Upregulation of functionally active vascular endothelial growth factor by human cytomegalovirus

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Human cytomegalovirus (HCMV) infection is known to modulate host gene expression and has been linked to the pathogenesis of vasculopathies; however, relevant pathomechanisms are still unclear. It was shown that HCMV infection leads to upregulation of vascular endothelial growth factor (VEGF) expression in human foreskin fibroblasts and coronary artery smooth muscle cells (SMC). Activation of VEGF transcription by HCMV infection was confirmed by transient-expression experiments, which revealed that a short promoter fragment, pLuc135 (–85 to +50), is sufficient for activation. Site-directed mutagenesis of Sp1-recognition sites within this fragment abolished the upregulation of transcription. Functional VEGF protein is released into the culture supernatant of infected SMC. Incubation of endothelial cells with supernatants from HCMV-infected SMC cultures induced upregulation of VEGF receptor-2 expression on endothelial cells, as well as a significant upregulation of DNA synthesis, implicating cell proliferation. The mean incline of DNA synthesis at 48 and 72 h post-infection was 148 and 197 %, respectively. Addition of neutralizing antibodies against VEGF completely abolishes this effect. Supernatants from SMC cultures incubated with UV-inactivated virus induced a comparable effect. This virus-induced paracrine effect may represent a molecular mechanism for HCMV-induced pathogenesis, such as inflammatory vasculopathies, by inducing a proatherogenic phenotype in SMC.

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen that causes severe disease in immunocompromised individuals. Practically all organ systems, including blood vessels, can be affected. Many patients suffering from cardiovascular disease lack known risk factors and attention has focused increasingly on a potential role of infectious agents in the pathogenesis of vasculopathies. HCMV has been shown to predispose to an enhanced rate of restenosis (Zhou et al., 1996) and vasculopathy following organ transplantation (Srivastava et al., 1999). However, in the literature, the reported seroepidemiological data are controversial. Changes in HCMV-infected cells towards a potentially proatherogenic phenotype are probably based on HCMV-associated modulations of cellular gene expression (Zhu et al., 1998; Browne et al., 2001). Direct effects on HCMV-infected cells (Sedmak et al., 1994) and paracrine effects (Almeida et al., 1994; Murayama et al., 1997) have been demonstrated.

Cytokines and various growth factors, e.g. vascular endothelial growth factor (VEGF), are important regulators of vascular functions and integrity (Waltenberger, 1997). The VEGF protein family influences endothelial cell survival, angiogenesis and vascular permeability (Zachary & Gliki, 2001). In the pathology of herpetic stromal keratitis, herpes simplex virus-induced VEGF-associated angiogenesis has been shown to occur in ocular lesions (Zheng et al., 2001). Human herpesvirus 8 is known to be associated with the development of malignancies such as primary effusion lymphomas and Kaposi’s sarcoma, which are interlaced with newly formed vessels. Viral cytokine-induced VEGF expression that increased tumour cell growth was demonstrated in primary effusion lymphoma cell lines (Liu et al., 2001). Our study was designed to identify consequences of HCMV infection on peptide growth factor expression. We have demonstrated HCMV-induced modulation of VEGF production and shown that Sp1 sites on the VEGF promoter are involved in the activation of VEGF gene expression. This HCMV-induced effect was shown in fibroblasts, a commonly used cell type that is susceptible to permissive HCMV infection, as well as in smooth muscle cells (SMC),
a relevant cell type in plaque development in atherosclerosis. The mitogenic activity of the released VEGF protein on endothelial cells was shown. This HCMV-induced paracrine effect might be relevant in vasculopathies.

METHODS

Cells and infection. Human coronary artery SMC, umbilical cord endothelial cells (HUCV), and human coronary artery endothelial cells (HCAEC) (Clonetics, BioWhittaker) were cultured in SMC basall medium supplemented with 5 % fetal calf serum (FCS) and growth factors (SmGM BulletKit, BioWhittaker) and in endothelial cell basall medium supplemented with 5 % FCS and growth factors (EGMVM single aliquots, BioWhittaker), respectively. The laboratory HCMV strain AD169 was obtained from the ATCC. The low-passaged HCMV strain TB40E was kindly provided by Dr Ch. Sinzger, University of Tübingen, Germany. To allow direct visualization of infection, a green fluorescent protein (GFP)-expressing, low-passaged clinical HCMV isolate was used in experiments when neutralizing antibodies were applied to prevent infection. Virus stocks were produced on human foreskin fibroblasts (HFF) (Michel et al., 1996). For inactivation, HCMV stocks were placed in plastic dishes and irradiated with UV light (366 nm, 2 min, 200 kJ); successful inactivation was ensured through lack of cytopathic effect on HFF. For immunofluorescence experiments, cells were grown on tissue-culture chamber slides (Becton Dickinson). For Northern blot analysis, cells were cultured in 10 cm dishes (Greiner). To produce conditioned media, SMC were cultured in 25 cm² flasks (Greiner). At a confluence of 80–90 %, cells were grown on tissue-culture chamber slides (Becton Dickinson) and cultured in SMC basal medium supplemented with 1 % BSA and 1 % FCS. After 48 and 72 h, supernatants were harvested and centrifuged at 3000 r.p.m. for 10 min. Following UV inactivation, supernatants were stored at −70 °C.

RNA extraction and Northern blotting. Total RNA was extracted and 10 μg total RNA was separated as described previously (Minisini et al., 2003). RNA samples were obtained from uninfected cells as well as from cells infected with viable virus, UV-inactivated virus and GFP-expressing virus, pre-incubated with HCMV hyper-immunoglobulin (Flebogamma; Grifols). RNA was transferred onto a nylon membrane and immobilized by incubation for 2 h at 60 °C. For hybridization, a DNA probe specific for VEGF was amplified by PCR. Membranes were stripped for 20 min with 0-1 × SSC and 0-1 % SDS for rehybridization with a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Probes were labelled with [32P]dCTP by using a random-priming kit (Pharmacia). Hybridization was carried out at 42 °C for 18 h in the presence of 50 % formamide. Filters were washed to a stringency of 0-2 × SSC, 0-1 % SDS at 65 °C. Intensity of RNA signals was quantified by densitometry. Ratios of specific VEGF RNA levels were standardized according to the signals obtained with the GAPDH probe.

Plasmids and transient expression. For transient expression, constructs consisting of different 5′ regions of the VEGF promoter linked to the luciferase reporter gene were used as described previously (FinkenSeeder et al., 1997) and the dual luciferase reporter assay system (Promega) was used for internal standardization. Briefly, HFF were grown on 12-well plates and transfected with 30 μg of each of the indicated plasmids by calcium phosphate precipitation as described by Sambrook et al. (1989), followed by glycerol shock. Cells were harvested 24 h post-transfection and resuspended in 100 μl lysis buffer. Luciferase activity was determined with a Lumat LB 9507 luminometer (Berthold). For transient-expression experiments in mock- and virus-infected cultures, cells were infected with viable or UV-inactivated HCMV 2 h before transfection.

Analysis of VEGF protein by Western blotting. Western blot analysis for detection of VEGF expressed in mock- or HCMV-infected cells and cells incubated with UV-inactivated virus was performed as described previously (Michel et al., 1996). Cell lysates were extracted and separated by SDS-PAGE (12 % acrylamide). VEGF proteins were detected with the antiserum sc152 (Santa Cruz) and visualized by enhanced chemiluminescence (Amersham Biosciences).

Immunofluorescence. SMC or HFF cultured on chamber slides were washed three times with PBS and fixed with ice-cold methanol. Primary antibodies were directed against the HCMV immediate-early antigen (Argene Biosoft, clone E13, exon 2, major IE) or the VEGF protein (Santa Cruz). Antibodies were added at a dilution of 1:50 in PBS supplemented with 1 % BSA (Sigma). Slides were incubated for 45 min at 37 °C. After extensive washing, a fluorescein isothiocyanate-labelled donkey anti-rabbit antibody (Dianova) was added at a dilution of 1:50 and a tetramethylrhodamine isothiocyanate-labelled goat anti-mouse antibody (Dianova) was used at a dilution of 1:30 in PBS/BSA. As antibody controls, we used irrelevant primary antibodies (DAKO) or the primary antibodies were omitted. After 45 min incubation at 37 °C, slides were washed and mounted with glycerol gelatin. Analysis was performed by using a fluorescence microscope (Zeiss).

Production of conditioned media. Cell-free supernatants were produced in parallel on non-infected and TB40E-infected SMC and on SMC incubated with UV-inactivated virus. Cultures were washed and cultured in SMC basall medium supplemented with 1 % BSA and 1 % FCS. After 48 h, supernatants were harvested and centrifuged at 3000 r.p.m. for 10 min. Following UV inactivation, supernatants were stored at −70 °C.

Fluorescence-activated cell sorting (FACS) analysis. HCAEC were harvested after 48 h incubation with conditioned media from HCMV-infected and non-infected SMC. Cells were detached and separated by using trypsin (BioWhittaker). Cells were fixed with 4 % paraformaldehyde and washed with PBS containing 3 % heat-inactivated FCS, 0-1 % sodium azide and 10 mM HEPES (FACS buffer). To minimize non-specific staining and to permeabilize the membrane, cells were incubated for 45 min at 4 °C with FACS buffer supplemented with 10 % human immunoglobulin (Flebogamma, 5 %; Grifols) and 0-1 % saponin. An irrelevant antibody (DAKO) or a specific mAb directed against an intracellular epitope of the VEGF receptor-2 (VEGFR-2) protein (Santa Cruz) was applied. After washing, a phycoerythrin (PE)-labelled goat antimouse secondary antibody (DAKO) was added. Cells were washed thoroughly following 30 min incubation on ice. Quantitative analysis was performed on a FACSscan flow cytometer (Becton Dickinson) using the CellQuest research software. In total, 1 × 10⁵ events per sample were collected and analysed.

Endothelial cell proliferation assay. HUVEC were seeded at a confluence of 50 % on 96-well plates (Greiner). Before adding the conditioned media produced on SMC, HUVEC were starved overnight. Starvation media were removed and conditioned media were added. Each sample was analysed in six replicates. To identify the mitogenic protein, neutralizing mAbs directed against either VEGF (RD Systems) or an irrelevant antigen were added to aliquots of conditioned media 1 h prior to the proliferation assay, according to the manufacturer’s instructions. HUVEC were stimulated by conditioned media for 24 h. During the final 12 h incubation, 1 μCi (37 kBq) [3H]thymidine per well (Amersham Biosciences) was added. After the stimulation period, medium was removed and cells were lysed and detached with 100 μl 0-1 % saponin and trypsin (5 g l⁻¹) in PBS. The lysates were transferred to a filter and scintillation counting was performed by using a Betaplate 96-well harvester (Pharmacia). For statistical analysis, the Mann–Whitney unpaired non-parametric test was used.
RESULTS

VEGF mRNA is increased after HCMV infection

For Northern blot analysis, RNA was extracted 24 and 48 h post-infection (p.i.) from fibroblasts infected with HCMV strain AD169, UV-inactivated virus or mock-infected cells. At both time points, increased VEGF signals at 3-7 kb were found in virus-infected cells and cells infected with UV-inactivated virus compared with mock-infected cells, as shown in Fig. 1. Expression of the housekeeping gene GAPDH in HFF is not altered by HCMV infection and was therefore used as input control. Pre-incubation of a GFP-expressing HCMV isolate with an HCMV-specific hyperimmunoglobulin preparation did not completely prevent infection of HFF and some infected cells could still be detected by the presence of intense green fluorescence. Therefore, a still higher expression of VEGF-specific RNA was observed, compared with mock-infected HFF (data not shown).

HCMV infection leads to activation of the VEGF promoter via Sp1-binding sites

To elucidate whether the known VEGF promoter is activated due to viral infection, transient-expression experiments were performed. For this functional analysis, fragments of the VEGF promoter ligated to the luciferase reporter gene were used. The constructs were transfected into mock-infected fibroblasts and into fibroblasts infected with viable and UV-inactivated AD169 (Fig. 2). As shown in Fig. 2, HCMV infection was responsible for VEGF promoter activation. Only the shortest VEGF promoter fragment, pLuc102, was not able to activate gene expression substantially. The highest activity compared with the empty plasmid was observed with construct pLuc135, showing that the region spanning nucleotide positions −85 to +50 is sufficient for activation, yet a virus-induced increase of luciferase activity compared with mock-infected cells was also seen with longer promoter fragments. After mutagenesis of the Sp1-recognition sites present in construct pLuc135/Sp1mut (Finkenzeller et al., 1997), the promoter activation was reduced significantly, suggesting that these sites are involved in this activation. However, further promoter elements probably play a role, as transfection of cells incubated with UV-inactivated virus did not lead to promoter activation (data not shown).

HCMV stimulates VEGF protein expression in infected HFF and SMC

Western blot analyses and indirect immunofluorescence assays of HCMV-infected and mock-infected cells were performed to test whether the increased transcriptional activity in virus-infected cells correlated with increased protein expression. As shown in Fig. 3, higher amounts of VEGF protein were observed in virus-infected fibroblasts compared with mock-infected cells at different time points after infection, beginning as early as 24 h p.i. Following incubation with UV-inactivated virus, upregulation of VEGF protein expression was observed, comparable to that seen for infection with viable virus (Fig. 3b). Indirect immunofluorescence assays using SMC and HFF (Fig. 4) also showed an increase in VEGF expression following HCMV infection. A strong green fluorescent staining for VEGF was only seen in infected cells that expressed the viral immediate-early antigen. Maximal modulation of VEGF protein in infected HFF and SMC was seen between 48 and 72 h p.i. (Fig. 4c, d). SMC infected with the AD169 strain showed similar upregulation, as did SMC infected with the TB40E strain (Fig. 4f). Mock-infected SMC (Fig. 4a) did not show staining for the VEGF protein, indicating an expression level below the limit of detection. Comparable results were observed following HCMV infection of HFF (Fig. 4g–j).

HCMV infection induces paracrine stimulation of endothelial cells

HCMV-induced upregulation of VEGF protein expression is followed by increased release of functional protein into the culture supernatant. Incubation of HCAEC with supernatants from HCMV-infected SMC leads to upregulation of VEGFR-2 expression, and thus to activation of endothelial cells (Fig. 5a). As a sign of an HCMV-induced increase in endothelial cell proliferation, incubation of
HUVEC with supernatants from HCMV-infected SMC was associated with a statistically significant upregulation of DNA synthesis. As SMC of three different individuals were used for the production of conditioned media, the non-infected cell control of each experiment was set as 100% to allow comparability. Incubation of HUVEC with supernatants from infected SMC harvested 48 h p.i. induced a mean upregulation of $[^{3}H]$thymidine uptake to 148 ± 23 ± 4% (mean value ± SD from four independent experiments), compared with the conditioned media from the non-infected control. At 72 h p.i., the stimulating molecule in the supernatants of HCMV-infected human coronary artery SMC cultures accumulated and a mean upregulation of 197 ± 65 ± 9% was observed. The paracrine HCMV-induced upregulation of endothelial cell proliferation was statistically significant at 48 ($P < 0.019$) and 72 ($P < 0.0001$) h p.i. As upregulation of VEGF mRNA (Fig. 1) and protein was also observed with UV-inactivated HCMV, supernatants of SMC infected with UV-inactivated HCMV were produced in parallel. A comparable release of functional mitogenic protein to the culture supernatants of SMC could be observed following incubation with UV-inactivated and viable HCMV (Fig. 5b).

Neutralizing antibodies against VEGF can abolish the paracrine HCMV-induced effect

To confirm the identity of VEGF as the mitogenic molecule in HCMV-infected SMC, supernatants were pre-incubated

- [Fig. 2. Transient expression in HCMV-infected and mock-infected HFF. Fibroblasts were infected 2 h before transfection. Cells were harvested and luciferase activity was determined. The nucleotide sequence at positions -2018 to +50 or deletion fragments of the human VEGF promoter were fused to the luciferase gene. Potential consensus binding sites for AP-1 (open boxes), AP-2 (open circles) and Sp1 (black boxes) are indicated. Deleted Sp1 sites are depicted by X. Values are given as fold increase over the basal promoter activity of mock-infected cells.]

- [Fig. 3. HCMV-induced upregulation of VEGF protein expression in HFF. Expression of the VEGF protein in HFF is modulated by HCMV infection. The kinetics of VEGF protein expression in HCMV-infected and mock-infected HFF are shown by Western blot analysis (a). Upregulation of VEGF protein by viable as well as UV-inactivated virus 48 h.p.i. is shown (b). Proteins were visualized by enhanced chemiluminescence.]
with neutralizing antibodies against VEGF. The HCMV-associated upregulation of DNA synthesis was neutralized completely by the addition of antibodies directed against VEGF (Fig. 5b). This demonstrates that the HCMV-associated upregulation of the VEGF protein in infected SMC results in the release of a functional protein that induces endothelial cell proliferation.

**DISCUSSION**

As HCMV is an obligate intracellular parasite, its survival depends on interactions with the host-cell machinery. HCMV uses various cellular signalling and regulatory pathways for replication and assembly. In HFF, a number of host-cell genes whose RNA levels have been altered in response to HCMV infection have been identified (Zhu...
et al., 1998; Browne et al., 2001). These modulations of host-cell gene expression might promote various disease processes. Although there is still no formal proof that HCMV plays a causal role in vasculopathies of non-immunocompromised individuals, increasing evidence supports the concept that HCMV may trigger the development of vascular lesions. Potential pathomechanisms induced by HCMV have been suggested by the results of a number of studies (Speir et al., 1994; Zhou et al., 1996; Streblow et al., 1999; Reinhardt et al., 2002). In the development of vascular disease, various peptide growth factors play an important role, among them VEGF. This prompted us to examine the influence of HCMV infection on the expression and release of the VEGF protein. The data presented here clearly demonstrate HCMV-induced increases in production (Fig. 3 and 4) and release (Fig. 5) of a mitogenic VEGF protein by infected SMC. As VEGF mRNA and protein levels are increased after HCMV infection, the regulation occurs at the level of activation of transcription (Fig. 1). Consistently with this, we observed VEGF promoter activation after transfection of HCMV-infected fibroblasts. Additionally, deletion analysis revealed that an 85 bp region upstream of the transcriptional start site of the VEGF promoter was sufficient for strong activation by HCMV. This activation is mediated in part by Sp1 binding, as shown with a VEGF promoter fragment carrying the same 85 bp upstream region with mutagenized Sp1-binding sites (Finkenzeller et al., 1997) (Fig. 2). The addition of UV-inactivated virus induced a similar increase in VEGF mRNA expression and release of mitogenic VEGF in SMC as were caused by viable virus (Fig. 1). Therefore, the mere contact of HFF and SMC and the uptake of viral structural proteins appear to be sufficient to induce enhanced VEGF expression. However, this must be mediated by an Sp1-independent mechanism, as transfection experiments showed no promoter activation in cells infected with UV-inactivated virus, thus indicating that more than one molecular mechanism must play a role in HCMV-induced VEGF upregulation. Binding of viral glycoproteins or components of the viral tegument to cellular structures that are involved in VEGF regulation may mediate the observed increase in VEGF.

Recently, immunohistochemical studies of human coronary artery tissues indicated that advanced atherosclerotic lesions contained a statistically significant number of intimal blood vessels, as well as higher numbers of VEGF-expressing cells within the intima (Chen et al., 1999). By double immunostaining, the majority of VEGF-expressing cells were identified as being SMC (Couffinhal et al., 1997; Chen et al., 1999). Within advanced lesions, VEGF could even be detected in intraplaque microvasculature. These authors suggested that, in vivo, VEGF induces neointimal angiogenesis and intimal hyperplasia; therefore, VEGF might promote progression of vasculopathies (Inoue et al., 1998; Moulton et al., 1999, 2003). Supporting data were published by Celletti et al. (2001), who demonstrated that the application of recombinant human VEGF increased the rate and degree of plaque development in an animal model. Additionally, a strong correlation has been found between intragraft VEGF protein expression and the development of intimal thickening in a cardiac allograft model (Lemström et al., 2002). Further, VEGF induces increased expression of adhesion molecules on the surface...
of endothelial cells (Keck et al., 1989) and attracts and activates monocytes (Clauss et al., 1990; Barleon et al., 1996). Thus, by increasing VEGF release, HCMV infection might enhance chemoattraction of monocytes, thus promoting inflammatory processes within the vessel wall. Neovascularization, expression of adhesion molecules and recruitment of leukocytes to plaques are all factors that contribute to vascular disease (de Boer et al., 1999). As no animal model for HCMV infection exists, due to its strict species specificity, direct evidence of HCMV-induced VEGF expression in vivo is not feasible. However, in humans, potential involvement of HCMV in the pathogenesis of vasculopathies is supported by the detection of viral antigen and nucleic acids in SMC cultured from coronary artery plaque material (Melnick et al., 1983). HCMV nucleic acid could be identified in DNA extracted from atherosclerotic femoral arteries and abdominal aortas (Hendrix et al., 1991). Viral DNA was detected in 90% of severe and in 53% of minimal atherosclerotic lesions. By using an artery organ-culture model for HCMV infection, we could demonstrate the presence of HCMV-infected SMC in the vascular media and intima (Reinhardt et al., 2003), thus establishing SMC as a viral target cell within the vessel wall.

In conclusion, we have demonstrated a previously unknown HCMV-induced modulation of the cellular VEGF gene. An increased amount of functional VEGF protein is produced and released by HCMV-infected SMC. This induced a paracrine stimulation of endothelial-cell activation and proliferation. Concerning the pathophysiologically relevance of this virus-induced change of the SMC phenotype, we can only hypothesize that increased VEGF protein levels in the environment of HCMV-infected SMC may enhance inflammatory processes or potentially promote plaque angiogenesis, finally supporting the development of virus-induced vasculopathy.

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