Antibody Treatment Promotes Compensation for Human Cytomegalovirus-Induced Pathogenesis and a Hypoxia-Like Condition in Placentas with Congenital Infection

Ekaterina Maidji,* Giovanni Nigro,† Takako Tabata,* Susan McDonagh,* Naoki Nozawa,* Stephen Shiboski,‡ Stefania Muci,† Maurizio M. Anceschi,§ Natali Aziz,¶ Stuart P. Adler,∥ and Lenore Pereira*

From the Department of Cell and Tissue Biology,* School of Dentistry, and the Department of Epidemiology and Biostatistics,‡ School of Medicine, University of California–San Francisco, San Francisco, California; the Department of Pediatrics,† University of L’Aquila, L’Aquila, Italy; the Department of Obstetrics, Gynecology and Perinatology,§ La Sapienza University of Rome, Rome, Italy; the Department of Obstetrics and Gynecology,¶ School of Medicine, Stanford University, Stanford, California; and the Department of Pediatrics,∥ Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia

Human cytomegalovirus (HCMV) is the major viral cause of birth defects worldwide. Affected infants can have temporary symptoms that resolve soon after birth, such as growth restriction, and permanent disabilities, including neurological impairment. Passive immunization of pregnant women with primary HCMV infection is a promising treatment to prevent congenital disease. To understand the effects of sustained viral replication on the placenta and passive transfer of protective antibodies, we performed immunohistological analysis of placental specimens from women with untreated congenital infection, HCMV-specific hyperimmune globulin treatment, and uninfected controls. In untreated infection, viral replication proteins were found in trophoblasts and endothelial cells of chorionic villi and uterine arteries. Associated damage included extensive fibrinoid deposition, fibrosis, avascular villi, and edema, which could impair placental functions. Vascular endothelial growth factor and its receptor fms-like tyrosine kinase 1 (Flt1) were up-regulated, and amniotic fluid contained elevated levels of soluble Flt1 (sFlt1), an antiangiogenic protein, relative to placental growth factor. With hyperimmune globulin treatment, placentas appeared uninfected, vascular endothelial growth factor and Flt1 expression was reduced, and sFlt1 levels in amniotic fluid were lower. An increase in the number of chorionic villi and blood vessels over that in controls suggested compensatory development for a hypoxia-like condition. Taken together the results indicate that antibody treatment can suppress HCMV replication and prevent placental dysfunction, thus improving fetal outcome. (Am J Pathol 2010, 177:1298–1310; DOI: 10.2353/ajpath.2010.091210)
immunity to HCMV before conception rarely deliver infants with symptomatic infection. Symptomatic infants (25% of those born with congenital infection) can have both temporary symptoms and permanent birth defects. Symptoms such as intrauterine growth restriction (IUGR) and hepatosplenomegaly, which originate in insufficient placental functions, improve after birth. In contrast, viral replication in the fetal brain may cause neurological damage and permanent disabilities.

A critical hurdle in pregnancy maintenance is the embryo’s acquisition of a supply of maternal blood. Cytotrophoblasts, which are specialized epithelial cells of the placenta, perform the mechanics of this process. These cells follow two tightly regulated pathways that give rise to the differentiated cytotrophoblasts in anchoring and floating villi. In floating villi, which are attached at only one end to the tree-like fetal portion of the placenta, villous cytotrophoblasts differentiate by fusing with the multinucleated syncytiotrophoblasts that are in direct contact with maternal blood and participate in nutrient, waste, and gas exchange. In anchoring villi, which are attached at one end to the fetal portion of the placenta and at the other end to the uterus, a subpopulation of cytotrophoblasts aggregate into cell columns and invade the maternal decidua. Cell column cytotrophoblasts include undifferentiated proliferating cells and differentiated invasive cells. A subset of differentiated cells invades the first third of the myometrium (interstitial invasion) and breaches portions of the maternal arterioles that span these regions (endovascular invasion). By mid-gestation, endovascular cytotrophoblasts, which have completed an epithelial-to-endothelial transition, replace the endothelial cell lining and result in a hybrid vasculature composed of fetal and maternal cells. Cytotrophoblast proliferation and differentiation are strictly regulated by oxygen tension. The cells also express substances that influence vasculogenesis and angiogenesis, including vascular endothelial growth factor family ligands vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and receptors VEGF receptor 1 (Flt1) and VEGF receptor 2. VEGF is expressed downstream in the transcriptional network that regulates cellular responses to oxygen tension, and its expression changes as cytotrophoblasts differentiate. By mid-gestation, when the uterine-placental vasculature has been established, normoxic conditions prevail in healthy pregnancies and VEGF expression declines.

Studies of early gestation placentas showed that determinants of placental infection include the avidity of HCMV-specific antibodies in the circulation and virion receptors expressed on cytotrophoblasts. In the pregnant uterus, initial infection in the uterine vasculature spreads to invasive cytotrophoblasts that remodel the arterioles. In the adjacent placenta, syncytiotrophoblasts internalize IgG-virion complexes that are transcytosed by the neonatal Fc receptor. Syncytiotrophoblasts of placentas from women strongly seropositive for HCMV (ie, with high-avidity IgG) contain virion structural proteins and DNA in the presence of low-avidity IgG, HCMV replicates in underlying cytotrophoblasts, causing focal infection that spreads to the villous stroma and fetal capillaries. Productive infection is regulated by proteins that serve as virion receptors, epidermal growth factor receptor and integrin αv, expressed on villous cytotrophoblast progenitors, and integrins α1β1 and αvβ3, induced on invasive cells. Infected cytotrophoblasts impair differentiation, down-regulate key integrins and adhesion molecules, and produce cmvIL-10, a viral cytokine that reduces matrix metalloproteinase 9 activity and degradation of the extracellular matrix. In blood vessels of chorionic villi, infected endothelial cells up-regulate integrin αvβ6, which activates transforming growth factor-β and increases extracellular matrix deposition. Based on the profound dysregulation observed in vitro, HCMV replication at the uterine-placental interface could impair vascular remodeling and cause fibrosis, occluding blood flow and further reducing exchange between the maternal and fetal circulation.

It was reported that hyperimmune globulin (HIG) containing HCMV-specific, high-avidity antibodies infused into the maternal bloodstream after primary infection significantly reduced virus transmission and congenital disease. IgG avidity in circulation increased after infusion and was higher among treated mothers than among untreated mothers at delivery. Ultrasound analysis showed that untreated placentas were thicker than those of women receiving HIG treatment. To understand the underlying basis for the differences, we performed semi-quantitative immunohistological analysis of placentas and measured hypoxia-related factors from women with primary HCMV infection during gestation who did or did not receive HIG treatment to prevent congenital disease.

Materials and Methods

Study Group

Placental biopsy specimens and amniotic fluid were described previously. Approval to obtain placentas from Moffitt Hospital was acquired from the Institutional Review Board of the University of California–San Francisco. Primary infection was identified by seroconversion in previously HCMV-seronegative women or by detection of HCMV IgM and very low levels of IgG with low avidity (<25%). Congenital infection was confirmed by isolating the virus or detecting viral DNA in fetal urine or saliva by real-time PCR (Amplimedical-Bioline, Turin, Italy). Symptomatic disease included neurological involvement with microcephaly, periventricular calcifications, cerebral dysplasias, chorioretinitis, and auditory impairment. Head and abdominal circumferences less than the 10th percentile for fetuses of a similar age were evidence of IUGR. HCMV disease at ≥2 years manifested as mental and/or motor retardation (IQ <70) and auditory or visual impairment. HCMV-specific IgG, IgM, and IgG avidity was evaluated by enzyme immunoassays (ELISAs) from Radim (Pomezia, Italy) and Diasorin (Saluggia, Italy). The HIG treatment group consisted of women who received monthly treatment with HIG within 6 weeks of seroconversion, from diagnosis of infection until delivery. The
control group consisted of HCMV-seronegative pregnant women.

**Placental Biopsy Specimens**

Biopsy specimens from 26 placentas from women with untreated congenital CMV infection, women receiving HIG prevention, and healthy controls were selected for analysis based on following criteria: i) primary infection occurred in first trimester of pregnancy, ii) consent to receive HIG was obtained, iii) treatment was begun soon after seroconversion, and iv) women were nonsmokers during pregnancy. These criteria were met in 18 women with untreated infection, 3 women receiving HIG treatment, and 5 controls. The numbers of placentas used in our analyses represented the total number available for study. Biopsy specimens (two to five each) were taken from different sites in the placenta center, fixed in 10% formalin, and embedded in paraffin. Serial 5-μm-thick tissue sections of the paraffin-embedded biopsy specimens were used in all experiments.

**Microscopic Evaluation of Pathology**

Tissue sections were deparaffinized in xylene and rehydrated in graded alcohols and stained with H&E. Tissue morphology was evaluated without group identifiers and reviewed independently by two investigators blinded to the clinical outcome (E.M. and L.P.) with assistance from a clinical pathologist. Biopsy specimens were examined for histological abnormalities associated with congenital HCMV infection, hypoxia-associated injury, and compensatory development (described in Results). Immunostaining was performed for HCMV proteins and specific hypoxia-induced cellular proteins. Each manifestation of pathology was rated from 1 to 3 based on frequency of appearance within a biopsy specimen.

**Immunohistochemistry**

Tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase was quenched with 3% H2O2 in methanol for 10 minutes. HCMV antigens were retrieved by heating tissue slides in 10 mmol/L sodium citrate, pH 6.0, for the detection of cytokeratin 7, CD31 (PECAM-1), CD34, CD68, VEGF, and Flt1. Nonspecific binding was reduced by incubation in PBS containing 1% bovine serum albumin and 5% normal host serum of secondary antibody. We used primary rabbit polyclonal antibodies to von Willebrand factor (Novocastra Laboratories, Newcastle, UK), VEGF (A20, Santa Cruz Biotechnology, Santa Cruz, CA), PECAM-1 (H-300, Santa Cruz Biotechnology), and Flt1 (Abcam, Cambridge, MA). Mouse monoclonal antibodies were CD68 (clone KP1, Zymed Laboratories, South San Francisco, CA), CD34 (BD Biosciences Pharmingen, San Diego, CA), cytokeratin 7 (clone OV-TL 12/30; Dako, Carpinteria, CA), and HCMV immediate early 1 and 2 proteins (UL123) and DNA-binding protein (UL44) (clones CCH2/DDG9, Dako). HCMV proteins were detected with high-pH Target Retrieval Solution (Dako). Primary antibodies were incubated overnight at 4°C in a humidified chamber. Labeling with secondary antibody was performed using two methods: the biotin/avidin system (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) and direct labeling using Fab fragments of secondary antibodies (PicTure-Double Staining Kit, Invitrogen, Carlsbad, CA). Immunostaining with mouse IgG was developed using a diaminobenzidine (DAB) substrate kit (Vector Laboratories). For double immunostaining, goat anti-mouse IgG-horseradish peroxidase was used with DAB to produce one stain (brown) and goat anti-rabbit IgG-alkaline phosphatase was used with Fast Red to produce a second stain (red). Counterstaining was done with hematoxylin (Vector Laboratories). Specificity of each immunohistochemical reaction was verified by using nonimmune rabbit or mouse IgG (Vector Laboratories) as the primary antibody and secondary antibody labeling by the two methods described above. Immunostaining was reproduced from 3 to 10 times. Images were obtained using a Nikon Eclipse 50i microscope.

**Quantification of Chorionic Villi and Blood Vessels**

Cytokeratin 7-stained tissue sections were used to quantify chorionic villi. Two sections taken at different levels of the tissue block were analyzed from each placenta. Three to five placentas per group were examined. One hundred to 200 microscopic fields were scanned at ×200 magnification, and the number of villi per mm2 was counted. Placental sections double-stained for CD34 and CD31 were used to count the blood vessels. Tissue sections were scanned at ×400 magnification, and the number of blood vessels per villus was counted for 100 villi.

**Image Analysis**

Semi quantitative image analysis of VEGF and Flt1 immunostaining was performed using DAB-labeled (see above) tissue sections. Five to six images (×200 magnification) from randomly selected areas were obtained using a Nikon Eclipse 50i microscope. Illumination conditions of the bright-field optics and camera exposure were maintained constant throughout acquisition of all images. Background correction was performed before digital acquisition. ImageJ (National Institute of Health, Bethesda, MD) was used to perform semi quantitative analysis of the digitalized images. Conventional bright-field RGB color images were split and the blue component (greater contrast DAB staining) was selected as described previously31 and used for further analysis. The mean staining intensity (mean gray value of the pixels in the selection) was measured for each image as described previously32,33. In brief, the mean gray value was measured as the sum of the gray values of all of the pixels in the selection divided by the number of pixels. Because the darker staining correlates with the lower gray value, the gray-scale images were inverted to make the staining
intensity more intuitive: increased VEGF or Flt1 staining intensity correlated with greater gray value.

Quantification of Angiogenic Factors

Amniotic fluid was centrifuged, aliquoted, and stored at −70°C. Soluble (s) Flt1 and PI GF concentrations were measured with a commercial quantitative sandwich ELISA (Quantikine, R&D Systems, Minneapolis, MN). Free VEGF was detected using a quantitative sandwich ELISA (ChemiKine, catalog no. CYT214, Chemicon International, Temecula, CA). The sensitivity of the assays was as follows: sFlt1, 3.5 pg/ml; PI GF, 7 pg/ml; and VEGF, 26.6 pg/ml. Inter assay and intra-assay coefficients of variation were 3 to 10%. All measurements were performed in duplicate by investigators blinded to clinical information. The sFlt1/PI GF ratio, used as an indicator of balanced angiogenic factors, was computed by using the formula, log10[sFlt1/PI GF × 100].

Statistical Analysis

Quantitative data for statistical analysis of the number of chorionic villi and blood vessels were obtained by screening 100 to 200 microscopic fields per slide for three to four placentas per group. The average counts of the villi in placentas from the untreated and HIG treatment groups were compared with those in uninfected controls by using Poisson regression analysis. The analyses were controlled for the fact that each individual contributed multiple measurements. sFlt1 and PI GF concentrations in amniotic fluid were summarized using group-specific means and 95% confidence intervals, and the results are displayed graphically. Comparisons between groups were performed using one-way analysis of variance with Bonferroni correction of P values for multiple comparisons. To account for possible nonnormality of outcome distributions and for the relatively small sample size, we performed additional analyses using log-transformed outcomes and basing confidence intervals and P values on Bootstrap resampling using 1000 replications. These additional analyses did not alter any findings of significance compared with the results based on the assumption of normality, so only the latter results are reported.

Results

Viral Replication and Histopathological Changes in Infected Placentas

In the first set of experiments, we examined biopsy specimens of placentas from women with untreated congenital HCMV infection or after HIG treatment for patterns of viral replication by immunostaining and histopathology. Detection of infected foci required examination of numerous tissue sections cut from different levels within the specimen blocks. Analysis of placentas from the untreated infected group showed viral replication proteins immunostained in cy tomegalic cells, trophoblasts, and endothelial cells (Figure 1). Focal patterns of infected cells were widely scattered throughout the chorionic villi, and cy tomegalic cells with owl-eye nuclear inclusions were infrequent (Figure 1A). Immunostaining for HCMV proteins revealed positive cells, indicating viral replication in endothelial cells in the uterine artery (inset, original magnification, ×400) near an intramural fibrinoid (thrombi). C: HCMV-infected trophoblasts (inset, original magnification, ×200) surround the villus core of adjacent chorionic villi. D: Fetal leukocytes in blood vessels of the villus core contain HCMV-infected-cell proteins (inset, original magnification, ×200). BV, blood vessel; ST, syncytiotrophoblasts; VC, villus core.
Histological analysis showed that pathological changes were found only in placentas with untreated congenital HCMV infection. Typical damage is shown in sections stained with H&E (Figure 2). Necrosis and extensive stromal fibrosis were present in villi that contained many hemosiderin-laden macrophages, indicating infarcted blood vessels and long-term hemolysis (Figure 2, A–D). Syncytial knotting was frequently observed (Figure 2B). Large areas contained many chorionic villi that were encased in fibrin and had few or no blood vessels (Figure 2, C and D). Large areas of fibrinoid deposition were often surrounded by hydroidic villi with compressed capillaries at the periphery (Figure 2, E and F). The presence of severely hydroidic villi suggested inflammation. We occasionally detected villi composed almost entirely of plasma cells (chorionic villitis) (Figure 2, G and H). In marked contrast, pathological changes from viral replication were absent from the HIG treatment group, except for small perivillous fibrinoid plaques (data not shown).

Total histopathology scores were calculated for placentas with untreated infection, with or without fetal transmission, or HIG treatment and controls on the basis of two criteria. i) Infection-associated pathology included detection of HCMV replication proteins, necrosis, calcification, fibrosis, and large fibrinoids as well as inflammation indicated by plasma cells, hemosiderin-laden macrophages, leukocytic infiltration, and edematous villi. ii) Hypoxia-associated pathology included extensive syncytial knotting (Tenny-Parker changes), dilated and congested blood vessels, and avascular fibrinoid-encased villi, increased microvascular density (ie, hypoxic hypercapillarization) and increased numbers of chorionic villi indicated compensatory development. Each pathological feature was rated on a scale of 1 to 3 according to the frequency of defects and compensation observed within a biopsy specimen, and then a cumulative score was obtained for each specimen. Table 1 summarizes the histopathological results.

Values quantifying changes related to infection and hypoxia were comparable in untreated congenital infection with seroconversion in the first trimester (16 ± 1.7 and 16 ± 3.6, respectively) and the second trimester.
(14 ± 2.5 and 13 ± 1.5, respectively). All values for infection- and hypoxia-associated changes were higher than values obtained for controls (4 ± 1.5 and 5 ± 0.6, respectively). Interestingly, values for infection-associated changes were lower than those for hypoxia-associated changes in untreated infection without virus transmission (7 ± 2.5 and 13 ± 3, respectively) and in the HIG treatment group without virus transmission (5 ± 1.2 and 12 ± 0.6, respectively). Notably, hypoxia-associated values in all congenitally infected placentas, with or without virus transmission, and in the HIG treatment group were consistently higher than those in controls.

Comparable total histopathology scores were found for untreated congenital HCMV infection with seroconversion in the first trimester (32 ± 5.3) and the second trimester (28 ± 2.3). The scores for untreated congenital infection with seroconversion in the first trimester were significantly higher than those in the HIG treatment group (P = 0.007) and in controls (P < 0.001).

The scores for untreated congenital infection with seroconversion in the second trimester were significantly higher than those in controls (P = 0.002) but only marginally significant compared with those in the HIG treatment group (P = 0.054). Taken together, the results indicate that placentas with untreated congenital infection in the first and second trimesters sustain considerable damage from viral replication and hypoxia combined.

Compensatory Development in Congenitally Infected Placentas

Focal infection in the uterine vasculature could compromise cytotrophoblast remodeling of the arterioles and reduce maternal blood flow to the developing placenta, leading to hypoxia. Compensatory development, which manifests as increased numbers of chorionic villi and capillaries in the villus core, is found in placentas with physiological hypoxia from high altitudes and in placentas from women who smoked during gestation.

Preliminary analysis of H&E-stained tissue sections revealed the presence of many immature chorionic villi and capillaries in the villus core, is found in placentas with physiological hypoxia from high altitudes and in placentas from women who smoked during gestation.

We detected CD31 in all blood vessels, including small capillaries in placentas from controls (Figure 4A), untreated infected placentas (Figure 4, B and C), and HIG-treated placentas (Figure 4D). Intense CD34 expression was found in blood vessels of control placentas (Figure 4A), untreated infected placentas (Figure 4, B and C), and HIG-treated (Figure 4D) placentas. Large and medium-sized blood vessels, but not small capillaries, strongly expressed von Willebrand factor (data not shown). We then counted the number of blood vessels per villus in each group. The graph in Figure 4E presents the ratio of the average number of blood vessels in the untreated infected and HIG treatment groups relative to controls. Average counts in the untreated infection group were 1.8 times higher than those in the controls (P < 0.001). Average counts in the HIG treatment group were 1.4 times higher than those in controls (P = 0.02) and 1.41 times higher than those in the untreated infection group (P = 0.1). Quantitative analysis of chorionic villi and capillaries in the villus core showed that placentas with untreated infection and those with HIG treatment developed significantly more capillaries and villi than did the controls. Taken together, the results suggest that compensation for a hypoxia-like state in congenitally infected placentas includes remodeling of the placenta surface and angiogenesis, developmental changes that would increase the surface area for exchange of substances between the maternal and fetal circulation.
Up-Regulation of Factors Associated with Hypoxia in Congenitally Infected Placentas

Increased microvascular density in congenitally infected placentas suggested that hypoxic conditions could up-regulate the angiogenic factor VEGF and its receptor Flt1, which control vascular development.41,42 We next examined VEGF expression in decidua and placentas from control, untreated infection, and HIG treatment groups (Figure 5). Double immunostaining was used to identify syncytiotrophoblasts and villous and invasive cytotrophoblasts, which expressed cytokeratin and coexpressed VEGF. The specificity of each reaction was verified by using nonimmune rabbit IgG as a negative control (data not shown). Control placentas had almost no immunoreactivity to VEGF (Figure 5, A and B). In contrast, untreated infected placentas showed strong VEGF staining localized to decidual cells, syncytiotrophoblasts, cytotrophoblasts, and endothelial cells (Figure 5, C and D). In the HIG treatment group, VEGF was expressed at moderate levels in syncytiotrophoblasts, cytotrophoblasts, and endothelial cells (Figure 5, E and F). Moreover, abundant macrophages in the villus core, especially those proximal to capillaries, expressed VEGF at moderate levels (Figure 5F).

Immunohistological analysis of Flt1 expression in control, untreated infection, and HIG treatment placentas is shown in Figure 6. Double immunostaining was used to identify syncytiotrophoblasts and villous and invasive cytotrophoblasts that coexpressed Flt1. The specificity of each reaction was verified by using nonimmune rabbit IgG as a negative control (data not shown). In controls, strong intracellular Flt1 staining was found in decidual cells and invasive cytotrophoblasts (Figure 6A); syncytiotrophoblasts, villous cytotrophoblasts, and endothelial cells showed moderate intracellular Flt1 expression (Figure 6B), and macrophages showed strong membrane and cytoplasmic staining (Figure 6B, inset). In untreated infection, Flt1 was broadly expressed in a diffuse staining pattern in decidual cells (Figure 6C), syncytiotrophoblasts, cytotrophoblasts, and endothelial cells (Figure 6D). In the HIG treatment group, strong Flt1 staining was found in decidual cells, and weak expression was found in cytotrophoblasts, syncytiotrophoblasts, and endothelial cells (Figure 6, E and F).

Next, we compared the intensity levels of VEGF and Flt1 expression in control, untreated infection, and HIG treatment placentas. Control placentas showed weak VEGF expression: the mean value of staining intensity was 77.1 (Figure 7A). In contrast, untreated infected placentas showed the highest VEGF staining intensity (129.1), followed by the HIG treatment group (114.4). Importantly, we found that all congenitally infected placentas, untreated (P < 0.001) and HIG-treated (P = 0.005)
Dysregulated Angiogenic Factors in Amniotic Fluid of Congenitally Infected Fetuses

Amniotic fluid is composed of substances undergoing bidirectional diffusion from fetal circulation before keratinization of skin, as well as the surface of the amnion, placenta, and umbilical cord. Having found strong immunostaining of VEGF and Flt1 in cytotrophoblasts (Figures 5–7) and foci of viral replication in placentas from untreated infection (Figure 1), we considered that amniotic fluid could contain dysregulated angiogenic factors resulting from HCMV-infected cells and possible placental hypoxia.

A physiological balance between proangiogenic (VEGF/PIGF) and antiangiogenic (sFlt1) factors that control normal placental development is reflected by sFlt1/PIGF ratios. sFlt1 derived from the placenta forms complexes with VEGF and PIGF, reducing their functions.

Next, we measured VEGF, PIGF, and sFlt1 in amniotic fluid from 38 cases of untreated congenital HCMV infection, 9 cases from the HIG treatment group, and 7 controls. Amniotic fluid from fetuses with untreated infection contained dramatically elevated levels of sFlt1 (10.1 to 243.7 ng/ml) (Figure 8A); sFlt1 values were substantially lower in amniotic fluid from the HIG treatment group (1.1 to 44.5 ng/ml), and values were lowest in controls (1.1 to 7.1 ng/ml). PIGF levels in amniotic fluid varied in untreated infection (30 to 257 pg/ml), HIG treatment (45 to 148 pg/ml), and control (82 to 281 pg/ml) groups (Figure 8B). VEGF was not detected (data not shown).

The high variability of sFlt1 and PIGF within the groups, we reasoned that the ratio of sFlt1/PIGF ratio would be a more reliable index of the status of angiogenic factors in amniotic fluid. Amniotic fluid from untreated infection and from the HIG treatment group contained significantly elevated sFlt1/PIGF ratios compared with controls (P < 0.001) (Figure 8C). Although the mean sFlt1/PIGF ratio in the HIG treatment group decreased compared with that in the untreated infection group, the differences were not significant (P = 0.135).

Next, we determined whether sFlt1/PIGF ratios in amniotic fluid could predict poor fetal outcome, including temporary symptoms that resolve soon after birth (IUGR...
and hepatosplenomegaly) and permanent birth defects (neurological impairment).12 Each clinical manifestation associated with congenital infection—IUGR, fetal infection (CMV DNA positive) and brain disease—was scored as 1. Absence of a symptom was scored as 0. A comparison of fetal symptoms at birth (“outcome score”) (Figure 8D) and sFlt1/PIGF ratios (Figure 8C) showed that amniotic fluid from symptomatic babies with untreated congenital infection had significantly elevated sFlt1/PIGF ratios compared with controls (P < 0.001). In addition, the sFlt1/PIGF ratios in the asymptomatic infection with HIG treatment group were significantly higher than those in controls (P < 0.001). The decrease in the ratio between the untreated infection and HIG treatment groups was not significant (P = 0.135) and may be due to the small sample size. Taken together, the results suggest that an elevated sFlt1/PIGF ratio in amniotic fluid could indicate a hypoxia-like environment in congenital HCMV infection.

Discussion

In this study, we showed that a hypoxia-like condition in placentas congenitally infected with HCMV up-regulates VEGF expression and, combined with HIG treatment, promotes compensatory development, increasing the number of chorionic villi and the vasculature that improves fetal outcome. Previous studies of early-gestation placentas infected with HCMV revealed virus replication in uterine and fetal blood vessels, villous cytотrophoblast progenitors, and differentiating cells.19,20,22,25 Others found HCMV antigens in trophoblasts, stromal cells, and endothelial cells of placentas from symptomatic congenital infection.51,52 We reported that HCMV infects the decidua and spreads to the placenta.23 Infected cells include endothelial cells of the uterine vasculature21 invasive cytотrophoblasts,19 and decidual cells and uterine glandular epithelial cells.20 Infected cytотrophoblasts invading the decidua could impair differentiation/invasion through a constellation of events19,26,27,53 that compromise cytотrophoblast remodeling of uterine arteries, diminish maternal blood flow, and contribute to a hypoxia-like condition. We found infected uterine arteries with intramural fibrinoids and vasculitis of blood vessels in the villous core that could lead to thrombosis and calcification,20 reducing blood return and resulting in placental edema. Extremely thick placentas with microcalcifications and focal ischemia have been reported in cases of severe congenital infection.29,54—57 Because the placenta lacks a lymphatic system,58 reduced blood flow could cause hydropic villi and, in severe cases of congenital infection, fetal hydrops.57,59,60

Some temporary symptoms of congenital HCMV infection, such as thombocytopenia, extramedullary hematopoiesis (blueberry muffin syndrome), and hepatomegaly, are probably fetal compensation for hypoxia.61—64 The reversible nature of these symptoms was shown by treatment of congenital infection with HCMV-specific HIG.28,65—67 In contrast, sensorineural hearing loss correlates with symptomatic congenital infection at birth and prolonged virus shedding.68,69 Together, the transient clinical manifestations could result from reduced nutrient and oxygen transport to the fetus, whereas permanent neurological damage could be caused by both HCMV replication in fetal brain and hypoxic conditions in utero. Infection-induced damage could lead to hypoxia-like conditions and associated compensatory development (substantially increased numbers of chorionic villi and capillaries) in placentas with HIG treatment. HCMV infection in the uterine vasculature (decidua) results in fibrinoids (thrombi), spreads to cytотrophoblasts, and impairs invasiveness that together reduce maternal blood flow. Viral replication in cytотrophoblasts, endothelium, and stromal fibroblasts results in fibrosis from deposition of extracellular matrix proteins by integrin αvβ6-mediated transforming growth factor-β activation27 and impairment of extracellular matrix degradation by down-regulation of matrix metalloproteinase activity.26 Eventually, transport of nutrients and oxygen to the fetal compartment would be impaired. As fetal demands for oxygen increase, the intraterine environment becomes hypoxic, and adaptation takes the form of dilated blood vessels congested with fetal leukocytes and erythrocytes. In hydropic villi, capillaries were located at the villous periphery, close to syncytiotrophoblasts to optimize transport to the fetal bloodstream (Figures 2, E and F, and 4B). Such vascular changes were not observed in placentas with HIG treatment, in which capillaries were distributed throughout the
villus core of many immature villi (Figure 4D). Importantly, the overall surface for exchange between the maternal and fetal circulation was dramatically increased with HIG treatment in early gestation, a time when the abundant cytotrophoblast stem cell population enables plasticity and remodeling of chorionic villi. It follows that a greater syncytiotrophoblast surface area and microvascular mass will increase blood flow and improve the efficiency of oxygen, nutrient, and IgG transport from the maternal to fetal circulation.

Others reported that placentas with symptomatic congenital HCMV infection contain more immature villi. Quantitative analysis showed that the number of chorionic villi significantly increased with HIG treatment relative to those of controls, but untreated infection had only marginal increases (Figure 3D). Under hypoxic conditions in first-trimester placentas, villous cytotrophoblasts proliferate, differentiate, and invade the uterine wall, regulated by oxygen tension. When a hypoxic environment persists, cytotrophoblast stem cells divide and form new villi. Our results suggest that a prolonged hypoxia-like state associated with congenital infection could extend this process. In pregnant women from high altitudes with low oxygen, the relative volume of cytotrophoblasts increases and placentas weigh significantly more than they do at sea level, suggesting adaptation by development of a more extensive peripheral villous tree. Placental weights also increase in heavy smokers, but the cytotrophoblast stem cell population is prematurely depleted and the number of anchoring villi is reduced. In addition, capillary densities increase within placental terminal convolutes, suggesting an adaptive angiogenic response in chorionic villi. Placentas with congenital HCMV infection increase in weight and thickness, and HIG treatment leads to some reduction in size, suggesting that irreversible enlargement could result from a combined increase in the numbers of chorionic villi and villous capillaries. Like quiescent endothelium, blood vessels in control placentas expressed CD34 but those in untreated and HIG-treated placentas failed to express CD34 or did so weakly, suggesting a proliferating endothelium.

Balanced VEGF and PI GF levels throughout gestation are necessary for normal placental development. In the first trimester, physiological hypoxia favors VEGF expression, which influences branching angiogenesis. In the third trimester, normal oxygen levels correlate with strong PI GF expression and nonbranching angiogenesis, whereas VEGF levels decline. Cytotrophoblasts cultured under hypoxic conditions strongly up-regulate VEGF expression and down-regulate PI GF. VEGF expression is dramatically up-regulated in placentas from women who smoke during gestation and enormously enlarged blood vessels develop at the periphery of villi. In parallel with increased numbers of immature villi (Figure 3) and blood vessels (Figure 4), VEGF expression was significantly up-regulated in syncytiotrophoblasts, cytotrophoblasts, and blood vessels of infected placentas, with or without HIG treatment, relative to controls (Figure 7). Taken together, the results suggest that induced VEGF expression could contribute to the adaptation found in congenitally infected placentas. Quantitative analysis strongly suggests that the compensatory development was a response to biologically active VEGF up-regulated by a hypoxia-like condition from viral damage at the uterine-placental interface. It is also possible that VEGF expression was up-regulated by the inflammatory cytokines, TNF- and interleukin-6, associated with HCMV replication, especially considering the higher levels of VEGF that were found in untreated congenital infection. VEGF expression could also be a downstream effect of hypoxia-inducible factor 1 induction by activation of a nuclear receptor, peroxisome proliferator-activated receptor (PPAR), that modulates cytotrophoblast differentiation/invasion. HCMV-infected cytotrophoblasts induce transcriptional activity of PPAR that binds to the viral immediate-early promoter, resulting in enhanced replication and reduced cell invasion. Mononuclear phagocytes, PPAR activation trans-represses the metalloproteinase 9 promoter. Whether downstream effects of HCMV-induced PPAR activation in cytotrophoblasts also reduce metalloproteinase 9 protein that degrades the extracellular matrix, contributing to reduced cell functions remains to be tested.

Placental development is regulated by levels of pro- and antiangiogenic factors reflected in sFlt1/PI GF ratios. In amniotic fluid, sFlt1 forms complexes with VEGF and PI GF that could reduce free, functional angiogenic factors. In healthy pregnancies, increased sFlt1 levels positively correlate with PI GF levels, whereas in preeclampsia they have a negative correlation. Interestingly, in a case of severe congenital HCMV infection with mirror syndrome, maternal symptoms that resemble preeclampsia, sFlt1 levels in serum increased precipitously. We found sFlt1/PI GF ratios to be significantly elevated in amniotic fluid from untreated congenital infection and HIG treatment compared with controls (Figure 8C). Moreover, sFlt1/PI GF ratios tended to decrease in the HIG treatment group relative to those in the untreated infection group. Angiogenic factors and antagonists are dysregulated in various pregnancy complications involving the placenta. Our studies suggest that congenital HCMV infection could induce a hypoxic state and compensatory development similar to that in pregnancies at high altitude and when the mother smoked during gestation. In preeclampsia, sFlt1 is elevated and PI GF is reduced in the maternal circulation and amniotic fluid. VEGF expression is reduced in cytotrophoblasts cultured from these placentas, which could explain the failure of preeclamptic placentas to undergo compensation found in smokers.

Our results suggest that HIG treatment reduced viral replication in the placenta and subsequent injury from persistent infection. As reported, significantly fewer babies had congenital infection or disease after HIG treatment. In vitro, HIG reduces HCMV infection of fibroblasts, neutralizes virus-induced intracellular NF-kB and phosphatidylinositol 3-kinase signaling pathways, and has neutralizing activity that blocks virus entry into epithelial cells. HIG treatment is associated with increased HCMV-specific IgG concentration and avidity in maternal circulation after infusion. At delivery, IgG avidity in placentas with treatment is higher than that in those with...
untreated infection and provides an immediate source of protective antibodies that evolved over the course of gestation. A recent study of placentas from uncomplicated deliveries in seropositive women showed that the concentration of high-avidity, HCMV-specific IgG with virus-neutralizing activity in the fetal bloodstream is equal to or higher than the concentration in the maternal circulation. Thus, antibodies from HIG infusions could first reach the placenta, suppress viral replication, and eventually reach the fetal bloodstream, facilitated by neonatal Fc receptor transcytosis across syncytiotrophoblasts. High-avidity, HCMV-specific antibodies could prevent viral transmission and fetal infection or symptomatic disease. Administration of HIG after maternal seroconversion in early gestation, when cytotrophoblasts proliferate, could reduce viral injury and accelerate and prolong development to compensate for hypoxia-like conditions. Together with serological evidence of primary maternal infection, our findings suggest that an elevated sFlt1/PIGF ratio precedes fetal transmission and the presence of HCMV DNA in amniotic fluid. Accordingly, these proteins could serve as early biomarkers of placental infection to identify at-risk pregnancies that could benefit from HIG treatment to prevent infection and congenital disease.

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