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HCMV infection triggers smooth muscle cell proliferation in a 3D human coronary *in vitro* model

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Abstract

Background: Although a role of the human cytomegalovirus (HCMV) in atherosclerosis and restenosis is probable, clinical studies are not conclusive. The present study investigates in a threedimensional (3D) human coronary transfilter co-culture model the effect of cell-free and cell-associated HCMV-infection.

Methods: Human coronary endothelial cells (HCAEC) and HCMSMC were seeded on both sides of a polycarbonate filter membrane. HCMV-infection was carried out by HCMV-infected MO. As controls MO-attack without HCMV-infection, cell free HCMV-infection, and the transfilter co-culture model without MO-attack and without HCMV-infection (Mock) were used.

Results: At day 1, day 4, day 7, and day 14 the effects of HCMV-infection on MO adhesion and chemotaxis and on reactive proliferation of HCMSMC was studied. Cell-associated HCMV infection of HCAEC and HCMSMC was less intense and postponed in comparison to cell-free HCMV infection. Endothelial adhesion of MO after cellbased HCMV infection was decreased in comparison to non-infected MO, no clear effect was found on chemotaxis. Both after cell-associated and cell-free HCMV infection proliferation of HCMSMC was significantly increased.

Conclusions: In the 3D human coronary transfilter co-culture model both cell-free and cell-associated HCMV-infection significantly increased proliferation of HCMSMC. If we assume that HCMV is involved in the pathogenesis of atherosclereosis and restenosis, the effect of antiviral treatments should be studied in experimental and clinical studies.

Keywords: HCMV, restenosis, atherosclerosis, transfilter co-culture model

Introduction

More than a decade ago Danesh *et al.,* raised, in the light of increasing evidence that chronic infection is associated with atherosclerosis, the question: Is there a link between chronic infections and coronary artery disease? [1] Specific organisms, including herpesviruses, enhance atherosclerosis in the apolipoprotein E (ApaE)-deficient mouse [2,3]. Epidemiological studies have suggested an interaction between infection with the human cytomegalovirus (HCMV) and diabetes in promoting vascular disease [4]. HCMV has been associated with atherosclerosis, transplantation vasculopathy, vascular allograft rejection, and restenosis after angioplasty [5,6].

Recently the group of Bentz *et al.,* [7] demonstrated that HCMV infection triggers proliferation, migration, and morphogenesis in human endothelial cells. While it appears that HCMV contributes to the development of restenosis and atherosclerosis, there is no definitive proof that the virus causes as well an increased proliferation of smooth muscle cells. Rather, HCMV infection of human fibroblasts appears to trigger an opposite effect, namely, cell cycle arrest. In previous studies HCMV infection was shown to induce arrest of cell growth either in late G1 or in G2/M [8,9]. These findings conflict with reports that connected HCMV infection with the ability to promote an environment conducive to cell proliferation [10] and the induction of cellular proliferation [11]. In a recently described human ex vivo organ culture model of HCMV-infection [12] reactive cell proliferation after cellfree productive HCMV-infection was delayed but not increased [13].

The threedimensional (3D) human coronary transfilter co-culture model mimics the inner layers of the vessel wall [14,15]. Endothelial cells and smooth muscle cells are cultured on both sides of a polycarbonate filter, through the filter pores the cells can communicate with eachother and move from one side to the other. Recently we have established this model [16,17] with human coronary endothelial (HCAEC) and smooth muscle cells (HCMSMC). Adding of monocytes (MO) to the endothelial side of the model significantly stimulates cell proliferation of co-cultured HCMSMC [16]. In the current study we carry out both cell-free and cell-associated HCMV-infection of the 3D human coronary transfilter co-culture model and investigate the effect on MO-adhesion/chemotaxis and the reactive proliferation of HCMSMC.

Methods
Cell culture

Human coronary media smooth muscle cells (HCMSMC, Cambrex Bio Science, Vervier, B) were cultured in Smooth Muscle Cell Growth Medium (Cambrex). For identification of HCMSMCs, monoclonal antibodies against smooth muscle a-actin were used (Sigma, Taufkirchen, D). Human coronary endothelial cells

(HCAEC, Cambrex) were grown in Endothelial Growth Medium (Cambrex). For identification of endothelial cells, antibodies against von Willebrand factor (Dakopatts, Hamburg, D) were used. MO were isolated from residual leukocytes of single donors using the MACS cell-isolation kit (Milteny Biotec, Bergisch-Gladbach, D).

The 3D transfilter co-culture model

The 3D human coronary transfilter co-culture model mimics the inner layers of human coronary arteries [16,17]. The internal elastic membrane is represented by a polycarbonate filter with a thickness of 10 μm and a pore size of 5 μm (Whatman, Göttingen, D). Filters were fixed in a specially designed frame and inserted in a siliconized culture dish. On both sides of the filters cell cultures were established, direct contact of the cultures was made possible through the pores of the filter.

HCMSMC were seeded on one side of the filter at a density of 2.5 x 10^4 cells/cm². After 24 h cells had attached to the surface and frame and filters were turned upside down. HCAEC were seeded on the opposite side of the filter at a density of 2.5 x 10^4 cells / cm². Both HCAEC- and HCMSMC-cultures were supplied with the appropriate culture medium and cultured for 14 days. At day 14 leukocyte attack was carried out by adding 3 x 10^5 MC on the endothelial side of the units.

HCMV-infection

In the current study MO-attack in the 3D human coronary transfilter co-culture model was carried out by HCMV-infected MO. As controls MO-attack without HCMV-infection, cell free HCMV-infection, and the transfilter co-culture model without MO-attack and without HCMV-infection (Mock) were used.

For cellfree infection the endotheliotropic clinical HCMV isolate TB40E was used. 10 MOI were added on the HCAEC side of the co-culture model. This isolate was kindly provided by Dr. Ch. Sinzger, University of Tübingen. Virus stocks were produced in human foreskin fibroblasts [12], infectivity was determined by plaque titration of stock virus [18]. Ten-fold dilutions of virus stocks were carried out in quadruplicate. After staining for viral late antigen (monoclonal antibody AAC10, DAKO, Glostrup, Denmark) viral infectivity was determined by plaque couting and was expressed as plaque forming U/mL. Cell-associated HCMV infection was carried out with the same HCMV-strain as applied for cellfree HCMV-infection. For HCMV infection 3 x 10^5 MO were incubated with 10 MOI (multiplicity of infection) overnight.

Adhesion and chemotaxis assays

At day 1, day 4, day 7, and day 14 the effects of HCMV-infection on MC adhesion and MC chemotaxis were studied. Filters were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 3 h at room temperatures; each filter was divided in 4 segments. One segment was used for the identification of MO. MO were identified with primary antibodies directed against CD68 (Dakopatts). TRITC-labeled antibodies (goat

anti-mouse, Dianova) were used as secondary antibodies. The number of cells with positive staining against CD68 was detected horizontally after adhesion on the surface of HCAEC and after transmigration on the HCMSMC side of the 3DLA-units. For each investigation 10 microscopical fields of three different filters were analyzed (magnification, 40x).

Identification of HCMV

Identification of HCMV in the 3D transfilter co-cultures was carried out at day 1, day 4, day 7, and day 14 after infection with monoclonal antibodies against HMCV early antigen (Clon CCH2, Dakopatts). Alexa-Fluor 488-labeled antibodies (goat-anti-mouse, MoBiTel Göttingen) were used as secondary antibodies. The number of cells with positive staining against early antigen was detected horizontally on the surface of both the HCAEC- and the HCMSMC-side of the filter. For each investigation 10 microscopical fields of three different filters were analyzed.

Cell proliferation assays

At day 1, day 4, day 7, and day 14 the effects of HCMV-infection on reactive proliferation of HCAEC and HCMSMC was studied. 18 h before fixation, 5 bromo-2´deoxyuridine (BrdU, 20 μmol/L, Serva) and 2´deoxy-cytidine (20 μmol/L, Serva) were added to the culture medium of the transfilter co-culture units. One segment was embedded in Epon (Araldite, Serva) and was polymerized at 60°C for 3 days. For histological examination, semithin sections were mounted on poly-L-lysine-coated slips. One section was stained with toluidine blue (Merck). In another section, araldite was dissolved from the sections using potassium hydroxide dissolved in methanol and propylene oxide immediately before immunological staining. Proliferation was subsequently detected with monoclonal antibodies directed against BrdU (Dakopatts). For secondary antibodies, biotinylated goat anti-mouse antibodies were used. HCAEC were identified in a double staining with von Willebrand factor (Dakopatts) and a biotinylated goat anti-rabbit secondary antibody. In each group 10 sections of three filters were analyzed (magnification, 40x). The percentage of BrdU-positive HCMSMC and anti-von Willebrand factor positive HCAEC was calculated in relation to the total cell number.
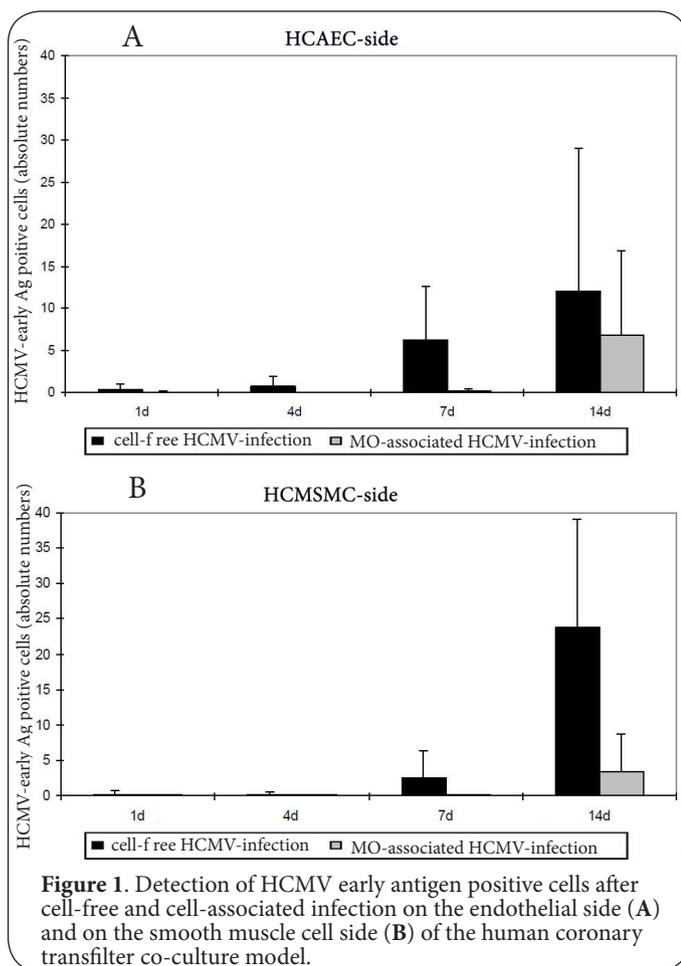
Statistical analysis

The Mann-Whitney rank-sum test was used to investigate the significance of differences in HCMV identification and adhesion/chemotaxis assays of MO and in the proliferation assays of HCAEC and HCMSMC. Results are expressed as mean±SD. Differences were considered significant at $p<0.05$.

Results

### Description of HCMV-infection in the 3D transfilter co-culture model

Time course and localization of HCMV early antigen was studied after cell-free and MO-associated HCMV-infection

Figure 1. Detection of HCMV early antigen positive cells after cell-free and cell-associated infection on the endothelial side (**A**) and on the smooth muscle cell side (**B**) of the human coronary transfilter co-culture model.

Figure 2. Adhesion (**A**) and chemotaxis (**B**) of monocytes (MO) on the the endothelial side respectively to the smooth muscle cell side in the human coronary transfilter co-culture model.

at day 1, day 4, day 7, and day 14. Both cell-free and cell-associated infection of the endothelial side and the smooth muscle cell side of the transfilter co-culture model with HCMV were successful. Cell-associated HCMSMC infection of both endothelial cells and smooth muscle cells was less intense and postponed in comparison to cell-free HCMV-infection (**Figure 1A**,**1B**, **4**).

After cell-free infection little numbers of HCMV were detected on the endothelial side of the filters (**Figure 1A**). 7 days and 14 days after cell-free HCMV-infection the number of HCMV endothelial cells was increased 21-times ($p<0.001$) and 40-times ($p=0.001$) in comparison to the number of HCMV detected at day 1. After cell-associated infection very little numbers of virus were detected in endothelial cells at day 1, day 4, and day 7 after infection. At day 14 after infection the number of HCMV was strongly increased in comparison with the low numbers of virus detected until day 7 ($p<0.001$).

On the smooth muscle side of the transfilter co-culture model (**Figure 1B**) very little numbers of infected cells were detected after cell-free HCMV infection at day 1 and day 4. At day 7 a small but significant increase of HCMV positive HCMSMC was detected ($p<0.05$) in comparison to the number of HCMV positive cells at day 1. At day 14 a strong increase of HCMV-positive cells was found ($p<0.001$ in comparison to the number of HCMV positive cells at day 1). After cell-associated HCMV-infection no virus was detected at day 1, day 4, and day 7 after infection. At day 14 after cell-associated HCMV-infection a significant increase of HCMV-positive cells was detected ($p<0.001$ in comparison to the number of HCMV positive cells at day 1).
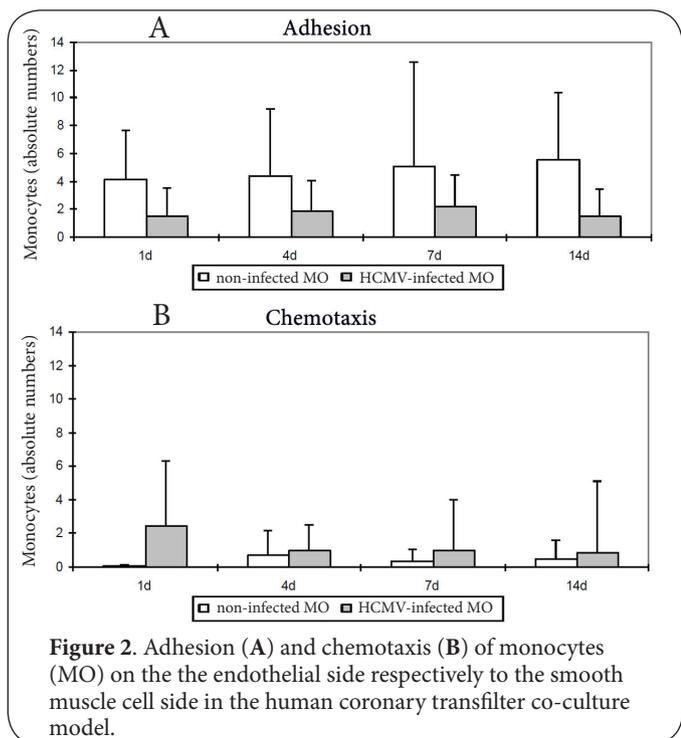
Adhesion and chemotaxis of monocytes
Adhesion of HCMV-infected MO on the endothelial side of the filters was decreased in comparison to non-infected MO (**Figure 2A**,**4E**), reaching statistical significance at day 1 and day 14. Chemotaxis of MO to the HCMSMC-side of the filters was very limited both in HCMV-infected and non-infected MO (**Figure 2B**).

MO-adhesion after cell-associated HCMV-infection was reduced in comparison to non-infected MO by 63% ($p<0.001$) and 57% ($p=0.05$) at day 1 and day 4 and by 58% (n.s.) and 72% ($p<0.001$) at day 7 and day 14. Chemotaxis of MO was 0, 0.7, 0.35 and 0.5 in the non-infected group and 2.47, 0.98, 1, and 0.85 in the HCMV-infected group. At day one the differences between non-infected MO and HCMV-infected MO were significant ($p<0.001$).

Cell proliferation of HCAEC and HCMSMC
Reactive cell proliferation of HCAEC and HCMSMC was studied after cell-free and MO-associated HCMV infection at day 1, day 4, day 7, and day 14. On the endothelial side of the filter very little proliferation was detected, no matter whether cell-free or cell-associated HCMV-infection was carried out (**Figure 3,4C** and **4F**). On the smooth muscle side of the
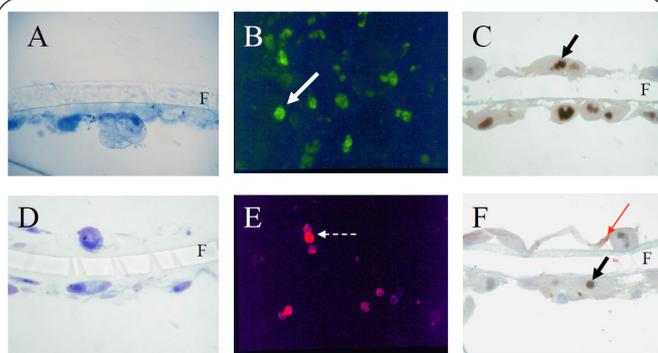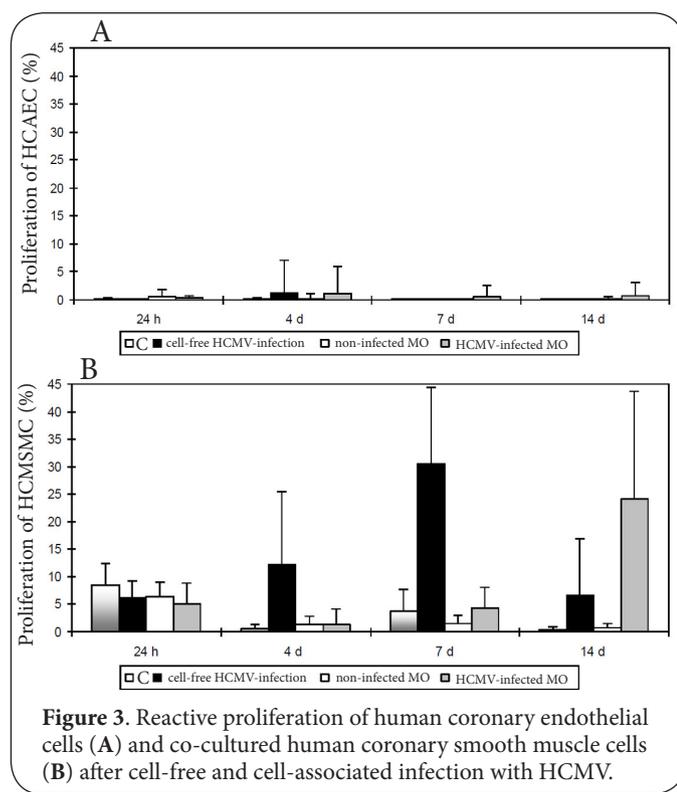
Figure 3. Reactive proliferation of human coronary endothelial cells (**A**) and co-cultured human coronary smooth muscle cells (**B**) after cell-free and cell-associated infection with HCMV.

Figure 4. Cell-free (**A,B,C**) and cell-associated (**D,E,F**) HCMV infection of the human coronary transfilter co-culture model: (A) 14 days after cell-free HCMV infection, toluidin-blue staining, frontal cut through the filter membrane of the transfilter co-culture model, F = filter. (**B**) 14 days after cell-free HCMV infection, staining against HCMV early antigen, view from the top on the endothelial side of the filter membrane, F = filter. (**C**) 14 days after cell-free HCMV infection, positive staining against BrdU for the analysis of reactive cell proliferation and against von Willebrand factor for the identification of endothelial cells, frontal cut through the filter membrane, black arrow = BrdU-positive human coronary smooth muscle cells, F = filter. (**D**) 14 days after cell-associated HCMV infection, toluidin-blue staining, frontal cut through the filter membrane of the transfilter co-culture model, F = filter, bar = 40 μm. (**E**) 7 days after cell-associated HCMV infection, staining against CD68 for identification of monocytes, view from the top on the endothelial side of the filter membrane, white arrow. (**F**) 14 days after cell-associated HCMV infection, positive staining against BrdU (black arrow) for the analysis of reactive cell proliferation and against von Willebrand factor (red arrow) for the identification of endothelial cells.

filters significantly increased levels of cell proliferation were detected, both after cell-free and cell-associated HCMV-infection. Cell-associated HCMV-infection caused a delay of reactive cell proliferation in comparison to cell-free HCMV-infection (**Figure 3**).

At day 1 the number of proliferating HCMSMC was very low in HCMV-infected cells and controls. Four and seven days after cell-free HCMV-infection proliferation of HCMSMC was increased by 97% (n.s.) and by 395% (p<0.001) in comparison to the number of proliferating cells at day 1. At day 14 the number of proliferating cells returned to the baseline level found at day 1 (p<0.01). After cell-associated HCMV-infection a delayed stimulation of cell proliferation was detected, cell proliferation was significantly decreased by 63% at day 4 (p<0.001), reached baseline levels at day 7 and was significantly increased by 370% at day 14 (p<0.001). In untreated controls and after adding of not-stimulated MO no increase of cell proliferation was detected (**Figure 3**).

Discussion

In the current study we investigated the effect of cell-free and MO-associated HCMV-infection in a 3D human coronary transfilter co-culture model. Three major findings were determined: First, cell-associated HCMV infection of HCAEC and HCMSMC was less intense and postponed in comparison to cell-free HCMV infection. Second, endothelial adhesion of MO after cell-associated HCMV infection was decreased in comparison to non-infected MO, no clear effect was found on chemotaxis. Third, both after cell-associated and cell-free HCMV infection proliferation of HCMSMC was highly increased.

Cytomegalovirus is a ubiquitous virus, as evidenced by the fact that the majority of adults throughout the world are seropositive for CMV (1). In immunocompromised subjects HCMV infection can cause serious diseases. With the exception of the mononucleosis-like syndrome it is generally believed that HCMV does not cause disease in healthy immunocompetent individuals. This may, however be incorrect. If HCMV plays a role in atherosclerosis and restenosis, it is critical to understand how HCMV is delivered to the vascular lesion and how the virus is activated from latency (5). In the current study we describe both cell-free and cell-associated HCMV-infection. Cell-free HCMV infection mimics the situation after HCMV infection of an individual. The endothelial layer of its vessel surface will get direct contact with the virus. Cell associated HCMV infection describes an alternative possibility suggested by recent studies focusing on the monocyte (5). Circulating monocytes have been shown to harbor CMV DNA (5). If the circulating HCMV-infected monocyte attacks the endothelial surface of the vessel wall we have a situation similar to the model of cell-associated HCMV infection.

In the current study cell-associated HCMV infection in HCAEC and HCMSMC was less intense and postponed

in comparison to cell-free HCMV infection. This result was probably caused by the fact that the amount of virus load in cell-free HCMV infections was higly increased in comparison to the virus load in cell-associated HCMV infection. A comparable effect was already described following cell-free and cell-associated HCMV infection in the human arterial organ culture model (12).

Adhesion of HCMV-infected MO was reduced in comparison to non-infected MO. Altannavch et al., [21] studied the effect of HCMV on expression of ICAM-1, VCAM-1, and ELAM-1 in HUVEC and described that HCMV increases expression of ICAM-1. These data are in contrast to data of the current study. For adhesion of MO expression of adhesion molecules is important not only at the surface of endothelial cells but although at the surface of MO. Gredmark et al., [22] recently demonstrated that HCMV infection of MO blocks the cytokine-induced cell differentiation, as a powerful tactic to avoid immune recognition at early phases of infection. Although there are no data in the literature on expression of adhesion molecules in HCMV infected MO, it is possible that expression of adhesion molecules is decreased. Multifaceted interactions as increased expression of adhesion molecules in endothelial cells and decreased expression in MO can only be detected in complex *in vitro* models as the transfilter co-culture model.

Although a role of HCMV in atherosclerosis and restenosis is likely, the experimental proof is difficult. Earlier reports connected HCMV infection with the induction of cellular DNA synthesis and with the induction of an environment conducive to cell proliferation [10]. In a recent report the group of Dhaunsi reported that HCMV increases the serum induced proliferation of HCMSMC [11]. However there is no proof that the virus causes an increased proliferation of smooth muscle cells in a more complex environment as the transfilter co-culture model. The coronary transfilter co-culture model allows a direct contact of HCAEC and HCSMC. Therefore interactions of these two cell types can be studied in an elegant manner, *e.g.* following cell-based or cell-associated HCMV infection, respectively.

Moreover, HCMV infection of human fibroblasts appears to trigger as well an opposite effect, namely, cell cycle arrest. In previous studies HCMV infection was shown to induce arrest of cell growth either in late G1 or in G2/M [8,9].

In the 3D human coronary transfilter co-culture model reactive cell proliferation of co-cultured HCMSMC was increased significantly 3.9 times after cell-free HCMV infection and 3.7 times after cell-associated HCMV-infection in comparison to controls. The data proof that, in the complex experimental conditions of the human coronary transfilter co-culture model, cell proliferation is triggered after cell-associated and cell-free HCMV infection. Some evidence supports a link between the reactivation of latent HCMV following coronary angioplasty, p53 activation, and restenosis [23-25], as HCMV was preferentially detected in a subset of restenotic lesions that exhibited high levels of p53 protein

[25]. Dhaunsi et al., [11] suggested a role of NAPDH oxidase in cytomegalo-induced proliferation of HCMSMC.

In the present study maximal reactive cell proliferation occurred at day 7 after cell-free HCMV infection and at day 14 after cell-associated HCMV infection. This delay of reactive cell proliferation can be partially explained by the fact that MO-based HCMC infection of both endothelial cells and smooth muscle cells was less intense and postponed in comparison to cell-free HCMV-infection. Moreover the data demonstrate that rather low numbers of HCMV are sufficient to trigger a strong proliferative response, reactive cell proliferation was almost identical after cell-free and cell-associated HCMV-infection.

Data of clinical studies comparing serum IgG and IgM antibodies to HCMV with the occurrence of restenosis following coronary intervention are contradicting. Both a clear correlation [26,27] between HCMV seropositivity and restenosis and no correlation at all [28,29] have been reported. Serum IgG and IgM antibodies to HCMV in individuals proof merely the presence of HCMV but do not indicate the ability or intention of the virus to stimulate [10,11], postpone [13], or inhibit cell proliferation [8,9]. A different activity of HCMV in seropositive patients may be the explanation for the contradicting results in clinical trials, comparing seropositivity of individuals with the occurrence of restenosis or atherosclerosis. Recently Melnick et al., [30] suggested that upregulation of ERK phosphorylation is necessary for initial HCMV induced pathogenesis. The presented models are suitable for further studies on stimulatory and inhibitory effects of HCMV-infection in human coronary cells.

The 3D human coronary transfilter co-culture model is, despite its complexity, merely an *in vitro* model. Although HCAEC and HCMSMC are cultured as closed cell layers and are able to communicate with eachother, the model is a limited imitation of the human vessel wall. The effect of the adventia *e.g.* is completely missing. Human organ culture models are more complex, the reaction of the complete vessel wall consisting of endothelial layer, medial layer and adventitial layer can be studied. On the nother hand human organ culture models are limited by the fact that the nutrition vessels of the artery are cut during the preparation procedure. This may explain the fact that HCMV-infection (13) or perfusion with monocytes (31) did not trigger reactive cell proliferation, as recently described by our group.

HCMV is ubiquitous and persists after infection troughout the lifetime of the host. Although primary infection is usually asymptomatic, reactivation in immunocompromised individuals can cause severe disease. An immune system however is completely missing in the system. Taken together, data of the literature and the results of the present study demonstrate that infection with HCMV may influence the host cell cycle machinery in many different ways, depending on its actual interest. In the current study a link between HCMV infection and increased cell proliferation can be proofed, both after cell-free and cell-associated infection.

If we assume that HCMV is involved in the pathogenesis of atheroscleraosis, restenosis, and transplant vasculopathy the effect of antiviral treatment strategies should be elucidated in further studies.

Competing interests

The authors declare that they have no competing interests.

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Publication history

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