Human cytomegalovirus infection impairs endothelial cell chemotaxis by disturbing VEGF signalling and actin polymerization

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Aims Human cytomegalovirus (HCMV) infection has been linked to the pathogenesis of vasculopathies; however, its pathogenic relevance remains to be established. A prerequisite for vascular repair is endothelial cell migration. We evaluated the influence of HCMV on chemokinesis and chemotactic response of human coronary artery endothelial cells (HCAEC) towards vascular endothelial growth factor (VEGF).

Methods and results A virus dose-dependent reduction in chemokinesis and VEGF-dependent chemotaxis was observed ($P < 0.05$). UV-inactivated virus did not inhibit chemotaxis or chemokinesis, indicating that viral gene expression is mandatory. We identified two HCMV-induced mechanisms explaining the reduction of chemotaxis: first, a non-ambiguous reduction of VEGFR-2 protein was observed, due to decreased transcription. This protein down-modulation could not be inhibited by Ganciclovir. The remaining VEGFR-2 expressed on infected HCAEC was able to stimulate cell activation. Second, HCMV infection influences actin polymerization in HCAEC as shown by FACS analysis: actin polymerization was significantly reduced to 53 and 51% ($P < 0.05$) compared with non-infected HCAEC at 24 and 72 h p.i., respectively. Genetically and pharmacologically eliminated VEGFR-2 function resulted in a significant ($P < 0.05$) reduction of VEGF-induced activation of actin polymerization.

Conclusion We demonstrated a significant reduction of the chemotactic mobility of HCMV-infected HCAEC mediated by down-modulation of the VEGFR-2 and by inhibition of actin polymerization. This VEGF resistance of HCMV-infected endothelial cells is likely to promote atherogenesis.

Keywords Human cytomegalovirus • Endothelial cell • Vascular endothelial growth factor • Chemotaxis • Signal transduction

1. Introduction

One of the key factors associated with early atherogenesis is endothelial dysfunction. It has been shown that an impairment of endothelial function secondary to diabetes, hypercholesterolaemia, or smoking is associated with endothelial dysfunction defined as reduced production of nitric oxide (NO).1 Endothelial function is an important denominator of vascular function and is critical for the maintenance of the structural and functional integrity of the arterial wall.2

Vascular endothelial growth factor (VEGF)-A has been recognized as an important stimulator of the endothelium via induction of migration, proliferation3 and, finally, survival4 of endothelial cells (EC). It has become evident that there is a continuous baseline level of VEGF-A in the blood stream, which was found to be elevated under pathological conditions, e.g. after acute myocardial infarction.6 In fact, blockade of VEGF action results in endothelial apoptosis and vascular pathology.4 Several risk factors for inducing vascular injury are known.7 However, since many atherothrombotic events occur in patients lacking accepted risk factors, it is likely that other so far undefined mechanisms might be involved. Infections have been recognized to trigger vascular inflammation. Among other infectious agents, a highly species-specific human cytomegalovirus (HCMV) has been associated with the pathogenesis
of atherosclerotic lesions, either by representing an ‘injury factor’ or by interfering with the repair processes. This is especially relevant since HCMV infection in vivo affects smooth muscle cells (SMC) and EC, both involved in atherogenesis. Interestingly, similar to the murine CMV model, a relationship between coronary heart disease in humans and the number of acquired infectious agents (pathogen burden) was described. One group even postulated the cumulated ‘herpesviral burden’ as an important predictor for cardiovascular disease.

In plaque formation, the local expression of growth factors and their receptors play key roles in maintaining endothelial and vascular integrity. Of note, the PDGF-α has recently been identified as a specific, functional entry receptor for HCMV into SMC. Additionally, EGFR was identified as a cellular receptor for HCMV entry into target cells. Besides interacting with EGFR, the binding of HCMV to the integrin αβ3 is required for viral entry. The virus-induced crosslinking of these molecules leads to the activation of PI3-K as well as Src and consequently to an increase in downstream signalling. This may result in a potent mitogenic stimulus and contribute to HCMV-induced cell migration and inflammation.

HCMV is known to modulate cellular gene expression. Diverging sets of genes are affected in different host cells. For example, dendritic cells substantially down-regulate chemokine receptors, whereas chemokine receptor down-modulation is only moderate in macrophages. Nevertheless, both cell types are severely impaired regarding their migratory capabilities, since macrophages’ migration is reduced additionally by the HCMV-induced release of macrophage migration inhibitory factor. Further, PDGF-β, the most important stimulus for SMC proliferation and migration, is up-regulated in HCMV-infected SMC. By this mechanism, HCMV may directly induce a proatherogenic phenotype in infected SMC. In SMC, we observed a further potentially important proatherogenic mechanism induced by HCMV: HCMV up-regulates cellular VEGF gene expression via Sp-1 binding. This para-crine effect might enhance inflammatory processes within the plaque by attracting monocytes or may lead to plaque softening by promoting intra-plaque vascularization.

In this study, we have asked the question whether HCMV infection can affect the function of EC. We therefore analysed the functional endothelial response towards VEGF-A, an important physiological stimulus. We have found that the endothelial response towards VEGF-A is greatly impaired following HCMV infection of the endothelium. This impairment is based on a significant reduction of VEGF-2 expression, while the expression of other endothelial proteins such as adhesion molecules and other growth factor receptors was not or only mildly reduced. Since it is known that motogenic responses of VEGF-2 signalling are dependent on actin polymerization in EC, we analysed the specific effects of HCMV infection on actin reorganization. Actin polymerization was found to be diminished in infected EC. These data provide a novel molecular basis for the proatherogenic action of HCMV.

2. Methods

2.1 Cells and infection

Human coronary artery endothelial cells (HCAEC) (Lonza) were cultured in endothelial cell basal medium supplemented with 5% fetal calf serum (FCS) and growth factors (EGM-MV singleQuotes, Lonza). HCAEC were used in passage 5 or 6. All dishes were coated with rat collagen (Sigma). Human coronary artery smooth muscle cells (HCASMC) were obtained from Lonza. The low-passaged HCMV strain TB40E was kindly provided by Dr. Ch. Sinzger, University of Tübingen. Virus stock production and UV inactivation were performed as published recently. The study conforms to the Declaration of Helsinki.

HCAEC were HCMV or mock infected at a confluency of 70–90% with a multiplicity of infection (MOI) as indicated. Additionally, for immunoblot analysis, infections were performed in the presence of 100 μM Ganciclovir (GCV, Roche).

2.2 Production of conditioned media

Mock-infected and TB40E-infected HCAEC (MOI 5) or HCASMC (MOI 1) were cultured for 72 h in endothelial or smooth muscle cell basal medium supplemented with 1% FCS. The conditioned media (CM) were centrifuged (2000 rpm, 10 min) and filtered twice (0.1 μm) to remove free virus from the supernatants.

2.3 Migration

VEGF-A (Relatech), insulin-like growth factor (IGF-1, PeproTech), or hepatocyte growth factor (HGF, PeproTech) were used as chemoattractants at a concentration of 10 ng/mL. Chemotaxis and chemokinesis were assessed as previously described using the modified Boyden chamber assay. To evaluate paracrine effects induced by HCMV, we incubated naive HCAEC in CM from mock-infected and HCMV-infected cultures for 24 h. All samples were assessed in triplicate.

2.4 Quantitative reverse transcription–polymerase chain reaction

Total RNA was isolated from mock-infected, TB40E-infected (MOI 5) HCAEC and from HCAEC incubated with UV-inactivated virus (MOI 5) using the RNeasy mini kit (Qiagen) and converted to complementary DNA (cDNA) by reverse transcription with the Omniscript RT kit (Qiagen), according to manufacturer’s protocols. cDNA was amplified in an optical thermal cycler (Biorad) using the Taq PCR mastermix kit (Qiagen) and specific primers for the genes of interest. GAPDH and β-actin served as normalization controls. The following primers were used:

- VEGFR-2 forward, 5′ CGG AGA AGA ACG TGG TTA AA 3′
- VEGFR-2 reverse, 5′ CGA GCA TCT CTT TTT CTG AC 3′
- GAPDH forward, 5′ GCC TCA AGA TCA TCA GCA AT 3′
- GAPDH reverse, 5′ GGA CTG TGG TCA TGA GTC CT 3′
- β-actin forward, 5′ ATC CTC ACC CTG AAG TAC CC 3′
- β-actin reverse, 5′ CAC GCA GCT CAT TGT AGA AG 3′

All samples were assessed in triplicate.

2.5 Immunoblot analysis

Immunoblot analysis of VEGFR and signalling molecules was performed as recently described. Antibodies recognizing VEGFR-2 were from R&D systems.

2.6 Cell proliferation assay

Using a commercially available assay (CellTiter 96, Promega), we determined the extent of cell activity following stimulation with VEGF-A, IGF-1, and RANTES. HCAEC were cultured in 12-well plates (Greiner). Four or 60 h p.i. mock-infected and TB40E-infected HCAEC were put in starvation medium containing 1% FCS. Cells were stimulated for 12 h or were left unstimulated, and then Owen’s reagent was added 22 or 70 h p.i. to stimulated and unstimulated HCAEC. The bioreduction of the colour compound was stopped after 2 h. The absorbance was recorded at 490 nm. The assays were performed in duplicate.
2.7 Knockdown of VEGFR-2 and VEGFR-2 kinase inhibition in HCAEC

HCAEC were transfected with Lonza HCAEC nucleofector kit according to manufacturer’s instructions. siRNA (smart pool) targeting VEGFR-2 was from Dharmaco. Transfected cells were seeded in 6-well dishes with coverslip inserts (whenever cells were used for microscopy). After 40–48 h, cells were serum-starved for 6 h before VEGF-A stimulation. For pharmacological inhibition of VEGFR-2 kinase, HCAEC were treated with 60 μM SU1498 (EMB Millipore) for the last 2 h of starvation before stimulation with VEGF-A.

2.8 Immunofluorescence

HCAEC cultured on chamber slides were fixed with 4% paraformaldehyde. Cells were permeabilized with ice-cold acetone. The primary antibodies were directed against the viral immediate early antigen (Argene). Specific as well as irrelevant control antibodies (Sigma) were added to a dilution of 1:100 in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA; Sigma). Slides were incubated for 45 min at 37°C. After washing, the FITC-labelled goat anti-mouse antibody (Dianova) was added at a dilution of 1:50, and TRITC-labelled phalloidin (Sigma) was added at a dilution of 1:100 in PBS/BSA. After 45 min incubation at 37°C, slides were viewed with a fluorescence microscope (Zeiss). For analyzing the impact of VEGFR-2 depletion and VEGFR-2 kinase inhibition on VEGF-A-induced actin polymerization in HCAEC, cells grown on coverslips were fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton-X-100. F-actin was stained using 30 μg/mL (in PBS/BSA) FITC-labelled phalloidin (Sigma) for 40 min. Images were taken using fluorescence microscope (Leica). Fluorescence intensity of the F-actin staining was quantified using ImageJ software.

2.9 Actin polymerization assay

The amount of polymerized actin was quantified as recently published. In brief, 4 or 60 h p.i. mock-infected and TB40E-infected HCAEC (MOI 5) were put in starvation medium containing 1% FCS. Twenty-four or 72 h p.i. the amount of polymerized actin was quantified in unstimulated and stimulated HCAEC. All stimulations were performed at 37°C. The agonist VEGF-A was diluted to result in a final concentration of 50 ng/mL. HCAEC were added to the stimulant and mixed gently. To stop actin polymerization after 15 or 30 s and 1 and 5 min, 4% paraformaldehyde was added. Cells were washed with PBS then permeabilized by incubation with 0.1% Triton-X in PBS for 10 min. One millilitre of FACS buffer (2% FCS, 0.01% sodium azide in PBS) was added and cells were pelleted by centrifugation. The pellet was resuspended in FACS buffer containing 1.5 μg/mL FITC-labelled phalloidin (Sigma). Cells were stained for 30 min in the dark. Quantitative analysis was performed on a FACScan flow cytometer (Becton Dickinson) using the CellQuest Pro software. A total of 10 × 10⁶ events/sample was collected and analysed.

2.10 Statistical analysis

We have used either unpaired t-tests or one-sample tests for comparing two data sets and the one-way analysis of variance (ANOVA) followed by the Tukey comparison or the Holm–Sidak method when comparing several data sets. The exact test used is indicated in the figure legends. The Graph Pad Prism software (Graph Pad Prism) and SigmaPlot were applied for statistical analysis. The alpha level was 0.05.

3. Results

3.1 HCMV infection of HCAEC is associated with an impaired cellular response

HCAEC were cultured to subconfluence and then assayed in a modified Boyden chamber for ligand-stimulated migration (chemotaxis). In mock-infected HCAEC, VEGF-A (10 ng/mL) led to a significant and reproducible stimulation of chemotaxis (Figure 1A). In HCMV-infected HCAEC, however, we observed a significantly reduced VEGF-A-induced chemotaxis. This inhibition of VEGF-A-induced HCAEC migration seemed to be virus dose dependent and correlated with the MOI. Following infection with MOI 1, a significant reduction of VEGF-induced chemotaxis was observed 72 h p.i. (P<0.05). This inhibitory effect increased with higher MOI (Figure 1A). Likewise, chemokinesis appeared to be reduced at MOI 1 and significantly at MOI 25 (P<0.05), indicating an additional negative effect of HCMV infection on HCAEC mobility (Figure 1A).

Other growth factor stimuli were also tested in HCAEC chemotaxis, but fibroblast growth factor 2, IGF-1, as well as insulin (Figure 1B, only IGF-1 data are shown) failed to induce migration. Using HGF (10 ng/mL) as chemoattractant in HCMV-infected HCAEC 24 h p.i., the stimulation of migration was significantly higher compared with unstimulated infected cells (P<0.05), however still reduced compared with mock-infected HCAEC (Figure 1B).

3.2 The expression of VEGFR-2 in HCAEC following HCMV infection is increasingly reduced in an MOI- and time-dependent fashion

Using western blot analysis, the expression of VEGFR-2 was assessed in HCAEC following HCMV infection. Quite clearly, compared with the mock-infected HCAEC, the expression of VEGFR-2 is already reduced to 78% when using an MOI of 1 and increasingly reduced with an MOI of 5 or 25 (Figure 2C). In fact, using an MOI of 25, almost no VEGF-R2 expression is detectable. A difference in cell numbers was not responsible for the reduction (Figure 2B). To find out whether there is a general decrease of protein expression or whether this is specific for VEGFR-2, we have performed Western blot analysis in HCMV-infected HCAEC for other membrane bound proteins such as ICAM-1 as well as for structural proteins of the cytoskeleton such as vimentin (Figure 2A and C). When using a low MOI especially within the first 48 h p.i., as shown in Figures 2 and 3A, ICAM-1 expression was even increased, consistent with published data. Irrespective of the MOI, the expression of ICAM-1 protein did not decrease substantially during infection with an observation period of up to 72 h. The same was true for the expression of vimentin, which was moderately reduced in HCMV-infected cells. The changes in VEGFR-2 expression following HCMV infection appear to be partly specific for VEGFR-2, as another receptor tyrosine kinase, IGF-1Rβ, was only moderately affected by HCMV infection (Figure 3A).

3.3 A decrease in transcription is responsible for the down-regulation of the VEGFR-2 expression

The underlying mechanism for the HCMV-induced down-regulation of VEGFR-2 could be identified. Using quantitative reverse transcription–polymerase chain reaction, a non-ambiguous lower level of specific mRNA expression induced by HCMV infection could be shown 24 h p.i. following infection with viable TB40E (MOI 5) compared with the mock-infected control (Figure 3B).
3.4 Viral gene expression is essential for the inhibition of migration and the down-regulation of the VEGFR-2

HCAEC were incubated with UV-inactivated virus to clarify whether viral gene expression is required for HCMV-induced disturbance of HCAEC function or whether the mere contact of HCAEC with viral proteins is sufficient for this effect. Following incubation with UV-inactivated virus (MOI 5), the capability to migrate (Figure 1D) as well as the level of VEGFR-2 mRNA expression remained unchanged compared with mock-infected HCAEC (Figure 3B). Thus, viral gene expression is essential to impair HCAEC function.
Interestingly, inhibition of viral DNA replication by Ganciclovir did not prevent HCMV-associated VEGFR-2 reduction, indicating that viral immediate early or early genes are responsible (Figure 3A). Furthermore, HCMV infection does not induce paracrine effects impairing HCAEC migration since incubation with CM from HCMV-infected HCAEC did not reduce chemokinesis (Figure 1C). In contrast to HCMV infection of HCAEC, HCMV infection of HCASMC induces paracrine stimulation of cell migration by stimulating VEGFR-2 expression in uninfected HCAEC (Figure 3C).

### 3.5 Metabolic activity following stimulation with VEGF-A is partially conserved in HCMV-infected HCAEC

The metabolic activity of TB40E-infected HCAEC was assessed using a colorimetric method. As demonstrated in Figure 4, HCAEC proliferation was decreased in HCMV-infected cells (MOI 5) at 24 as well as 72 h p.i. ($P < 0.05$), compared with mock-infected cells. However, cell activity increased following VEGF-A stimulation, suggesting that the remaining...
VEGFR-2 molecules are intact and present on the cell surface. Twenty-four hour p.i. HCMV-infected HCAEC showed comparable levels of cell activity as uninfected HCAEC following stimulation with IGF-1. These results are in agreement with our protein expression analysis at 24 h p.i. (Figure 3A), demonstrating comparable amounts of IGF-1R-β in uninfected and HCMV-infected HCAEC. Additionally, following stimulation of surface receptor not belonging to the tyrosine kinase receptor family by RANTES, no differences in cell activity between infected and mock-infected HCAEC were observed (Figure 4). This indicates that certain cellular pathways are still undisturbed by HCMV infection at 24 h p.i. and capable to induce metabolic activity in infected cells.

3.6 HCMV infection leads to impaired actin polymerization

To elucidate the molecular mechanism responsible for HCMV-induced reduction of spontaneous migration and chemotaxis in HCAEC, we analysed the composition of actin, a major component of the cytoskeleton involved in cell motility. To visualize long filamentous polymers called F-actin, indispensable for migration,22 we used fluorescence-labelled phalloidin. As demonstrated in Figure 5A, HCMV-infected HCAEC no longer possess cytoplasmatic F-actin containing protuberances which are important in chemokinesis independent of VEGF-A stimulation. In contrast, these are clearly visible in mock-infected cells, indicating that HCMV leads to a rearrangement of F-actin fibres in HCAEC. Interestingly, not only the distribution of F-actin was changed in HCMV-infected cells, but also the absolute amount of filamentous actin, since quantitative FACS analysis of the F-actin content in uninfected and HCMV-infected HCAEC (MOI 5) revealed significant differences at 24 and 72 h p.i. (P, 0.05) (Figure 5B). The total amount of structural proteins such as G-actin and vimentin by HCMV infection did not change significantly (Figure 5C). To analyse whether the deregulation of VEGFR-2 expression/activity by HCMV is contributing to impairment of VEGF-A-induced F-actin polymerization, we have genetically and pharmacologically eliminated VEGFR-2 function in HCAEC. siRNA-mediated VEGFR-2 knockdown resulted in significant down-regulation of VEGFR-2 mRNA (Figure 6A) and protein (Figure 6B). These cells were then analysed for their capacity to induce actin polymerization upon VEGF-A stimulation. VEGFR-2 knockdown did not appreciably affect basal F-actin levels; however, it resulted in a considerable reduction of VEGF-A-induced activation of actin polymerization (P < 0.05) (Figure 6C and D). Likewise, blockade of VEGF-R2 kinase activity by a specific inhibitor, SU1498, also led to a significant impairment of actin polymerization (P < 0.05) (Figure 6E and F). Consequently, we could
consistent with the idea that endothelial dysfunction acts as an initial driving force allowing the remodelling to happen. It is interesting to note that the HCMV-induced down-regulation of VEGFR-2 is much more pronounced than changes in ICAM-1- or IGFR-IR expression. These as well as other data sets in our current study have been reproduced several times; however, some data sets have only been repeated three times, which represents a limitation, at least with regard to further statistical analysis. Since inhibition of polymerization greatly affects the migratory phenotype, it might be relevant that polymerized actin expression is strongly reduced following HCMV infection with higher MOI, although total G-actin is not specifically altered in EC. These HCMV-induced changes are pointing towards a selective inhibition of molecules responsible for mediating specific endothelial functions. HCMV-induced HCAEC dysfunction is mediated mainly through the down-regulation of VEGFR-2 expression. Genetic and pharmacological inhibition of VEGFR-2 in HCAEC recapitulated HCMV-induced defective actin polymerization. Migration, especially chemotaxis, is an important aspect of endothelial function as it is needed for endothelial repair as well as for endothelial maintenance. In turn, endothelial dysfunction is associated with impaired repair and with a proatherogenic phenotype. Recently, Wilkinson and co-workers demonstrated a disruption of focal adhesions in HCMV-infected fibroblasts following infection with a laboratory-adapted HCMV strain at late time points during infection, indicating a potential HCMV-induced influence on the migration apparatus. Studying chemokinesis in HUVEC, Streblov et al. also published data suggesting an HCMV-induced impairment of cell movement. Our studies, using low-passage HCAEC and a low-passage clinical HCMV strain, could document an inhibition of migratory function within 24 h of infection. Comparable to our studies, Heiske et al. reported that only active HCMV infection is capable to modulate VEGFR-2 expression when analysing human fibroblasts. In contrast to our studies, they found an increase of VEGFR-2 expression following HCMV infection. Nevertheless, our current study demonstrates an additional indirect HCMV-induced mechanism relevant in cell migration, namely increased VEGFR-2 expression in HCAEC incubated with supernatants conditioned on HCMV-infected HCAEC. This might be due to the fact that diverging sets of genes are affected by HCMV infection in different host cells. Although many studies have been performed in the last decades, the specific viral genes involved are not known so far. HCMV expresses more than 160 viral genes. Their expression is highly regulated in a cascade fashion. By demonstrating that VEGFR-2 down-regulation cannot be influenced by adding Ganciclovir, an inhibitor of viral late gene expression, we can conclude that viral immediate early or early genes are responsible for the HCMV-induced reduction in chemotaxis in HCAEC.

While our present data support a direct inhibitory action of HCMV following endothelial infection, other recent studies identified gain-of-function effects. Botto et al. identified several factors, among them IL-6 within the cytomegalovirus secretome that promotes angiogenesis. Bentz et al. showed the activation of EGF-dependent angiogenesis. With regard to the effect of HCMV on atherogenesis, both data sets are not contradictory. It is well known that inflammation-associated angiogenesis occurs within the arterial wall during atherogenesis, leading to the formation of plaque angiogenesis, which in turn contributes to plaque vulnerability.

Our data provide a novel molecular basis for the proatherogenic action of HCMV. The mechanism of HCMV-related endothelial and vascular dysfunction as proposed in this article can explain why the infection...
Figure 5 Actin polymerization is impaired in HCMV-infected HCAEC. HCAEC were infected with the TB40E strain (MOI 5) in complete medium and were cultivated in starvation medium 24 h before actin analysis (1% FCS). (A) Immunofluorescence was performed 24 h p.i. on unstimulated and VEGF-A (10 ng/mL) stimulated HCAEC. Polymerized actin was visualized by incorporation of TRITC-labelled phalloidin. HCMV-infected cells are identified by nuclear staining against the viral immediate early antigen visualized by a FITC-labelled goat anti-mouse antibody. Representative pictures of three independent experiments are shown. The included size bars represent 50 μm. (B) The amount of polymerized F-actin in HCMV-infected (MOI 5) and uninfected HCAEC without stimulation was determined 24 and 72 h p.i. by FACS analysis. The mean fluorescence intensities of six independent experiments are shown. The amount of polymerized actin was significantly reduced 24 as well as 72 h p.i. (unpaired t-tests, * indicates P < 0.05). (C) Equal amounts of cell lysates were used to investigate G-actin protein expression by western blot analyses. No differences in the amount of total G-actin was observed between HCMV-infected and uninfected HCAEC 24 and 72 h p.i. Representative results from three independent experiments are shown.
Figure 6 Genetic and pharmacological inhibition of VEGFR-2 impairs VEGF-induced actin polymerization. (A) HCAEC were transfected with siVEGFR-2 and scrambled siRNA using nucleofector. After 48 h, RNA was extracted and the expression of VEGFR-2 mRNA was detected using reverse transcription–polymerase chain reaction. GAPDH was used as internal control. The data shown represent means ± SD of three independent experiments performed in triplicates. Statistical analysis is done using unpaired t-test. Statistical analysis was done using one-sample test, *indicates P < 0.05. (B) HCAEC were transfected with siVEGFR-2 and scrambled siRNA using nucleofector. After 48 h, VEGFR-2 was immunoprecipitated from 250 μg of protein and detected using western blotting. Twenty-five micrograms of the total lysates was used to detect β-actin. Images are representative of four independent experiments with consistent results. (C and D) Immunofluorescence was performed after 40 h after siRNA knockdown of VEGFR-2 on unstimulated and VEGF-A (15 ng/mL)-stimulated HCAEC. Polymerized actin was labelled with FITC-labelled phalloidin, and images were taken using fluorescence microscope. Representative pictures of three independent experiments are shown. The included size bars represent 100 μm. Fluorescence intensity was quantified using Image J software and is depicted in (D). The data shown represent means ± SD of three independent experiments performed in triplicates. Statistical analysis was done using one-way analysis of variance, followed by the Holm–Sidak method, *indicates P < 0.05. (E and F) Immunofluorescence was performed on serum-starved-unstimulated and VEGF-A (15 ng/mL)-stimulated HCAEC. VEGFR-2 kinase activity was blocked by treating the cells with 60 μM SU1498 for 2 h. Polymerized actin was labelled with FITC-labelled phalloidin, and images were taken using fluorescence microscope. Representative pictures of three independent experiments are shown. The included size bars represent 100 μm. Fluorescence intensity was quantified using Image J software and depicted in (F). The data shown represent means ± SD of three independent experiments performed in triplicates. Statistical analysis was done using one-way analysis of variance, followed by the Holm–Sidak method, *indicates P < 0.05.
with HCMV can be associated with vascular pathology. HCMV infection should therefore be further investigated in cases of premature atherosclerosis. Previously, HCMV infection has been suggested to be proatherogenic, but essentially by activating SMC rather than EC. 17

In summary, our novel data demonstrate that HCMV infection of human EC leads to endothelial dysfunction and an impaired potential for endothelial repair. This endothelial dysfunction is based on the down-modulation of VEGFR-2 and by affecting actin polymerization. These functional impairments of HCMV-infected EC are consistent with a proatherogenic phenotype supporting earlier reports of the involvement of HCMV in degenerative vascular disease.

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References


Figure 7 VEGF-A-induced actin polymerization is significantly impaired in HCMV-infected HCAEC. HCAEC were infected with the TB40E strain (MOI 5) in complete medium and cultivated in starvation medium 24 h before F-actin analysis (1% FCS). The kinetics of actin polymerization following VEGF-A stimulation in HCMV-infected and uninfected HCAEC was determined 24 (upper panel) and 72 h p.i. (lower panel) by FACS analysis. Results are shown as percentage in relation to the unstimulated control and are summarized from five independent experiments. The broken lines represent data obtained from uninfected cells and the solid lines represent data from HCMV-infected HCAEC at indicated time points after stimulation with 50 ng/mL VEGF-A. VEGF-induced actin polymerization was significantly reduced in HCMV-infected HCAEC compared with uninfected cells (unpaired t-tests, *indicates P < 0.05).


