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Placental pericytes and cytomegalovirus infectivity: implications for HCMV placental pathology and congenital disease

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Abstract

Problem—Placental pericytes are essential for placental microvascular function, stability, and integrity. Mechanisms of HCMV pathogenesis incorporating placental pericytes are unknown.

Method of Study—HCMV infected placental tissue was stained by dual-labeled immunohistochemistry. Primary placental pericytes, cytotrophoblasts, and villous fibroblasts were exposed to HCMV; infectivity was analyzed by microscopy and immunofluorescence. Cytokine expression was examined by Luminex assay. A HCMV-GFP recombinant virus was used to examine replication kinetics.

Results—Immunohistochemistry showed HCMV in trophoblast and the villous core with T-cell and macrophage infiltration. SBCMV-infected pericytes showed dysregulation of proinflammatory and angiogenic cytokines when compared to control cells. A tri-cell model of the villous floor showed a unique expression profile. Finally, we show pericytes infected *in vivo* with HCMV in placental tissue from a congenitally infected child.

Conclusions—Placental pericytes support HCMV replication, inducing proinflammatory and angiogenic cytokines that likely contribute to viral dissemination, placenta inflammation, and dysregulation of placental angiogenesis.

Graphical abstract

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Author contributions

DJA conceived of and designed the study and drafted the manuscript. DJA, HC LMR, DMA, and RAB performed the experiments. All authors have read and approved the final version of the manuscript.

Keywords

Angiogenesis; Cytokines; HCMV; Inflammation; virus

Introduction

Congenital human cytomegalovirus (HCMV) infections can result in abnormalities including vision loss, mental retardation, motor deficits, seizures, and hearing loss [1–5]. Forty percent of mothers with primary HCMV infection during gestation transmit the infection to their babies [6, 7]. Even more, 58% of transplacental transmission of HCMV occur in women who are seropositive with non-primary maternal infections [8]. Annually, 0.5 to 3.0% of all newborns in the US are infected with HCMV [9]. HCMV not only causes life-threatening disease in immunocompromised individuals [10, 11], but also HCMV-associated pathologies that lead to long-term health risks and represent an important health disparity in underserved communities. Higher infection rates are observed among non-Hispanic Blacks and Mexican Americans than among non-Hispanic Whites [12]. HCMV infection disrupts normal placental function and development, suggesting that congenital HCMV should be considered as an underlying cause of intrauterine growth restriction (IUGR) [13].

Placental pericytes are essential for endothelial cell proliferation and placental microvasculature stability and integrity, but have largely been ignored in placenta biology [14]. Pericytes are critical for placental vascular development and angiogenesis, being pluripotent cellular components of the capillaries and post-capillary venules abluminal to microvascular endothelial cells [15, 16]. The retina and brain have the highest density of vascular pericytes in the body [17]. Alcendor *et al.*, recently reported that primary human retinal vascular pericytes are fully permissive for HCMV infection and that retinal pericytes are more permissive for HCMV lytic replication compared to retinal microvascular endothelial and Müller cells [18]. They concluded that retinal pericytes likely serve as amplification reservoirs disseminating through the inner retinal barrier. Retinal pericyte

exposure to a clinical strain of HCMV resulted in dysregulation of proinflammatory angiogenic cytokines [18]. Similar findings were observed with brain pericytes [19].

HCMV placental pathogenesis models that include placental pericytes have not been reported. The signaling mechanisms between placental pericytes, cytotrophoblast, and villous fibroblasts are largely unknown [20]. This is the first report investigating the infectivity of human placental pericytes for HCMV, their potential role in viral dissemination in placental tissue, and the implications for HCMV-associated congenital disease.

Materials and Methods

Placental collection and trophoblasts isolation

HCMV-negative placentas were obtained from elective non-laboring caesarean sections after uncomplicated full-term pregnancies at Vanderbilt University Medical Center. These studies were approved by the Vanderbilt University Institutional Review Board. HCMV status was verified by immunochemistry (IHC) [21].

For cytotrophoblast isolation, placental nodes (cotyledon) were washed 3× with phosphate buffered saline (PBS), excised, and the decidual layer removed to expose villous tissue. Nodes were minced with a number 21 scalpel and individual 5 mm explants were spatially added to 100×20 mm dishes and cultured in trophoblast medium (ScienCell 10% fetal bovine serum, 1% penicillin/streptomycin Carlsbad, CA). Trophoblast medium was supplemented with 25 ug/ml of Fungizone (Gibco, Life Technologies, Grand Island, NY). Explant trophoblast outgrowth was determined by microscopy. Cytotrophoblast colonies were isolated by trypsinization (0.05% trypsin/EDTA (Gibco, Life Technologies, Grand Island, NY) for 10 min at 37°C) using cloning cylinders. Trypsinized cytotrophoblast were added to 4-well chamber slides containing trophoblast medium. Confluent cells were confirmed as trophoblasts by cytokeratin-7 staining (Millipore, Bedford, MA).

Placental tissue

IRB-approved placental tissues were obtained via collaboration with Dr. Ravit Boger at The Johns Hopkins University Medical Center. Disseminated cytomegalovirus infection in placental tissue was confirmed by a pathologist and subsequently reconfirmed by IHC staining for the HCMV major immediate early (MIE) proteins [22]. Tissue was formalin fixed and paraffin embedded, and 5 micron sections were placed on Chemate slides for dual-labeled IHC staining [21].

Cells and viruses

The primary isolate (termed SBCMV) was provided by Dr. Ravit Arav-Boger, Johns Hopkins University, and obtained from the urine of a congenitally infected infant with disseminated HCMV disease. Institutional review board (IRB) exemption for the use of this isolate was given by Johns Hopkins Hospital [19]. The HCMV-GFP recombinant virus was obtained from Dr. Gary Hayward, Johns Hopkins University. All infections with SBCMV clinical strain were performed at passage level 3. We are aware that high level passage in vitro can result in the acquisition of mutations that can impact viral tropism and replication

therefore our effort is to restrict passage level between 3 and 4 post initial cultivation by employing new freezing in subsequent experiments to avoid high passage level in culture. Primary human placental pericytes were obtained from PromoCell Corporation (Heidelberg, Germany) and cultivated in pericyte medium (PM) from ScienCell. Placental pericytes were maintained at passage level 3 in PM. Human villous fibroblasts were obtained from ScienCell and cultivated in fibroblast medium from ScienCell. All cells were trypsinized and plated in uncoated 100 cm² dishes or uncoated 4.2 cm²/well glass chamber slides at density 1×10⁶ and 2.5×10⁵ cells per dish and well, respectively. Heat-killed SBCMV was prepared by heating the inoculum to 65°C for 30 min in a water bath [29]. The mild heat inactivation that we employ is unlikely to cause a global effect on thermolabile viral proteins.

Immunohistochemistry

Archived placental tissue was dual-label IHC stained for HCMV-MIE, cytokeratin-7, CD3 (T-cell marker), and CD68 (macrophage marker) as previously described [21]. The placenta tri-cell culture was dual-labeled for antigenic biomarkers cytokeratin-7 (clone OV-TL 12/30, Millipore, Bedford MA), and CD31 (Millipore, Bedford MA) as previously described [18, 19]. Substrates for IHC were diaminobenzidine (blue color) and alkaline phosphatase (red color). Placental fibroblasts were unstained.

Cytomegalovirus infection of pericytes, cytotrophoblasts, and villous fibroblasts

Cytomegalovirus infection and RNA isolation have been previously described [19]. The SBCMV clinical isolate, Toledo lab-adapted strain of HCMV, and HCMV-GFP recombinant virus were cultivated in human foreskin fibroblasts. Placenta pericytes, cytotrophoblasts and villous fibroblasts were infected at a multiplicity of infection (MOI) of 0.1. Virus adsorption was allowed for 2 h and the infectious inoculum was replaced with fresh medium. Mock-infected cells included medium only with no virus.

Immunofluorescence

Chamber slide cultures containing SBCMV-infected or mock-infected pericytes, cytotrophoblasts, or villous fibroblasts were washed twice with PBS pH 7.4, air dried, and fixed in absolute methanol for 10 min. Cells were air dried for 15 min, hydrated in Tris buffered saline (pH 7.4) for 5 min, and incubated separately for 1 h with monoclonal antibodies to SMA, cytokeratin-7, and vimentin (Santa Cruz Biotech), all diluted 1:50 in PBS pH 7.4. For HCMV infection of placental pericytes, cells were incubated for 1 h with monoclonal antibodies to HCMV-MIE (MIE, MAB810, Millipore), the HCMV viral tegument protein pp65, (UL83, Vector Laboratories, Burlingame, CA) and the late virion tegument protein pp28 (Santa Cruz Biotech) both at 1:50 dilution. Immunofluorescence was performed as previously described [19].

Tri-cell placenta model

The placental tri-cell culture model, composed of primary pericytes, cytotrophoblasts, and villous fibroblasts, was established in chamber slides at a ratio of 1:2:2, respectively. The rationale for the 1:2:2 ratio supports the finding that pericyte density (with the exception of

the brain and retina) are found to be lower in other vascular beds. However, the pericyte cell density associated with the placental vasculature is unknown.

Initial cell cultivations were performed with medium recommended by the manufacturer. Pericytes were initially cultivated in complete PM and allowed to become confluent at cell density 2.5×10^5 . Cytotrophoblasts, initially cultivated at the same cell density in trophoblast medium (ScienCell), were added and the mixture cultivated in PM. After 48 h, villous fibroblasts cultivated in fibroblast medium at the same cell density were added to complete the tri-cell mixture with all three cell types growing in PM. The tri-cell mixture was then infected for 24 h with SBCMV at a MOI of 0.1. The tri-cell mixture was stained for live/dead cell viability using an assay kit (Life Technologies, Grand Island, NY).

Luminex analysis

The inflammatory and angiogenic cytokine analysis was performed with 200 μ l of supernatant from 3 pooled cultures of mock-infected, SBCMV-infected, and SBCMV heat-killed placental pericytes 24 h post-exposure using a Luminex instrument (Luminex Corporation, Austin, TX) and 100-plate viewer software. Luminex analysis was performed on supernatants as previously described [24]. Infections were performed in triplicate in chamber slides for 24, 48, 72 and 96 h post-infection. Replicate assays are inherent in the Luminex technology by counting 50 bead replicates per analyte and reporting the median. This is the equivalent of running 50 replicate assays per well. In addition, robotic pipetting was performed for all volume-critical steps, which minimizes well-to-well variability, and calibrators and controls were run in duplicate involving 3 levels of control per analyte in duplicate on every plate.

Virus replication

For virus replication, kinetics are the averages of triplicate samples performed in multi-well chamber slides as previously described [18].

Results

HCMV replication is enhanced in the villous core of placenta in vivo

Using dual-labeled IHC, for cytokeratin-7 to stain trophoblasts brown and HCMV-MIE to stain HCMV-infected cells red, we examined archival placental tissue from a neonate with disseminated HCMV disease and observed focal infection initially in syncytiotrophoblasts (Fig. 1A). On the same slide we observed virus in the underlying cytotrophoblast layer (Fig. 1B), and increased viral replication in the villous core region (Fig. 1C), which agrees with normal HCMV dissemination in the villous core. Infected cells are identified using black arrows.

HCMV induces T-cell/macrophage infiltrate at the site of infection in vivo

Using dual-label IHC staining for the T-cell biomarker CD3 (brown/white arrows) and the HCMV-MIE for HCMV-infected cells (red/black arrows) in archival tissue from the placenta depicted in Fig. 1, we observed T-cell infiltration localized at the site of infection, indicated by HCMV-infected cells staining red (designated by black arrows in Fig. 2A and B). We

stained for the macrophage antigenic biomarker CD68 and observed infiltrate at the site of infection (designated by white arrows in Fig. 2C and D).

Placental pericytes, cytotrophoblasts, and villous fibroblasts are fully permissive for HCMV infection

Normal placental pericytes, cytotrophoblasts, and villous fibroblasts had morphological characteristics similar to their respective cell types, as previously observed (Fig. 3). Placental pericytes, cytotrophoblasts, and villous fibroblasts stained positive for known antigenic biomarkers SMA, cytokeratin-7, and vimentin, respectively (Fig. 3A–3C). All three cell types showed characteristic HCMV cytopathology after 5 days post-infection with the Toledo lab-adapted strain of HCMV, and 7 days post-infection with SBCMV. (Fig. 3A–3C). All cell types expressed both the major immediate early and the pp65 late protein by immunofluorescent staining [25]. Pericytes also expressed the pp28 late antigen (insert Fig. 3A–7). All cell types supported lytic replication of a HCMV-GFP recombinant virus, as demonstrated by phase/fluorescent imaging (Fig. 3A–3C).

Proinflammatory and angiogenic cytokines are induced in SBCMV-infected placental pericytes

Dysregulation of proinflammatory and angiogenic cytokines plays a major role in HCMV placental pathology [26, 27]. We examined 24 h supernatants from SBCMV-infected primary human placental pericytes along with mock-infected cells and cells exposed to heat-killed virus for changes in secretion profiles using Luminex assays (Fig. 4). We observed increased MCP-1 secretion by pericytes exposed to SBCMV, and a marginal increase in MCP-1 from cells exposed to heat-killed virus, relative to mock-infected cells (Fig. 4A). The highest level of MCP-1 secretion was observed from pericytes exposed to replication competent virus (Fig. 4A). High levels of VEGF were observed in supernatant from pericytes exposed to SBCMV and heat-killed virus compared to mock-infected cells (Fig. 4B), and equal levels of VEGF were observed from SBCMV-infected and heat-killed virus exposed pericytes. There was a marginal increase in IL-8 levels from SBCMV-infected pericytes compared to mock-infected cells, and a reduction of IL-8 from heat-killed virus exposed pericytes (Fig. 4C). There was an increase in RANTES/CCL5 from SBCMV-infected pericytes compared to heat-killed virus exposed pericytes and mock-infected cells (Fig. 4D). RANTES levels were equal for heat-killed virus exposed pericytes and mock-infected cells. We observed reduced levels of the proinflammatory cytokine IL-6 from both SBCMV-infected and heat-killed exposed pericytes compared to uninfected controls (Fig. 4E). The greatest reduction occurred in cultures exposed to heat-killed virus. We observed no significant change in MMP9 and TIMP-1 (Fig. 4F and G) levels.

Placental pericytes are more permissive for HCMV infection compared to cytotrophoblasts and villous fibroblasts

Individual cellular components of the placenta tri-cell mixture were compared to determine their infectivity when exposed to HCMV-GFP. Individual cell types were infected with a MOI of 0.1 to model *in vivo* conditions; mock-infected cells served as controls. Infections were performed in triplicate in chamber slides for 24, 48, 72 and 96 h post-infection (Fig. 5A). The total GFP positive cells were counted by fluorescence microscopy and the average

value was used in the final analysis. After 24 h, pericyte cultures had a 4-fold increase in GFP positive cells relative to cytotrophoblasts and villous fibroblasts. After 48 h, pericyte cultures had a greater than 4-fold increase in GFP positive cells relative to cytotrophoblasts, and a greater than 2-fold increase relative to villous fibroblasts. This trend continued with pericytes being more permissive for HCMV infection at time points 72 and 96 h. When compared to cytotrophoblasts, pericytes had a 3-fold and 2.5-fold increase in GFP positive cells at 72 and 96 h post-infection, respectively. Villous fibroblasts were consistently more permissive for HCMV infection than cytotrophoblasts. Compared to villous fibroblasts, pericytes had a 1.6-fold and 1.7-fold increase in GFP positive cells at 72 h and 96 h post-infection, respectively. Infected cultures of villous fibroblasts, trophoblasts, and placental pericytes were examined for HCMV infectivity at 96 h post-infection, showing the highest number of GFP positive cells in pericytes compared to villous fibroblasts and trophoblasts (Fig. 5B). We observed the greatest degree of cell death and presence of multinucleated giant cells (insert) in pericytes compared to villous fibroblasts and trophoblasts (Fig 5B). We established the placental tri-cell model of primary placental fibroblasts, cytotrophoblasts and placental pericytes. All cell types were cultivated in PM media from Sciencell over a period of 7 days as shown by phase contrast imaging (Fig.5C–A). The tri-cell model was maintained for 7 days and showed greater >95% viability (Fig. 5C–B) via live/dead staining, and stained positive for the pericytes marker CD31 (stained red) and cytotrophoblasts with cytokeratin-7 (stained brown). Placental fibroblasts were unstained (Fig.5C–C).

Dysregulation of proinflammatory and angiogenic cytokines in a tri-cell model of the SBCMV-infected placenta

Supernatant from the placental tri-cell culture cultivated in PM only was exposed to SBCMV, heat-killed SBCMV, and medium only (mock-infected) and examined by Luminex assay at 24 h post-infection (Fig. 6). We observed higher levels of MCP-1, RANTES, IL-6, and TIMP-1, no change in VEGF or IL-8 levels, and lower levels of MMP9 in SBCMV-infected tri-cell cultures compared to mock-infected controls. Tri-cell cultures exposed to heat-killed virus had increased levels of MMP9 and TIMP-1, no change in VEGF or IL-8, and lower levels of MCP-1 IL-8, RANTES, and MMP9 relative to mock-infected control cultures. Tri-cell cultures exposed to heat-killed virus showed higher levels of MMP9 compared to those exposed to SBCMV.

We examined placental tissue dual labeled IHC from a child with congenital HCMV infection in utero and observed disseminated HCMV in the placenta villous (Fig 7A). We demonstrate dual labeled pericytes stained dark purple and positive for HCMV MIE antigen stained brown (Fig. 7B).

Conclusion

To our knowledge, this is the first report that demonstrates placental pericyte permissiveness for HCMV infection and identifies them as the most permissive cell type in the villous stroma relative to trophoblasts [28, 29] and villous fibroblasts. The results from these studies support our previous work showing that pericytes in vascular beds of the blood brain barrier and the inner blood retinal barrier (IBRB) are the most permissive cellular targets for

HCMV primary infection [19, 18]. Expression profiles of primary cells can change in culture; therefore, it is essential that tri-cell culture infection models be established using primary cells at low passage, and infections with clinical strains at low MOI, to better approximate clinical conditions.

We observe HCMV induction of T-cell/macrophage infiltrates at the site of infection in vivo. Pereira et al., 2005 finds that macrophages within the villous stroma contain cytoplasmic vesicles with viral structural proteins which argues that macrophages can restrict virus replication [30]. A report by Pereira et al., show HCMV virion uptake by villus core macrophages and dendritic cells within 60 minutes after infection in vitro (unpublished observation). This could occur in utero leading to passage of HCMV virions and/or IgG-virion complexes across tight junctions of cytotrophoblast progenitor cells. Subsequent interactions with IgG virion complexes binding to the Fc-gamma I receptor on macrophages would result in the removal of opsonized virions, suppression of proinflammatory responses and reduced viral dissemination in the villous [31]. The mechanism involved in suppressed HCMV infection by villous macrophages could provide insights in to new therapeutic interventions to prevent HCMV associated disease.

In addition, there are contrasting findings from a number studies regarding HCMV infection and replication of syncytiotrophoblasts [32]. Some studies suggest that syncytiotrophoblast do not support HCMV infection and replication. Studies using first trimester placental tissue explants, maintained either as primary histocultures or after culture on matrigel, syncytiotrophoblasts were found to be only rarely infected by HCMV [33, 34]. A number of studies support HCMV infection and replication in syncytiotrophoblasts [32, 35, 36]. A study by Schleiss et al., showed that syncytiotrophoblast cells could support productive HCMV infection in vitro by dual expression of IE72 immediate early protein, and the late HCMV structural protein, pp65 (ppUL83) [32]. These differences in syncytiotrophoblasts permissiveness for HCMV infection and replication could be the result of culturing procedures, differences in the gestational age of the placental tissue as well as differences in the expression profile of HCMV receptors between syncytiotrophoblasts and cytotrophoblasts [37, 38].

Our examination of the secretion profile of placental pericytes alone at 24 h post-exposure to SBCMV revealed increased expression of MCP-1. Hamilton *et al.*, using *ex vivo* placental histocultures infected with laboratory and clinical HCMV, also showed increased MCP-1 [39]. They suggested that HCMV infection alters the placental microenvironment and proposed that MCP-1 initiates placental and fetal injury [39]. We observed increased levels of VEGF in supernatants of placental pericytes exposed to SBCMV (Fig. 4). Studies have found increased levels of soluble VEGF/VEGF receptor-1 in supernatants taken from preeclamptic placental explants, implicating placental VEGF/VEGF receptor-1 in the inhibition of angiogenesis in preeclampsia [40]. We observed a marginal increase in the levels of RANTES exclusively in SBCMV exposed pericytes. Recent studies also showed increased RANTES/CCL5 expression in brain vascular pericytes exposed to HCMV [18]. We also showed that IL-6 levels for both SBCMV-infected pericytes and heat-killed virus were lower compared to mock-infected pericytes. Other investigators found that IL-6 levels

were suppressed in human fibroblasts undergoing active infection mediated in part by HCMV IE2 protein and post-transcriptional destabilization of IL-6 mRNA [41].

The secretion profile of the placental tri-cell mixture at 24 h post-exposure to SBCMV revealed increased expression of MCP-1 and no significant change in VEGF or IL-8. However, we saw increased levels of RANTES, IL-6, and TIMP-1 (Fig. 6). Finally, lower levels of MMP9 were observed with the lowest occurring in SBCMV-infected cells (Fig. 6). This finding correlates with increased levels of TIMP-1 observed in the SBCMV-exposed tri-cell mixture. HCMV infection of placental pericytes is a lytic infection that would result in pericyte loss at proximal sites within the placental vasculature. This would have implications for placental vascular permeability, and would support an increase in placental inflammation and angiogenesis as well as impaired tissue perfusion and microcirculatory abnormalities. In previous studies in which retinal pericytes alone were exposed to SBCMV after 24 hours, we observed lower levels of MMP9, IL-6, and TIMP-1 when compared to placental pericytes exposed alone to SBCMV [18]. However, in both retinal and placental pericytes exposed to SBCMV at 24h we observed higher levels of RANTES when compared to both mock-infected and heat-killed virus controls, as well as higher levels of IL-8 in SBCMV and heat-killed virus exposed pericytes compared to mock-infected controls [18]. The eye and placenta represent vastly different microenvironments trafficked by HCMV that will likely influence cytokine/chemokine expression profiles during infection.

We acknowledge the limitations of the 24-hour time interval; however, cytokine profiles can be unstable over time, and our intention was to examine early events triggered by HCMV during the course of infection. We are also aware that the marginal changes observed in the level of proinflammatory and angiogenic cytokines post HCMV exposure may not be biologically relevant and may require a more strategic timecourse with both medium and low multiplicities of infection.

These studies are a foundation for strategies to protect pericytes and their vascular beds from HCMV infection. In addition to obtaining data on HCMV congenital disease, an infection model of the placenta maintained under microfluidic conditions could open up the field, as the system could be easily adapted to study other abnormalities, including IUGR, preeclampsia, and diabetic placenta, and the effects of potential toxins [42]. This model could determine molecular signatures expressed pre/post-exposure, reveal new targets for diagnostics, and create strategies for gauging success of therapeutic interventions. This would offer predictive value and risk assessment for patients, with the potential to improve clinical outcomes.

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Abbreviations

αSMA	alpha smooth muscle actin
CCD	charge-coupled device camera
CCL-5	also known as RANTES
CD3	cluster designation 3 T-cell marker
CD31	cluster of differentiation 31
CD68	cluster designation 68 macrophage marker
CMV	cytomegalovirus
cytokeratin-7	trophoblasts marker
DAB	3,3-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HCMV	human cytomegalovirus
IHC	immunohistochemistry
IL	interleukin
IL-6	interleukin-6
IL-8	interleukin-8
IUGR	intrauterine growth restriction
MAB810	monoclonal antibody to human cytomegalovirus major immediate early proteins 1 and 2
MMP9	matrix metalloproteinase-9
MCP-1	monocyte chemotactic protein-1
MIE 1 and 2	human cytomegalovirus major immediate early gene/ proteins 1 and 2
MOI	multiplicity of infection
NG2	neuron-glial antigen 2
pp28	human cytomegalovirus phosphorylated nuclear protein expressed at late times during virus replication

pp65	human cytomegalovirus phosphorylated envelop protein expressed at late times during virus replication
RANTES	regulated upon activation normal T cell expressed and presumably secreted
SBCMV	primary HCMV isolate from a patient
TIMP-1	tissue inhibitor of metalloproteinase-1
UL83	HCMV phosphoprotein pp65
US28	HCMV-encoded chemokine receptor
VEGF	vascular endothelial cell growth factor

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HCMV Entry via Infection of Cytotrophoblasts and Dissemination in the Villous Core by Dual Labeled IHC **Fig. 1.**

Paraffin embedded placental tissue from neonates infected with CMV dual stained by IHC for cytotrophoblasts with cytokeratin-7 (brown) antibody and CMV with antibodies to the Major Immediate Early genes (MIE), (red). Black arrows show HCMV-infected cells staining red. Scale bar = 100 μ m.

CMV Infection in the Villous Core and T-cell Infiltration by Dual Labeled IHC

CMV Infection in the Villous Core and Macrophage Infiltration by Dual Labeled IHC

Fig. 2. Proinflammatory infiltrate at sites of HCMV infection in placental tissue. Paraffin embedded placental tissue from a neonate infected with CMV. (A and B). Cells dual stained by IHC for T-cells with CD3 antibody (brown/white arrows) and CMV with antibodies to the Major Immediate Early genes (MIE), (red/black arrows) (C and D) cells dual stained by IHC for macrophages with CD68 antibody (brown/white arrows) and CMV with antibodies to the Major Immediate Early genes (MIE), (red/black arrows). Scale bar = 100 μ m.

Fig. 3.

A Placenta pericytes from Promocell cultivated in PM medium from ScienCell were allowed to develop as monolayers in chamber slides. Cells were infected with different HCMV strains: 3A-1) uninfected subconfluent growth; 3A-2) Uninfected confluent growth; 3A-3) IFA stained with alpha actin smooth muscle antibody; 3A-4) Toledo CMV infected pericytes (lab strain); 3A-5) SBCMV infected pericytes (clinical strain); 3A-6) IFA of infected pericytes expressing CMV major immediate early gene proteins; 3A-7) IFA of CMV infected pericytes expressing the pp65 and pp28 (insert) late gene proteins; and 3A-8)

HCMV-GFP recombinant virus with a GFP cassette in the US28 coding region. **3B** Primary placenta cytotrophoblasts isolated from term placentas cultivated in trophoblast medium from ScienCell and allowed to develop as monolayers in chamber slides. Cells were infected with different HCMV strains: 3B-1) subconfluent growth; 3B-2) confluent growth; 3B-3) IFA stained with cytokeratin-7; 3B-4) Toledo CMV infected cytotrophoblasts (lab strain); 3B-5) SBCMV infected cytotrophoblasts (clinical strain); 3B-6) IFA infected cytotrophoblasts expressing CMV major immediate early gene proteins; 3B-7) IFA of CMV infected cytotrophoblasts expressing the pp65 late gene proteins; and (3B-8) HCMV-GFP recombinant virus with a GFP cassette in the US28 coding region. **3C** Primary human villous fibroblasts were cultivated in fibroblast medium from ScienCell. Cells were placed in chamber slides and infected with different HCMV strains. Mock inf = no virus; Toledo = Toledo strain of HCMV; SBCMV = clinical isolate of HCMV; US28-GFP Towne strain of HCMV with a GFP cassette in the US28 coding region; and HCMV MIE (major immediate early genes) and pp65 (late gene).

Fig. 4.

Luminex analysis of HCMV induction of soluble proinflammatory and angiogenic cytokines in placenta pericytes. Cytokine profiles of SBCMV-infected placental pericytes by Luminex analysis 24 h post-infection. Results from cells exposed to medium only are shown as solid black bars, cells exposed to heat-killed SBCMV are shown as shaded bars, and results from cells exposed to the SBCMV clinical isolate are shown as striped bars. Results are included for (A) MCP-1/CCL2, (B) VEGF, (C) IL-8, (D) RANTES/CCL5, (E) IL-6, (F) MMP9 and (G) TIMP-1. Results are given in picograms (pg) or nanograms per ml. Results were

determined by analyzing a minimum of 20 beads collected per assay and the median result is shown.

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Fig. 5.

A. Time course analysis of HCMV-GFP infection of placenta villous cells. A graph indicating the number of infected HCMV-GFP positive villous fibroblasts (open bars), trophoblasts (gray bars), and placental pericytes (black bars) per 1.25×10^5 total cells over the time course 12, 24, 48 and 96 h post-infection. **5B.** HCMV infected cultures of villous fibroblasts, trophoblasts, and placental pericytes at 96 h post-infection showing GFP positive cells in pericytes, villous fibroblasts, and trophoblasts **5C.** **(A)** Phase contrast image of a tri-cell culture mixture of villous fibroblasts, trophoblasts, and pericytes representing the

placental villous core. **(B)** A live/dead stain of the villous tri-cell culture. **(C)** Dual labeled IHC of the tri-cell villous culture. Villous trophoblasts stained with cytokeratin-7 antibody (brown), placental pericytes stained with CD31 antibody (red), and villous fibroblasts are unstained, color chart shown. All images have a total magnification of 200 $\times$.

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Luminex analysis of tri-cell model infected with SBCMV

Fig. 6. SBCMV induction of proinflammatory and angiogenic cytokines in the placental villous tri-cell culture. Cytokine profiles of the SBCMV infected placental villous tri-cell mixture by Luminex analysis at 24 h post-infection. Results from cells exposed to medium only are shown as solid black bars, cells exposed to heat-killed SBCMV are shown as gray bars, and results from cells exposed to the SBCMV clinical isolate are shown as stippled black bars. Results are included for: (A) MCP1; (B) VEGF; (C) IL-8; (D) RANTES; (E) IL-6; (F) MMP9; and (G) TIMP-1. Results are given in picograms (pg) per ml.

IHC Staining of Disseminated HCMV in Placental Tissue

Fig. 7. Immunohistochemical stain of placental tissue from a child with disseminated cytomegalovirus infection of the placenta. **(A)** cytomegalovirus infected tissue stained for the major immediate early protein (MIE) using 3,3-diaminobenzidine (DAB) colored brown. **(B)** Dual staining for NG2 proteoglycan for pericytes shown as dark purple and cytomegalovirus MIE stained with DAB colored brown.