Direct infection of primary endothelial cells with human cytomegalovirus prevents angiogenesis and migration

Rasmus K. L. Gustafsson, Hannah C. Jeffery, Koon-Chu Yaiw, Vanessa Wilhelmi, Ournia N. Kostopoulou, Belgis Davoudi, Afsar Rahbar, Melinda Benard, Thomas Renné, Cecilia Söderberg-Nauclér and Lynn M. Butler

1Clinical Chemistry, Department of Molecular Medicine and Surgery, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden
2Institute for Clinical Chemistry and Laboratory Medicine, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany
3Department of Medicine, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden
4INSERM UMR 1043, Hospital Purpan, Paul Sabatier University, Toulouse 31024, France

Human cytomegalovirus (hCMV) is a beta herpesvirus that establishes lifelong infection. Although the virus does not usually cause overt clinical symptoms in immunocompetent individuals it can have deleterious effects in immunocompromised patients, such as those on post-transplant medication or with HIV infection. hCMV is the most common congenital infection and can lead to serious fetal sequelae. Endothelial cells (ECs) are natural hosts for hCMV in vivo, therefore, investigations of how this cell type is modulated by infection are key to understanding hCMV pathogenesis. Previous studies have examined the effect of secretomes from hCMV-infected cells on EC angiogenesis, whereas the effect of direct infection on this process has not been so well investigated. Here, we show that placental ECs are viral targets during congenital infection and that vessels in infected tissue appear morphologically abnormal. We demonstrate that the clinical hCMV strain VR1814 impaired EC tube assembly in vitro angiogenesis assays and inhibited wound healing ability in scratch assays. Secretomes from infected cultures did not impair angiogenesis of uninfected ECs, suggesting that cell-intrinsic changes, as opposed to secreted factors, were responsible. We observed viral gene transcription dependent downregulation of the expression of angiogenesis-associated genes, including angiopoietin-2, TEK receptor and vascular endothelial growth factor receptors. An alternative clinical hCMV stain, TB40E showed similar effects on EC angiogenesis. Together, our data indicate that direct infection with hCMV can induce an anti-migratory and anti-angiogenic EC phenotype, which could have a detrimental effect on the vasculature development in infected tissues.

INTRODUCTION

Human cytomegalovirus (hCMV) is a ubiquitous beta herpesvirus that establishes lifelong infection in its host (Landolfo et al., 2003). Whilst this virus does not cause overt clinical disease in immunocompetent individuals, it can cause life-threatening infection in immunocompromised individuals, such as transplant and AIDS patients (Weber & Doerr, 1994). Furthermore, hCMV is the most common congenital infection (Lombardi et al., 2010), with transmission of hCMV from mother to fetus, occurring in 0.6% of US births, and of these around 20% of the children...
will get clinical symptoms, which can include hearing loss and mental retardation (de Vries et al., 2011; Dollard et al., 2007). The seroprevalence of hCMV increases with age and decreases with socioeconomic status (Dowd et al., 2009). In women of reproductive age, hCMV seroprevalence ranges between 45–100 %, being highest in Asia, South America and Africa and lowest in Western Europe and the United States (Cannon et al., 2010).

Endothelial cells (ECs), which line the lumen of all blood vessels, are an in vivo target of hCMV infection and important in viral pathogenesis (Alcendor et al., 2012; Jarvis & Nelson, 2007; Sinzger et al., 1995). Angiogenesis is the physiological process through which new blood vessels develop from pre-existing ones. This process, which is critically dependent on EC cytoskeletal remodeling and migration, is induced by chemotaxis under the influence of vascular endothelial growth factor (VEGFA) and VEGF-receptor signalling (Lamalice et al., 2007). In the presence of VEGFA signalling the angiopoietin (ANGPT)2/TEK receptor pathway also contributes to this process (Carmeliet & Jain, 2011). New vessel formation through EC angiogenesis is crucial for fetal development (Wittko-Schneider et al., 2014) and also for growth and repair in the adult (Xiong et al., 2010). Poor vessel growth can contribute to several pathologies, including vascular diseases (Caposio et al., 2011; Lamalice et al., 2007). Disrupted vessel growth in the placenta can hamper its functionality and in turn cause fetal growth restriction (Burton et al., 2009) and preeclampsia, with severe acute and long-term effects for both mother and fetus (Santillan et al., 2009), as well as pregnancy loss (Pereira et al., 2015).

Recent studies have shown how hCMV infection of various cell types can induce paracrine cytokine networks that influence EC function and indicate a pro-angiogenic effect (Alcendor et al., 2012; Dumortier et al., 2008; Fiorentini et al., 2011). Furthermore, hCMV infection of ECs has been reported to induce the secretion of pro-angiogenic and anti-apoptotic factors (Botto et al., 2011; MacManiman et al., 2014). Secretomes produced by infected lymphatic ECs have also been shown to be pro-angiogenic and to increase human endothelial cell migration (Fiorentini et al., 2011). Likewise, factors in the supernatants of infected fibroblasts increased the formation of capillary networks and greatly improved the duration of neovessel stability (Dumortier et al., 2008). Together, these reports provide support for the hypothesis that the presence of hCMV infection could potentiate angiogenesis. However, the direct consequence of hCMV infection of ECs on subsequent angiogenesis is less clear. Both increased (Bentz et al., 2008) and decreased (Yamamoto-Tabata et al., 2004) EC motility has been reported with hCMV infection. Furthermore, to our knowledge, possible temporal changes of the effect of infection on angiogenesis have not been considered. Here, we report that the clinical hCMV stain VR1814 inhibited in vitro wound healing and angiogenesis of primary ECs. This effect was not recapitulated by exposure to supernatants from virally infected cultures, indicating a direct, rather than paracrine mechanism. Indeed, expression of pro-angiogenic genes was inhibited by infection, and this was dependent on active viral replication. Interestingly, we saw similar, but milder, effects on angiogenesis and wound healing using an alternative clinical hCMV strain, TB40E. Overall, our study shows that hCMV infection of primary ECs can induce an anti-angiogenic phenotype. In heavily infected tissues in vivo, such as congenital infected placenta, this could result in inhibited vascular development, hampered circulation and compromised fetal development.

RESULTS

hCMV immediate early proteins are detectable in congenitally infected placenta

Placentas from congenital hCMV-infected fetuses or normal controls were stained for hCMV immediate early (IE) antigen or von Willebrand factor (vWF). hCMV-IE staining was absent in control uninfected placental tissue (Fig. 1a) but clearly present in hCMV-infected placental tissue. The degree of staining observed ranged from ‘extensive’ to ‘low’ between different donors (Figs 1d, g and i, respectively). hCMV-IE staining was particularly apparent in trophoblastic cells, but also in cells that formed a lumen morphology and were morphologically consistent with being ECs (Fig. 1d, magnified image). vWF staining highlighted EC lining blood vessels, revealing the extensive placental vasculature (Fig. 1c), which showed indications of abnormality in the hCMV-infected placenta, with enlarged irregular vessel lumens present (Fig. 1f, arrowed). As congenital hCMV infection resulted in expression of hCMV proteins in the placental ECs and the appearance of a morphologically abnormal vasculature, we choose to further study the effects of hCMV on EC behaviour.

hCMV VR1814 infection of primary ECs

Phase-contrast images revealed that hCMV VR1814 induced EC morphology changes (Fig. 2a), which were absent following mock or UV-inactivated hCMV VR1814 inoculation. To formally determine the level of infection we performed immunofluorescence staining using an antibody that targeted hCMV IE proteins. At 1 day post-inoculation (p.i.) the mean infection rate was 30 %, which increased to a maximum by 7 days p.i. (Fig. 2b, c). No IE positive cells were detected in cultures inoculated with UV-inactivated hCMV VR1814. hCMV-inoculated ECs were no less viable than mock-inoculated ECs at 7 days p.i., as measured by MTT assay (Fig. 2d) and trypan blue exclusion (data not shown).

hCMV VR1814 infection of primary ECs prevents tubulogenesis

To establish whether hCMV VR1814 infection of ECs had direct effects on angiogenesis we performed tube formation
assays at 1 and 7 days p.i. using polymerized growth factor reduced matrigel as the substrate. Assays were imaged for evidence of tube formation at 6 h post seeding. Mock treated cultures assembled into anastomosing tubules by this time point when seeded on matrigel after 1 or 7 days of culture, and enclosed lumens were formed by the connection of branches that extended from multi-direction branching nodes [Fig. 3a(i)]. At time points prior to 6 h post seeding, networks were not fully established and by later time points (12, 24 and 32 h) no additional networks had developed (data not shown). For hCMV VR1814-inoculated ECs, the angiogenic capacity was reduced by 40–50 % at 1 day p.i., compared to mock, measured by reduced branch [Fig. 3a(ii)] and branch point [Fig. 3a(iii)] formation (see Fig. S1, available in the online Supplementary Material, for quantification details). At 7 days p.i. hCMV-inoculated

![Image of Figure 1: Congenitally infected placenta can express high levels of hCMV immediate early (IE) protein. Representative pictures of normal placental tissue (a–c) or second and third trimester placental tissue from three of seven hCMV-congenitally infected fetuses (d–j). Placental tissue was stained with mouse anti-hCMV immediate early antigen antibody (a, d, g, i), secondary antibody only as control (b, e, h, j) or anti-human von Willebrand factor (vWF) antibody (c, f). Scale bars represent 100 μm. Arrows highlight hCMV IE stained cells lining a blood vessel (d) and abnormally shaped vWF positive vessels (f).]
hCMV inhibits EC angiogenesis and migration.
ECs did not form any tubules [Fig. 3a(ii, iii)]. This inhibition in tube formation did not simply represent an hCMV-induced delay, as no further network development was observed at 12, 24 or 32 h post seeding. To assess whether this effect was dependent on de novo viral gene expression the virus inoculum was UV-inactivated prior to inoculation of ECs. EC inoculated with UV-inactivated hCMV VR1814 had no effect on tube formation when compared to the mock-inoculated control (Fig. 3a.iv). As previous reports have shown that secretomes of hCMV-infected ECs can induce angiogenesis we tested if supernatants taken from hCMV VR1814 inoculated cultures at 1 day p.i. could modulate angiogenic responses. Supernatants from mock- or hCMV VR1814-inoculated ECs were collected and filtered to remove viral particles. Fresh ECs were preconditioned overnight in these secretomes before they were transferred to angiogenesis slides and cultured for 6 h. No difference in tube formation was found between ECs treated with secretomes from mock- or hCMV VR1814-inoculated cultures (Fig. 3b), in terms of branch number (Fig. 3b.ii) or branch point formation (Fig. 3b.iii).

Fig. 2. hCMV VR1814 infection of primary ECs. ECs were mock or hCMV VR1814 (MOI 3) inoculated and cultured for 1 or 7 days before being (a) imaged with ×10 magnification phase-contrast microscopy, (b) stained for the expression of hCMV immediate early protein and imaged with ×40 magnification confocal microscope, which was used to (c) quantify % infection. (d) EC viability was measured using MTT assay. Images are representative of 3–10 experiments. Data are mean ± SEM for 3–6 experiments. Each experiment used ECs from a different donor ***P<0.001. UD=undetected.

Fig. 3. Effect of hCMV VR1814 and the hCMV VR1814 EC secretome on EC tube formation. ECs were (a) mock or hCMV VR1814 (MOI 3) inoculated and cultured for 1 or 7 days or (b) treated for 24 h with day 1 or day 7 filtered supernatants from mock or hCMV VR1814 inoculated ECs, before they were transferred to angiogenesis slides and cultured for 6 h. [a(i) and b(i)] images were captured with ×4 magnification phase-contrast microscopy and the number of [a(ii) and b(ii)] branches and [a(iii) and b(iii)] branch points were quantified. [a(iv)] ECs were mock or UV-inactivated hCMV VR1814 inoculated and cultured for 1 or 7 days before they were transferred to angiogenesis slides and cultured for 6 h. The number of branches and branch points are expressed relative to the mock control. Images are representative of 3–5 experiments. Data are mean ± SEM for 3–5 experiments. Each experiment used ECs from a different donor *P<0.05, **P<0.01, ***P<0.001.
**hCMV VR1814 infection of primary ECs inhibits wound healing**

As cellular migration is an important prerequisite for tube formation, we sought to determine if hCMV VR1814 inoculation of ECs inhibited *in vitro* wound healing in scratch assays. At 1 day p.i. mock- or hCMV VR1814-inoculated EC monolayers were scratched using a pipette tip and images of the wound were taken at 0 and 24 h after the scratch was made. EC cultures inoculated with hCMV VR1814 showed inhibited capacity to migrate into the scratched gap, compared to mock or UV-inactivated hCMV VR1814 [Fig. 4a(i, ii)]. A similar observation was made in a previous study (Yamamoto-Tabata et al., 2004). The authors suggested that human interleukin 10 (hIL-10) or the viral homologue (cmvIL-10) could have a role in the wound healing observed when ECs were infected with hCMV VR1814, as they observed inhibitory effects when ECs were treated with recombinant hIL-10 or cmvIL-10. To examine if this could possibly provide an explanation for our observations, we measured secreted

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**Fig. 4.** Effect of hCMV VR1814 infection and the hCMV VR1814 secretome on EC wound healing. ECs were (a) mock or hCMV VR1814 (MOI 3) inoculated and cultured for 1 day or (b) treated for 24 h with day 1 filtered supernatants from mock or hCMV VR1814 inoculated ECs, before a scratch was made in the monolayer prior to (i) imaging with ×4 magnification phase-contrast microscopy and [a(ii)] quantification of the % gap closure 24h later. Images are representative of 3–5 experiments. Data are mean ± SEM for 3–5 experiments. Each experiment used ECs from a different donor. **P<0.01.
hIL-10 by ELISA and cmvIL-10 mRNA by PCR in mock- or hCMV VR1814-inoculated ECs. Secreted hIL-10 was low with no observable difference in quantity between mock or hCMV VR1814-inoculated ECs (Fig. S2). PCR revealed that mRNA encoding cmvIL-10 could be detected in hCMV VR1814-inoculated ECs at 1 day p.i., but not in the untreated controls or those treated with UV-inactivated hCMV VR1814 (Fig. S2). To assess whether the impaired wound healing capacity was due to autocrine signalling (such as a contribution of this encoded cmvIL-10), or to cell-intrinsic effects, supernatants from ECs inoculated with mock, UV or non-UV-inactivated hCMV VR1814 were collected and filtered to remove viral particles. Fresh ECs were preconditioned overnight in these secretomes prior to the scratch assay. No difference in gap closure was found between ECs treated

Fig. 5. Effect of hCMV VR1814 on the expression of angiogenesis relevant gene transcripts by ECs. ECs were inoculated with mock or UV- or non-UV-inactivated hCMV VR1814 (MOI 3) and cultured for 1, 3 or 8 days prior to collection for mRNA analysis. The relative mRNA expression of (a) vascular endothelial growth factor receptor 1 (VEGFR1), (b) vascular endothelial growth factor receptor 2 (VEGFR2), (c) TEK receptor tyrosine kinase (TEK), (d) vascular endothelial growth factor A (VEGFA) and (e) angiopoietin-2 (ANGPT2) was measured for UV- or non-UV-inactivated hCMV VR1814 inoculated ECs versus mock-inoculated cultures at each time point by qPCR. Target gene expression was normalized to 18S. Data are mean ± SEM of 3 to 8 experiments. Each experiment used ECs from a different donor. *P<0.05, **P<0.01, ***P<0.001.
with secretomes from mock, UV or non-UV-inactivated hCMV VR1814 [Fig. 4b(i)], with all conditions resulting in full gap closure after 24h. This indicated that the inhibition of wound healing was due to the direct effects of virus infection on EC behaviour, rather than the induction of secreted factor(s).

**hCMV VR1814 infection modulates mRNA expression of regulators of vessel morphogenesis**

Key regulators of angiogenesis and vessel stability are the VEGF-VEGFR and the angiopoietin (ANG)-TIE-2 signaling pathways (Carmeliet & Jain, 2011; Huang et al., 2010). To test whether hCMV infection influenced either of these pathways the relative expression of *VEGFA*, *VEGFR1*, *VEGFR2*, *ANGPT2* and *TEK* mRNA transcripts in ECs inoculated with mock, UV or non-UV-inactivated hCMV VR1814 was determined by quantitative PCR (qPCR) at 1, 3 and 8 days p.i. *VEGFR1* and *VEGFR2*, exhibited a reduced expression at 3 days p.i., and this suppression was even more pronounced at 8 days p.i. (Figs 5a, b). TEK expression was significantly suppressed by hCMV VR1814-inoculation at all time points tested, although again these effects became increasingly pronounced with prolonged culture (Fig. 5c). We also observed similar hCMV-mediated suppression of angiogenic gene expression in aortic ECs (data not shown). The transcription of *VEGFA*, the ligand for the VEGFRs, was actually increased by hCMV VR1814-inoculation at all time points tested (Fig. 5d). As this finding was something of a paradox to our observations that supernatants did not appear to behave in a pro-angiogenic manner (Fig. 3b), we also measured VEGFA at the protein level. In agreement with the mRNA data, we observed a significant increase in VEGFA protein secreted from hCMV VR1814 inoculated ECs.
ECs at 1 day p.i., but at modest levels (21.2 ± 8.1 pg ml⁻¹ for hCMV VR1814 versus 14.3 ± 0.7 pg ml⁻¹ for mock P < 0.05) and only a trend towards increased levels at 3 and 7 days p.i. The TEK ligand ANGPT2 was significantly suppressed by hCMV VR1814-inoculation at all time points tested, although again these effects became increasingly pronounced with prolonged culture (Fig. 5e). All of the described modulations in gene expression were absent when virus inoculum was UV-inactivated prior to inoculation, suggesting that viral gene transcription was required (Fig. 5a–e).

**Fig. 7.** Effect of hCMV TB40E on EC angiogenesis and wound healing. ECs were mock or hCMV TB40E (MOI 3) inoculated and cultured for 1, 7 or 8 days before (a) immunocytochemistry staining for hCMV immediate early protein expression, which were used for (b) quantification of % infection, (c) measurement of tube formation at 7 days p.i., (d) measurement of wound healing at 7 days p.i. and (e) measurement of relative mRNA expression of angiogenesis relevant gene expression. Images are representative of 3–4 experiments. Data are mean ± SEM for 3–4 experiments. Each experiment used ECs from a different donor. *P < 0.05, ***P < 0.001
**Hypoxia does not affect hCMV VR1814 modulation of mRNA expression of regulators of vessel morphogenesis**

Hypoxia is known to induce angiogenesis *in vivo* and *in vitro* (Krock et al., 2011). To determine the impact of hCMV infection on the angiogenesis-related response of ECs to hypoxia, mock- or hCMV-inoculated ECs were incubated under conditions of 1 or 20 % oxygen (hypoxia and atmospheric oxygen, respectively) at 7 days p.i., selected as the time point for examination since the inhibitory effect of hCMV on tubulogenesis was maximal at this time point. Under conditions of hypoxia VEGFA mRNA expression was increased in both untreated and hCMV-inoculated EC cultures, suggesting both cultures were able to respond to hypoxia (Fig. 6b). However, the magnitude of the change in VEGFA expression was significantly inhibited with infection (2.5-fold increase for hCMV-inoculated versus 5.2-fold increase for mock-inoculated cultures). Other genes that have been shown to respond among the hypoxia regulated targets of either HIF-1α or HIF-2α in ECs and which have functional relevance to cell migration and angiogenesis were also examined for changes in mRNA expression with hypoxia and hCMV inoculation (ANGPT2, VEGFR-1, TEK, CD82 and CXCL12) (Hirota & Semenza, 2006; Krock et al., 2011; Nagao & Oka, 2011; Skuli et al., 2009; Yang et al., 2013). However, in contrast to the response seen for VEGFA, none of these factors were upregulated under hypoxia by either mock- or hCMV-inoculated cultures (Fig. 6). With the exception of CXCL12 expression, which was not altered by hCMV, all transcripts were strongly suppressed by hCMV and remained so under hypoxia (Fig. 6).

**hCMV TB40E inhibits EC angiogenesis, wound healing and expression of angiogenesis relevant transcripts**

To examine if the inhibition of angiogenesis and wound healing was specific to the hCMV VR1814 strain we tested the effect of inoculation with an alternative hCMV clinical strain, TB40E. ECs were inoculated with hCMV TB40 at an MOI equivalent those used for hCMV VR1814 in the previous experiments. Immunocytochemistry staining revealed that TB40E infection levels measured by IE protein expression were comparably lower than those seen with VR1814 (Figs 7a, b), with an average of 15 % at 1 day p.i. and 27 % at 7 days p.i., compared to 30 % at 1 days p.i. and 100 % at 7 days p.i. for hCMV VR1814 (Fig. 2c). Therefore, we used TB40E-inoculated cultures at 7 days p.i. in the functional assays, as the infection rate was comparable to hCMV VR1814-inoculated cultures at 1 day p.i. (when a functional inhibition in angiogenesis and wound healing was observed). Similarly to hCMV VR1814, hCMV TB40E impaired EC angiogenesis (Fig. 7c) and wound healing (Fig. 7d). Similar to the qPCR results with hCMV VR1814 infection at 1 day p.i., the VEGF pathway mRNAs were not modulated, but ANGPT2 expression was inhibited (Fig. 7e).

**DISCUSSION**

Angiogenesis is the physiological process by which new blood vessels form from existing vessels through a process of EC activation, migration and proliferation (for review see (Otrock et al., 2007)). ECs are targets of hCMV *in vivo* and believed to be important in various hCMV pathogeneses (Jarvis & Nelson, 2002). Various studies have presented opposing views on the general role of hCMV in angiogenesis, indicating that the effect of the virus on this process is likely to vary depending on culture conditions, time points of analysis and whether direct infection or the influence of the secretome is studied. In line with this observation, we provide to our knowledge the first demonstration that direct infection of ECs with the clinical hCMV strains VR1814 and TB40E results in the inhibition of both tube formation in angiogenesis assays and EC wound healing in scratch assays. Consistent with these functional modifications hCMV suppressed transcription of key angiogenic genes.

Here, we showed that placental ECs are positive for hCMV IE proteins expression during congenital infection and that vessels appear morphologically abnormal, which could be indicative of modified angiogenic processes. hCMV congenital infection is the leading cause of hearing loss and developmental problems in developed countries (Cheeran et al., 2009). The exact mechanisms underlying the development of these hCMV-induced clinical manifestations are not well understood, but some of the clinical features are consistent with placental insufficiency and subsequent fetal hypoxia (Adler et al., 2007) or loss (Pereira et al., 2015). Interestingly, hCMV infections contracted postnatally do not induce symptoms that are associated with placental insufficiency, suggesting that infection in this compartment plays a critical role in the pathogenesis of congenital hCMV disease (Adler et al., 2007). Formation of new blood vessels through angiogenesis is crucial for fetal development (Wittko-Schneider et al., 2014), but the consequences of EC infection in congenital infection are not well studied.

The inhibitory effect of the virus on angiogenesis that we observed in this study could be seen as early as 1 day p.i. At this time point, hCMV-infected ECs do not express viral late antigens (Jeffery et al., 2013) and thus have not yet entered the later stages of the lytic cycle. This, in combination with our previous work, which showed that at 1 day p.i. ECs can retain specialized leukocyte recruitment function (Jeffery et al., 2013), suggests that the loss of angiogenic function is not simply due to hCMV-induced cellular ‘disruption’. The degree of angiogenic inhibition increased with time post-inoculation, which could be due to the increasing number of ECs that are positive for hCMV, or further modification in cellular gene expression.
Again, our earlier reported work confirms infected ECs retain viability at this time point, despite the expression of late viral antigens (Jeffery et al., 2013). Previous studies are consistent with our observations, where infection with hCMV VR1814 was shown by a scratch assay to impair human umbilical vein ECs (HUVECs) migration, an important pre-requisite to angiogenesis (Yamamoto-Tabata et al., 2004). However, other studies have reported that infection with other hCMV strains promoted wound recovery, migration and angiogenesis. This was observed in human microvascular ECs infected with TB40E or E-UL-32 strains (Bentz et al., 2008) and HUVECs infected with Towne strain (Zhang et al., 2013). Further variations exist between these various studies, Bentz et al. (2008) and Zhang et al. (2013) used EGM EC medium in their assay, which contains a number of additional supplements, including VEGF, whilst Yamamoto-Tabata et al. (2004) used Dulbecco’s with 1 % serum. Thus, it is likely that the different culture conditions, the presence or absence of serum starvation, the hCMV strains studied, the MOI used, the vascular bed from which the ECs were sourced and/or the time points of observation contribute to this variation reported for the influence of direct EC hCMV infection on angiogenesis.

In our assessment of the effects of soluble factors, 24h secretomes collected at 1 day post hCMV VR1814 inoculation did not affect tube formation by HUVECs on matrigel or in wound healing assays. This result is contrary to observations of some previous studies which found that secretomes of hCMV-infected HUVECs (Botto et al., 2011), as well as those of hCMV-infected fibroblasts (Dumortier et al., 2008) and lymphatic ECs (Fiorentini et al., 2011) had pro-angiogenic effects, promoting tube formation and wound healing by HUVECs. These differences may be explained by the different time points post-inoculation at which supernatants were collected. Botto et al. (2011) and Fiorentini et al. (2011) both attributed the enhanced angiogenesis they observed to IL-6 present in the secretomes collected from infected cultures at 96 h post-infection. A recent study revealed that hCMV pUL7, a viral protein found in the secretome of infected ECs, induces angiogenesis and IL-6 secretion (MacManiman et al., 2014). We have previously demonstrated that the effect of direct hCMV infection on HUVEC IL-6 secretion is in fact biphasic (Jeffery et al., 2013); IL-6 was not elevated in the first 24 h, was increased to a maximum at 4 days p.i. and thereafter decreased until reaching baseline levels (equivalent to mock-infected cultures) by 7 days p.i. Thus, the secretomes tested in the present study may not be expected to induce angiogenesis, based on the temporal profile of IL-6 secretion after hCMV infection. In addition, in the studies mentioned above, where pro-angiogenic effects of hCMV secretomes were observed, the ECs were serum starved prior to assay, unlike in the current study, which could further explain discrepancies in the results. Another study showed that recombinant viral IL-10 (cmvIL-10) could actually have inhibitory effects on wound healing (Yamamoto-Tabata et al., 2004).

hCMV VR1814-inoculated ECs were positive for cmvIL-10 transcripts in our study, but the secretome did not inhibit wound healing. It is possible that the concentration of cmvIL-10 protein in our conditioned supernatants was significantly lower that the 100 ng ml⁻¹ of recombinant cmvIL-10 protein was used in the Yamamoto-Tabata et al. (2004) study. Furthermore, the plethora of other virally induced proteins in the conditioned supernatants makes a direct comparison with the influence of a single recombinant protein difficult. Thus, the apparent influence of hCMV encoded proteins and conditioned secretomes on EC angiogenesis and wound healing likely depends on a myriad of factors, including infected cell type, time points of supernatant collection, concentration of relevant factors and culture conditions.

As we did not observe an effect of secretomes from hCMV VR1814 inoculated ECs on angiogenesis, we concluded that the inhibition of tube formation we observed depended on cell-intrinsic changes, rather than soluble factors. We observed reduced expression of both VEGFR1 and VEGFR2 in hCMV VR1814 inoculated ECs. VEGFR1 has been shown to be essential for vasculature formation, with a role in cell–cell interactions and EC-basement membrane interactions (Cai et al., 2003; Fong et al., 1995), and EC migration (Orecchia et al., 2003). VEGFA binds with highest affinity to VEGFR1 (Holmes et al., 2007) but VEGFR2 is also likely to have a role. Mice deficient in VEGFR2 do not produce differentiated endothelial cells or organized blood vessels, whereas VEGFR1 deficient mice exhibit mature, differentiated ECs, but have disorganized vessels (Zachary & Gliki, 2001). Paradoxically, we observed an increase in VEGFA protein production by hCMV VR1814 infected ECs at 1 day p.i., but no effect of this secretome on tube formation by uninfected ECs in the angiogenesis assay. This could be due to the relatively modest level of VEGFA production, which was in the pg ml⁻¹ range, whilst ECs are treated with VEGFA at ng ml⁻¹ levels to induce angiogenesis in vitro (Mandriota & Pepper, 1997). We did not observe an increase in VEGFA transcription following EC hCMV TB40E infection and previous studies did not observe a hCMV VR1814 induced increase in VEGFA following infection of lymphatic ECs (Fiorentini et al., 2011). We observed a substantial reduction in the expression of ANGPT2 and transcripts encoding its receptor, TEK, in hCMV VR1814 infected cells, which likely contributes to the inhibition by limiting ANGPT2-TEK induced vascular remodeling necessary for angiogenesis (Felcht et al., 2012; Wong et al., 1997). We observed a decrease in only ANGPT2 expression in hCMV TB40E infected ECs. As we observed inhibition of angiogenesis in both hCMV VR1814 inoculated ECs at 1 day p.i. and hCMV TB40E inoculated ECs, in the absence of modulation of transcripts encoding VEGF-VEGFR signalling, ANGPT2-TEK mediated signalling may be more important in this setting. Although these observations do not provide a conclusive explanation of the mechanism by which hCMV inhibits angiogenesis and...
migration in our study, they are consistent with the functional changes we observe and provide, to our knowledge, the first evidence that direct hCMV infection inhibits expression of angiogenesis-associated transcripts. The relevance of the consequences of hCMV secretome mediated effects versus direct hCMV infection should be considered in terms of the specific clinical context. The direct modulation of EC behaviour by infection may be less relevant if a very small number of cells are infected (perhaps during reactivation in the immunocompetent host, for example), whereas a modified secretome could have important consequences on the behaviour of bystander cells. In the case of the extensive widespread hCMV infection, such as that observed in the congenital situation, direct EC infection (and the consequences on cellular behaviour) is more likely to play a significant role in the development of pathology.

Overall, this study demonstrates that placental ECs are viral targets during congenital infection and that vessels in infected tissue exhibit abnormal morphology. To our knowledge, this is the first study to describe the effect of both early and prolonged hCMV infection on the ability of primary human ECs to undergo angiogenesis and migration. It provides evidence suggesting that hCMV infection can inhibit angiogenesis and thus may impair wound recovery in infected tissue. In addition, in settings of congenital infection, the virus might lead to complications associated with intrauterine growth retardation due to impaired vascularity and placenta function.

METHODS

Immunohistochemistry

Placenta collection. Second and third trimester placenta of seven hCMV-congenitally infected fetuses with brain damage were obtained at the Paule de Viguier Hospital, Toulouse, as previously described (Benard et al., 2014). Briefly, the diagnosis of congenital hCMV infection was based on maternal hCMV serology and pathological ultrasound examinations of the fetal brain. Growth restriction defined fetuses whose weight was below the tenth percentile for its gestational age. Pregnancies were terminated by request of the parents and with acceptance of the prenatal diagnosis centre of Toulouse. Termination of pregnancy was performed with feticide and thereafter, vaginal delivery. Fetal and placental tissues were obtained after informed parental consent and according to the procedures approved by the Regulations of the French Ministry of Health. Placentae were fixed in 10% buffered formalin and embedded in paraffin. Fetal brain damage was confirmed by anatomopathological examination. Six second and third trimester placenta from pregnancies without congenital hCMV infection were included as controls, in accordance with French ethical guidelines. These control placentae were collected after preterm delivery (between 24 and 34 weeks of gestation), due to impaired vascularization and placenta function.

Histopathological examination. Paraffin embedded placental tissue sections (4 μm) were de-waxed using Xylene and rehydrated in an ethanol series. Immunohistochemistry staining was performed on the tissue sections as previously described (Qiu et al., 2008). Briefly, the sections were incubated with the following antibodies: mouse anti-hCMV IE antigen (MAB810 Chemicon) and anti-human von Willebrand factor (vWF, IgG1, Dako). Polyclonal rabbit antibodies (R&D systems) and secondary antibodies only served as controls. Visualization was achieved using horseradish peroxidase detection system (BioGenex) with the chromogen diaminobenzidine (Innexon Biosciences). Finally, all slides were counterstained with haematoxylin (Vector Laboratories) and mounted in permanent mounting medium (Dako).

Isolation and culture of human umbilical vein ECs. Primary ECs were isolated from umbilical cords using collagenase (Sigma-Aldrich) as previously described (Cooke et al., 1993) and cultured at 37 °C, 5 %CO2 until confluent. Cells were cultured in Medium 199 (Gibco, Invitrogen, Life Technologies) supplemented with 20% Fetal Bovine Serum (FBS), 2.5 μg ml-1 amphotericin B, 50U ml-1 penicillin, 50 μg ml-1 streptomycin, 1 mg ml-1 EGF, 28 μg ml-1 gentamicin and 1 μg ml-1 hydrocortisone (all Sigma). Primary cultures were dissociated with trypsin/EDTA (Sigma) and passaged into tissue culture multi-well plates that were pre-coated with gelatin (Sigma) (0.5% gelatin in PBS). Seeding density was based on 2.4 × 106 cells/6-well or 1 × 105 cells/12 well and yielded 80-90% confluent monolayers for infection overnight.

hCMV production

The hCMV clinical strains VR1814 (a kind gift from Dr Giuseppe Gerna, University of Pavia, Italy) and TB40E (a kind gift from Dr Giardia Frascaroli, Institute of Virology, Germany) were used in these studies. The virus was propagated in HUVECs and supernatants containing extracellular free virus were collected at maximum cytopathic effect. The cellular material was clarified and ultracentrifuged at 28 000 r.p.m. for 1 h at 4 °C (Beckman, Optima L-90K Ultracentrifugation). The viral pellet was resuspended in 0.1 M sucrose phosphate buffer to increase the viral titre as previously described (Frascaroli & Sinzger, 2014). The virus titre was determined by a 50% tissue culture infectivity dose (TCID50) assay as previously described (Reed & Muench, 1938).

Inoculation of ECs with hCMV and preparation of secretomes

Cultures were inoculated with mock or hCMV VR1814 or TB40E strains at a multiplicity of infection (MOI) of 3 based on seeding density. To inoculate, cells were incubated at 37 °C with the virus diluted in culture medium. Conditioned supernatants were generated from cultures in 12-well plates and cleared of free virus by filtration through 0.1 µm pore filters.

Immunofluorescence

For immunofluorescence staining assays ECs were seeded in culture slides (Ibidi, µ-slide VI 0.4) and infected with hCMV strains VR1814 or TB40E. At 1 or 7 days p.i. the slides were fixed in a 1:1 mixture of acetone and methanol at −20 °C for 10 min and washed three times in PBS before they were blocked using Dako Protein Block (Dako Cytomation), Fc receptor blocker (Innovec) and 2.5% normal horse serum (Vector laboratories). Thereafter, the slides were stained with a mouse anti-hCMV-IEA antibody (Argene, bioMérieux diagnostics) and visualized with an Alexa Flour 488 conjugated goat anti-mouse IgG antibody (Life Technologies).

MTT cell proliferation assay

Cell proliferation was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid assay (MTT, Roche) according to the manufacturer’s instructions. Approximately 104 viable cells were seeded in six replicate wells prior to inoculation with mock or hCMV VR1814 at MOI 3, and assayed at the indicated time points.

Angiogenesis assay

Assays were performed in angiogenesis slides (µ-slide angiogenesis, Ibidi) according to the manufacturer’s guidelines. Ten microtites of
growth factor reduced matrigel (BD) were dispensed into each well and set at 37 °C. Cells were seeded in duplicate or triplicate at 2 × 10^4 cells per well in 50 μl culture medium. Tube formation was monitored over time under phase-contrast using a ×4 objective lens. To test the role of secreted factors, primary, untreated HUVECs were cultured in conditioned culture supernatants for 18h before dissociation and plating into the assay suspended in more of the same culture supernatants. Cultures were imaged after 6h to quantify angiogenesis. Images were captured using a ×4 objective for quantification of number of branches and number of branch points.

Wound healing scratch assay
For wound healing scratch assays HUVECs were seeded in 12-well plates. When the monolayers had reached around 80% confluence they were inoculated with mock or hCMV VR1814 or TB40E strains, or with secretomes generated as described above. At 1 day p.i. EC monolayers were scratched using a pipette tip, the culture medium was replaced and images of a specific wound site were collected at t=0 and t=24 h.

Evaluation of cmv IL-10 mRNA expression using reverse transcriptase PCR
A total of 1.5 μg RNA was subjected to DNase treatment (RQ1 RNase-Free DNase, Promega) according to the manufacturer’s instructions then purified using RNeasy Mini kit (Qiagen). RNA concentration was measured with a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and converted to cDNA using the SuperScript III First-Strand Synthesis System with random hexamers (Invitrogen, Life Technologies) according to the manufacturer’s instructions. Approximately 20 ng of cDNA was then subject to conventional PCR to detect the presence of hCMV-IL-10 with the following primers: forward primer: 5'-TCGGTGATGGTCTCTT-CCTC-3'; reverse primer: 5'-ATGAAAGGTGACGCGGAGAT-3' with an expected amplicon size=159 bp (VR1814 with GenBank ID: GU179289.1). The primers target the known consensus of first exon of the hCMV-UL111A gene and were designed using online free Primer3 software (Koressaar & Remm, 2007; Untergasser et al., 2012). Beta-2 microglobulin (B2M) was used as a housekeeping gene, forward primer: 5'-TATCCAGCGTACTCCAAAGA-3'; reverse primer: 5'-GACAAGGTCAAGCTGCTC-3' with expected ampli- con size=165 bp (NM_004082). The optimum annealing temperatures for both primers were determined and PCR was conducted with Applied Biosystems Veriti Thermal Cycler as follows: initial denaturation 95 °C, 3 min; 40 cycles (cmv IL-10) or 35 cycles (B2M) at 95 °C, 30 s; 59 °C (cmv IL-10) or 61 °C (B2M), 40 s; 72 °C, 30 s; final extension at 72 °C, 7 min and hold at 4 °C. Bands were separated with 1.2% (w/v) agarose (UltraPure Agarose, Life Technologies) incorporated with 1X GelRed (Biotium) and visualized with Gel Doc XR Imaging System (Bio-Rad).

Evaluation of gene expression by quantitative real-time PCR
Total RNA was isolated using the RNeasy Mini Kit with QIAshredder (Qiagen) according to the manufacturer’s instructions. RNA concentration was measured with a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and converted to cDNA using the SuperScript III First-Strand Synthesis System with Oligo(dT)_18 or random hexamers (Invitrogen, Life Technologies) according to the manufacturer’s instructions. VEGFR1, VEGFR2, TEK, VEGFA and ANGPT2 were measured using the TaqMan Fast Universal PCR Master Mix with the relevant Applied Biosystems TaqMan MGB primers/probe mix. Reactions also included the primers/probe mix for 185 allowing measurement of the endogenous control in duplex (Applied Biosystems, Life Technologies). The PCR was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems) and data were analysed using SDS 2.4 software. Relative expression was calculated by the comparative CT method.

ELISA
Levels of secreted VEGFA or human interleukin-10 (hIL-10) protein in supernatants from mock or hCMV VR1814 inoculated ECs were measured by ELISA according to the instructions (R&D systems).

Statistics
Differences between treatments were evaluated by Students t-test using the software Prism (GraphPad). P-values are denoted as follows: */P<0.05, */*/P<0.01, */*/*/P<0.001.

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