the UL130 gene product has no membrane anchor of its own. The

UL131A gene product has a membrane anchor which is cleaved off during maturation (83) (Scrivano and Adler, unpublished data). These three proteins form an alternative complex to the glycoprotein complex III (gCIII) of the viral envelope which consists of gH/gL/gO. gO (UL74) is a highly variable glycoprotein which associates with the extracellular portion of the gH/gL complex (86, 87). Deletion of UL74 results in a strong defect in secondary envelopment of virus particles. Deletion mutants can no longer release virus into the supernatant, but cell associated spread in cell culture remains rather efficient (88). Whether gO plays a role in entry and if yes, which role, is currently unknown. Viruses with double deletions, deletion of gO and additionally of one of the UL128–131A genes, are not viable, indicating that for virus spread expression of one of the gH/gL complexes is essential (88). The gH/gL/pUL(128,130,131A) complex not only determines the tropism for endothelial and epithelial, but also for dendritic cells, macrophages and polymorphonuclear leukocytes (10, 51, 74, 89), cell types potentially involved in virus dissemination.

gH/gL complexes of other herpesviruses are known to associate with receptor binding proteins, examples being gQ of human herpesvirus 6 (HHV6), which binds to CD46, and gp42 of Epstein-Barr virus (EBV) which binds to HLA class II (90, 91). Deletion of these proteins results in a loss of tropism for the cell types expressing the respective receptors (92). For EBV and HHV6, like for HCMV, two alternative gH/gL complexes have been identified (93, 94). The best characterised gH/gL complexes are those of EBV, where a gH/gL/gp42 complex defines the tropism for B-cells and a gp42-negative gH/gL complex defines the tropism for epithelial cells (95). Interestingly, virus released from B-cells is gp42-negative which induces a switch in tropism. This means that virus entering B-cells is different from virus leaving B-cells.

Alternative gH/gL complexes determining cell tropism are thus a common feature of herpesviruses and are likely to play an important role in virus spread in a network of interacting cell types. Therefore, studies in simple cell culture systems are not an appropriate test system to study the role of these complexes. Yet, there are attempts to address the role of cellular networks in virus spread *in vitro*. In co-culture experiments infected neutrophils were mixed with microvascular cells and cytotrophoblasts and it could be shown that infection of cytotrophoblasts is only possible via vascular cells but not directly from neutrophils (18). There are also reports on infections of organotypic cultures like explants of placental villi or renal arteries in which spread of infection in different cell types was shown *in vitro* (96, 97).

## Virus entry pathways as determinants of virus dissemination?

Enveloped viruses like herpesviruses can enter their host cells either by fusion at the plasmamembrane or via endocytotic pathways where fusion of the viral envelope with a host membrane takes place in endosomes. HCMV encodes four envelope glycoprotein complexes gCI (gB homodimer), gCII (gM/gN), gCIII (gH/gL/gO) and gH/gL/pUL (128, 130, 131A) which are assumed to contribute to virus entry. The exact role of the various

glycoproteins for binding to the target cell, for activation of cellular signalling pathways and for fusion of the viral envelope with cellular membranes is still unclear. Also, the contribution of cellular surface proteins to these steps is still a matter of research. The first cellular surface proteins that were identified to interact with HCMV binding and entry are currently not considered to be functional receptors, among them a 90 kD gH-binding protein (98), the gB-binding protein annexin 2 (99–101) and CD13 (102). EGFR and, more recently, PDGF- $\alpha$ R have been reported to be activated by HCMV and participate in the entry process, probably via interaction with gCI (103, 104). The relevance of EGFR however is unclear as inhibition of this receptor did not consistently block HCMV infection (102, 105). Consistent findings by various researchers strongly suggest a role for certain integrin heterodimers as cellular receptors promoting HCMV entry (105, 106), and again gCI appears to be involved. Cellular interaction partners of gCII and gCIII are unknown. Regarding infection of EC, identification of the cellular counterparts of the two gH/gL complexes is particularly interesting as the presence of an intact UL128–131A gene region determines the capability of HCMV strains to penetrate and infect EC.

For long time, HCMV, like all other herpesviruses, was generally assumed to penetrate target cells by fusion at the plasma membrane. This has changed when endocytosis has been shown as an alternative entry route for a number of herpesviruses, including EBV, Herpes simplex virus (HSV) and HHV8 (107–111). Fusion of the viral envelope with a cellular membrane is inevitable during herpesvirus entry in order to release the viral capsid into the cytoplasm, irrespective of whether this occurs directly at the plasma membrane or after endocytosis. Still, the site of fusion is not irrelevant as it has functional implications: (i) Endocytosis helps viruses to overcome the barrier formed by the actin cortex. (ii) Viruses entering via the endosomal-lysosomal route may use acidification as a fusion trigger but are (iii) at risk of becoming degraded in the lysosome. With HCMV, there are clear electron microscopy data showing endocytosis by a variety of cell types including endothelial cells (112–114). For EC however, there is controversy regarding the endocytic pathway utilised by HCMV (pH-dependent versus pH-independent), regarding the efficacy of this entry mode (abortive route versus effective route) and regarding the question whether EC-tropic HCMV strains enter the cells by endocytosis at all or by fusion (75, 113, 115).

*In vivo*, HCMV isolated from different body fluids exhibited differences in cell tropism (16). *In vitro*, virus derived from fibroblasts and epithelial cells was found to use distinct pathways to enter epithelial cells (116). These findings indicate that virus progeny derived from different cell types may differ in its host cell tropism. A cell type specific switch of glycoprotein complex compositions and concomitant of cell tropism has been described for EBV progeny derived from different cell types (95).

It may also be true for HCMV and will probably be a topic of future research. Cell type specific virus progeny might contribute to sorting/routing of virus during establishment of an HCMV infection. While it is clear that endothelial cells support a complete productive replication cycle with endotheliotropic HCMV, this does not preclude a role of endothelial cells in virus dissemination of non-endotheliotropic virus by an alternative

mechanism. *In vitro*, particles of non-endotheliotropic HCMV strains have been found internalised by endocytosis and resistant to degradation for at least 24 h (113). Interestingly, infectious virus reappears in the cell culture supernatant at 24 h after infection of endothelial cells despite extensive removal of unbound input virus, while a similar reappearance does not occur with fibroblasts (Sinzger, unpublished observation).

Given HCMV can be internalised by endothelial cells via endocytosis and virus containing vesicles are not destined towards the lysosome, intact virus particles may be released again by the cell resulting in transcytosis of infectious particles through EC. For non-endotheliotropic virus strains, lacking the gH/gL/pUL (128, 130, 131A) complex, HCMV entry into EC and dendritic cells through endocytosis has been described (115, 117). For dendritic cells it could be shown, that entry is mediated by DC-SIGN and that, though cells are not productively infected, the virus can be transmitted from the dendritic cell to permissive cells.

While observations in monolayer cultures have increased our knowledge of the molecular mechanisms underlying EC infection, they are clearly limited regarding investigations into the role of EC in HCMV infection and disease, as they do not depict the complex interaction of various cell types at the interface between circulation and organ tissue. In particular, it is unclear (i) whether productive infection of EC is a prerequisite for HCMV to pass through the vascular lining, and if so (ii) whether cell-associated spread or release of cell-free virus is the major mode of transmission. New more complex cell culture models with polarised endothelial layers forming a barrier between leukocytes and fibroblasts are now necessary to address these questions regarding the pathogenetic role of EC. Regarding the issue of cell-free versus cell-associated spread, gO deletion mutants may be a valuable tool. These mutants have a severe defect in envelopment and virus release, but still can form infectious foci both in endothelial cell and fibroblast layers, given the UL128–131A gene region is intact (88). If cell-to-cell-spread of HCMV is sufficient for efficient haematogeneous dissemination, gO deletion mutants should be capable of crossing the vascular endothelial cell layer in either direction. Concerning the question whether productive infection of EC is necessary for viral dissemination, the comparison of UL(128–131A)-deficient mutants with endotheliotropic wild-type virus may help. If endocytosed virus particles bypass the lysosome and are released by subsequent exocytotic

events this may result in transcytosis of infectious HCMV through the EC layer. In a polarised cell culture model it may be tested whether transcytosis occurs at all and whether there is a preferential direction.

While complex cell culture systems suitable for such analyses are still to be established, a new approach in the murine CMV (MCMV) system may help addressing these questions.

## MCMV – a model to study the role of cell tropism *in vivo*

MCMV infection of mice resembles the human infection with respect to organ and cell tropism, pathogenesis during acute infection, establishment of latency and reactivation after immunosuppression, but it also exhibits a number of differences regarding the sites of latency and the viral genes required for reactivation when compared to HCMV infection (118–120). Yet, it is the leading animal model to study pathogenesis of HCMV infection. MCMV has an extensive sequence homology to HCMV. Some HCMV proteins can even substitute MCMV proteins in infection experiments (121). Using a cell-type-specific virus labelling system, it has recently been shown, that virus progeny derived from certain cell types can be quantitatively tracked during MCMV infection of mice (122). It could be shown that infection of endothelial cells is a frequent event within many organs. MCMV has a true functional homolog for HCMV gO (Scrivano and Adler, unpublished observation). gO deletion mutants are not dead, but can still grow in cell culture, indicating that also MCMV expresses alternative gH/gL complexes, though these complexes still have to be identified.

In the mouse model two crucial questions regarding the role of endothelial cells could be addressed. Converting the cell-type specific labelling to a cell-type specific inactivation of virus, it would be possible to answer the question whether productive infection of endothelial cells is essential for establishment of infection and pathogenesis. This would be an approach superior to deletion of a gH/gL complex mediating endothelial cell infection, as exclusively virus infecting endothelial cells could be targeted. Using an MCMV delta gO mutant which cannot release virus in the supernatant, the role of free virus for the spread of infection could be evaluated.

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