Human Cytomegalovirus-Caused Damage to Placental Trophoblasts Mediated by Immediate-Early Gene-Induced Tumor Necrosis Factor- α

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Infection of the fetal epithelium (trophoblast) lining the villous placenta by human cytomegalovirus (HCMV) accompanies placental inflammations and fetal intrauterine growth restriction. However, the consequences of infection on the villous trophoblast have not been explored. We show that HCMV infection of primary immature (cytotrophoblast-like) or mature (syncytiotrophoblast-like) cultures results in loss of half of the cells within 24 hours of virus challenge. Two-color immunofluorescence of HCMV immediate early (IE) gene expression and apoptosis (terminal dUTP nick-end labeling) revealed apoptosis only in uninfected cells. Antibody to tumor necrosis factor (TNF)- α completely inhibited infection-induced trophoblast apoptosis and cell loss, as did co-incubation with epidermal growth factor, known to inhibit trophoblast apoptosis. Transfection with HCMV immediate early- (IE)1-72 and IE2-86, but not IE2-55, expression plasmids induced paracrine trophoblast apoptosis inhibitable by epidermal growth factor or antibody to TNF- α . These results show that HCMV infection of villous trophoblasts leads to rapid loss of neighboring cells mediated by viral IE protein-induced TNF- α secretion. We propose that HCMV infection damages the placental trophoblast barrier by accelerating trophoblast turnover and decreasing its capacity for renewal. (*Am J Pathol* 2002, 161:1371–1381)

Human cytomegalovirus (HCMV), a member of the *Herpesviridae* family, is a common infection found in 50 to 90% of adults.¹ A primary infection is asymptomatic in immunologically healthy women but fetal infection by maternal HCMV is a serious problem. There is a 30 to 40% possibility of intrauterine transmission of HCMV to the fetus during a primary infection of the mother and a 0.2 to 0.5% possibility during a recurring or re-infection. The

overall frequency is 0.5 to 2% of all live births making HCMV the most common congenital infection. A congenital HCMV infection can result in abortion or stillbirth with symptomatic survivors displaying sequelae such as thrombocytopenia, hepatosplenomegaly, vision loss, sensorineural deficits, and mental retardation.

HCMV can establish productive, persistent, or latent infections in a variety of cells including those of epithelial origin.² HCMV gene expression occurs in sequential phases designated immediate early (IE), early (E), and late (L). IE gene expression is required for the transcription of early genes, which encode proteins essential for viral DNA replication. In turn, replication of viral DNA is a prerequisite for late viral gene transcription of structural proteins. Transcription of IE genes has been mapped to five regions on the human HCMV genome. The most abundantly transcribed IE region is the ie1/ie2 locus. This region encodes two viral proteins, IE1-72 and IE2-86, and IE2-55, a splice variant of IE2-86. The IE1-72 protein interacts with the cellular transcription factors nuclear factor (NF)- κ B, c-fos, and c-myc.^{3–5} The IE2-86 protein is a strong transcriptional activator that interacts with basal-transcriptional machinery and blocks cell cycle progression.⁶ Importantly, IE1-72 and IE2-86 regulate both viral gene and cellular gene expression. Through interactions with cellular factors such as p53 and NF- κ B, both viral proteins inhibit apoptosis^{7–9} and the induction of apoptosis by tumor necrosis factor (TNF)- α .¹⁰

In addition to the neurological damage caused by fetal infection, hematogenous infection of the placenta by HCMV is a major risk factor for fetal intrauterine growth restriction (IUGR),¹¹ which in turn is linked to cardiovascular disease later in life.^{12,13} Although there are several possible origins of IUGR, all lead to deficient oxygen and nutrient delivery by the placenta to the fetus.¹⁴ Villitis (inflammation of the villous placenta) characterizes placental infections by HCMV,¹⁵ is a risk factor for IUGR,¹⁶ and is accompanied by focal damage to the villous trophoblast, the major function of which is nutrient delivery from the maternal to the fetal circulations.¹⁷ Thus, villous

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trophoblast damage by HCMV likely contributes to IUGR, however, the mechanism of damage is unknown.

Immunohistochemical analysis of sections from term placentas displaying chronic villitis revealed IE^{18,19} but not E¹⁸ or L (p150)¹⁹ antigens, suggesting abortive infections.¹⁹ However, *in situ* hybridization revealed CMV DNA in stromal cells and in the trophoblast of term placentas with chronic villitis.²⁰ Placentas from first or second trimester abortions contain nuclear inclusions frequently in stromal cells²¹ and more rarely in trophoblasts¹⁵ with expression of early antigen pp65 in the trophoblast^{22,23} suggesting a permissive trophoblast infection. Importantly, pure populations of term and first trimester villous trophoblasts can be productively infected in culture,²⁴ observations that both confirm villous trophoblast infection and provide a model for studying HCMV-induced placental damage.

The villous trophoblast comprises two cell types: an extended syncytium spanning the intervillous surface, the syncytiotrophoblast (ST), and an underlying layer of mononuclear cytotrophoblasts (CT).¹⁷ Villous trophoblast apoptosis is a normal event in placental development²⁵ that is increased in placentas associated with IUGR.²⁶ Primary villous trophoblast apoptosis in culture occurs spontaneously and is stimulated by the inflammatory cytokines TNF- α and gamma interferon (IFN- γ)^{27,28} and by serum withdrawal.²⁹ Primary villous CT can be isolated³⁰ and differentiated in culture into syncytialized clusters by treatment with epidermal growth factor (EGF).^{27,31} Thus, cultures of mature and immature primary villous trophoblasts provide a good model for investigating the consequences of villous trophoblast infection by HCMV.

Using this model, we asked whether HCMV infection damages trophoblast cultures, and if so, how. We found that within 24 hours of infection more than half of both mature and immature trophoblasts were lost by apoptosis stimulated by TNF- α release but that only uninfected cells in the culture underwent apoptosis. We also show that expression of HCMV immediate early genes IE1-72 and IE2-86 are sufficient for these effects.

Materials and Methods

Cells

Human embryonic lung fibroblasts were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and propagated in Eagles' minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc., Grand Island, NY) and 50 μ g of gentamicin per ml. L929-8 cells were maintained in culture with Iscove's modified Dulbecco's medium (IMDM) (Life Technologies, Inc.) containing 15% FBS as previously described.³² The cervical carcinoma cell line HeLa and the colon carcinoma line CaCo were both obtained from ATCC and were propagated in 10% FBS in IMDM.

Human term villous CT were isolated from placentas obtained after normal term delivery or elective cesarean section from uncomplicated pregnancies and cryopreserved as previously described.^{33,34} After thawing, the

cells were washed in IMDM supplemented with 10% FBS, seeded in 96-well dishes (Nunc, Roskilde, Denmark) at 10^5 per microwell per 100 μ l of 10% FBS/IMDM and incubated for 4 hours at 37°C in a fully humidified atmosphere of 5% CO₂ in air. Nonadherent cells and debris were washed away with prewarmed IMDM and the cultures continued in 10% FBS/IMDM. All preparations contained <10 vimentin-positive cells after the 4-hour wash. All experiments were performed either with cells cultured for 24 hours without EGF (operationally termed CT-like cultures) or with cells that had been syncytialized by treatment with 10 ng/ml of EGF (Peprotech, Rocky Hill, NJ) for 5 days as previously described²⁷ (operationally termed ST-like cultures).

The adherence and spontaneous apoptosis of cryopreserved primary CTs from a single placenta are consistent in independent experiments performed at different times. However, these properties can be very different in cells from different placentas even if examined at the same time (unpublished observations). Each experimental data set (figure) consists of three independent experiments with cells from two different placentas (two experiments with one placental preparation and one from the other). Six different placental preparations were used in the experiments. The two placental preparations for a given figure were chosen for a single consistent property (the fraction of cells that adhered after 4 hours of culture) before the experiments were performed. The two placental preparations, thus chosen, showed a consistent basal frequency of apoptosis, either high or low, and HCMV infection consistently increased this basal frequency regardless of whether it was high or low. For example, even though spontaneous apoptosis frequencies for CT- and ST-like cultures varied considerably (1 to 17%) between Figures 3, 5, 6, and 7, the ratios of HCMV-induced to basal apoptosis frequencies across these experimental groupings were consistent (2.4 ± 0.7 , $n = 8$). Thus, the trends are consistent for cells from all placentas, even though absolute numbers may be different.

Virus Preparation, Culture Challenge, and Assessment of Infection

HCMV laboratory strain AD169 was passaged in confluent human embryonic lung cells in 2%FBS-MEM as previously described,²⁴ the lysate passed through 0.45- μ m-pore-size filters (MILLEX-HV; Millipore Products Division, Bedford, MA) and stored in liquid nitrogen until use. Viral titers were determined by inoculating confluent human embryonic lung fibroblast cultures in 96-well plates with dilutions of each virus preparation in serum-free MEM. The plates were then centrifuged for 45 minutes at 2500 rpm in a GCL-2 Sorvall centrifuge, the wells washed five times with warm MEM and the plates incubated for a further 18 to 20 hours in fresh 2% FBS-MEM. The cultures were fixed in ice-cold methanol and immunohistochemically stained for CMV IE antigen as described below. Each IE-positive nucleus was equated to an infection focus of infectious virus, and the titer of virus was deter-

mined within a linear dose-response concentration range as infection focus/ml.

Where indicated HCMV preparations were inactivated by exposure to UV light (30 W, germicidal, distance of 20 cm) on ice for 20 minutes. Virus-free supernatant was obtained by filtering HCMV batches through a 0.1- μ m-pore-size syringe top filter (Millipore). UV inactivation and complete filtration was assured by the absence of IE-positive nuclei in trophoblast cultures.

Modifications in HCMV challenge methods have increased trophoblast infection frequencies considerably compared to a previous publication.²⁴ The modified methods are summarized below: ST-like cultures were virus-challenged 5 days after plating and CT-like trophoblasts 1 day after plating. Both culture types were washed once with warm IMDM and challenged in 2% FBS/IMDM for 6 or 24 hours. The virus challenge was at a multiplicity of infection (MOI) of 10, calculated by first enumerating, in parallel cultures, the number of nuclei in CT- and ST-like cultures by 4,6-diamidino-2-phenylindole (DAPI) staining (see below) and then adding a 10-fold higher infection focus of virus (or an equal volume of UV-inactivated or virus-free supernatant from the same preparation). After the 6 or 24 hours of culture, the cells were washed twice with phosphate-buffered saline (PBS), fixed in ice-cold acetone:methanol (1:1) for 10 minutes at -20°C , and washed three times with PBS in preparation for immunofluorescence and/or terminal dUTP nick-end labeling (TUNEL) (see below). Cultures extending longer than 24 hours were washed at that time five times with warm IMDM, fresh 2% FBS-IMDM with (for ST-like cells) or without EGF (for CT-like cells) added and the media changed every 2 days for the duration of the culture. In some experiments (eg, Figure 6), EGF was added to both culture types during the virus challenge period.

Transfection of HCMV IE Expression Plasmids

All plasmids were propagated in *Escherichia coli* DH5 α , isolated by standard procedures and the plasmid DNA purified with a Qiagen Plasmid Maxiprep kit (Qiagen, Mississauga, Ontario, Canada). Plasmids pcDNA3-IE1-72, pcDNA3-IE2-55, and pcDNA-IE2-86, respectively, express the HCMV IE proteins, IE1-72, IE2-55, and IE2-86.⁴ The vector without inserts, pcDNA3 (Invitrogen, San Diego, CA), served as a negative control. Trophoblasts (both CT- and ST-like cultures) were prepared in micro-wells as described above and transfected with Lipofectamine 2000 (Life Technologies, Inc.)/plasmid DNA complexes as follows: 700 μ g of DNA in 25 μ l of Opti-MEM (Life Technologies, Inc.) was mixed with 0.5 μ l of 1 mg/ml Lipofectamine 2000 diluted with 25 μ l of Opti-MEM then added to a microwell containing 100 μ l of 2% FBS/IMDM. The transfection efficiencies for each plasmid type was determined by IE immunofluorescence as described above.

Immunofluorescence Staining

Three-color fluorescence analysis was performed to determine total nuclei number, the fraction and location of nuclei expressing HCMV IE proteins, and the fraction and location of nuclei containing nicked double-stranded DNA (a marker of apoptosis). After acetone:methanol fixation and PBS washing, the fraction of nuclei with nicked DNA was determined by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin DNA-nick end labeling³⁵) as previously described²⁸ with modifications to allow for simultaneous immunofluorescence analysis. After the reaction was terminated by adding double-strength 300 mmol/L sodium chloride plus 30 mmol/L sodium citrate (2 $\times$ standard saline citrate), the cells were washed three times with double-distilled water, nonspecific binding sites blocked with 3% skim milk/0.5% Tween 20/PBS for 30 minutes and primary antibody to CMV IE (detecting p72; Specialty Diagnostics, Dupont) or its IgG1 isotype control (DAKO, Carpinteria, CA) added and incubated at room temperature for 1 hour. The primary antibody was then removed, the cells washed five times with PBS and 50 μ l per well of streptavidin Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR) and Alexa Fluor 546 goat anti-mouse IgG conjugate (Molecular Probes) each diluted in 3% skim milk/0.5% Tween 20/PBS to 1 μ g/ml added and incubated for 1 hour at room temperature. The cells were then washed five times with PBS. To visualize all nuclei 100 μ l of 1.4 μ g/ml DAPI (Molecular Probes) was added to each well and allowed to sit for 10 minutes at room temperature. Cells were then washed with PBS five times and visualized with a fluorescence microscope (see below). The total number of nuclei (DAPI, blue), IE-positive (Alexa Fluor 546, red), and TUNEL-positive (Alexa Fluor 488, green) were determined per well by digital analysis as described below.

Digital Photography and Analysis

Flourescence was visualized with an inverted phase-contrast microscope (model DS-IRB; Leica, Heerbrugg, Switzerland) equipped for epifluorescence with a 50-W high-pressure mercury lamp driven by a Ludl power source (Ludl Electronic Products, Hawthorne, NY). Identical digital images of each well were taken with a DAPI filter (blue), a rhodamine filter (red), and a fluorescein isothiocyanate filter (green) using a SPOT digital camera (Diagnostic Instruments, St. Sterling Heights, MI). The red and green images were superimposed using an imaging program, Image-Pro Plus (Media Cybernetics, Del Mar, CA). Three images, each containing $\sim$ 900 nuclei, were taken in each of triplicate wells.

TNF Bioassay and Neutralization

Supernatants from infected and uninfected cells were saved and frozen at -20°C until analysis. The supernatants were thawed and assayed for TNF activity using recombinant human TNF- α (a gift from Hofmann La Roche, Basel, Switzerland) standards in the L929-8 bio-

Figure 1. HCMV-IE protein expression in virus-challenged trophoblasts as a function of time after challenge. CT-like and ST-like cultures were challenged at a MOI of 10 with HCMV strain AD169 and cultured as described in Materials and Methods. At the times indicated, IE-positive nuclei were visualized with an Alexa Fluor 547 conjugate and total nuclei with DAPI as described in Materials and Methods. Depicted is the mean $\pm$ SD of two independent experiments with two different placentas, each containing three replicate cultures.

assay as previously described.³² The lowest level of detection is 0.5 pg/ml. To neutralize biologically active TNF- α released in trophoblast cultures, 20 μ g/ml of polyclonal anti-human TNF- α antibody (anti-TNF- α ; ICN, Aurora, OH) was added to the culture at the time of virus challenge. After incubations as noted in individual figure legends, cells were washed with PBS three times, fixed with acetone:methanol (1:1), and TUNEL analysis and immunofluorescence were performed as described above.

Statistical Analysis

Experiments for each figure were performed at least three times on trophoblasts isolated from two different placentas. Differences between experimental groups for the two cell types (CT- or ST-like cells) were evaluated by one-way analysis of variance with pair-wise multiple comparison procedures (Tukey test) using the SigmaStat program (Jandel Scientific, San Rafael, CA). Results were considered to be significant at $P < 0.05$.

Results

HCMV Infection of Trophoblasts, but Not Other Epithelial Cells, Induces Cell Loss within 24 Hours of Virus Challenge

Both immature (CT-like) and mature (ST-like) trophoblasts were challenged with HCMV laboratory strain AD169 and the infection allowed to progress throughout an 11-day period (Figure 1 shows a typical infection time course). Cells were stained with DAPI to determine total nuclei per well and the frequency of infection was determined by immunostaining for HCMV IE antigens. Six

Figure 2. Nuclei loss from trophoblast cultures after HCMV infection. CT-like and ST-like cultures were given medium alone (control), treated with TNF- α /IFN- γ , or challenged with filtered, UV-inactivated, or active HCMV strain AD169 virus preparations at a MOI of 10. After 24 hours, the number of nuclei remaining in CT-like (top) and ST-like (bottom) cultures was determined by DAPI staining. Depicted is the mean $\pm$ SD of three independent experiments (each with duplicate microwells) using cells from two different placentas. Experimental groups within each panel labeled with different letters (a or b) are significantly different ($P < 0.05$).

hours after virus addition there were few detectable IE-positive nuclei in either culture type. However, 24 hours after the addition $\sim$ 25% of nuclei in CT-like cultures were IE-positive and 3% of nuclei in ST-like cultures. By day 11 of culture $>$ 70% of nuclei remaining in CT-like cultures were IE-positive compared to $\sim$ 10% of nuclei in the ST-like cultures.

Because of the differing proportions of multinucleated cells in CT-like and ST-like cultures,³¹ cell loss was characterized in both culture types by counting nuclei and comparing infected to control cultures (Figure 2). Three negative control groups were examined: mock-infected control (control), virus preparations filtered free of virus particles (filtered), and UV-inactivated virus preparations (UV-inactivated). The positive control was treatment with the inflammatory cytokines TNF- α and IFN- γ , known to stimulate both cell loss and apoptosis.^{27,28} Six hours after challenge, for a given culture type, all groups contained the same number ($P > 0.05$) of nuclei (average $\pm$ SD for CT-like cultures, $4.1 \times 10^4 \pm 1.2 \times 10^3$ /well; for ST-like cultures, $6.2 \times 10^4 \pm 4.2 \times 10^3$ /well). However, 24 hours after challenge both HCMV infected CT- and ST-like cultures lost approximately half of their nuclei compared to untreated control cultures ($P < 0.05$) (Figure 2). Treat-

Figure 3. HCMV-induced trophoblast apoptosis 24 hours after challenge. CT-like and ST-like cultures were given medium alone (control), treated with TNF- α /INF- γ , or challenged with filtered, UV-inactivated, or active HCMV strain AD169 virus preparations at a MOI of 10. After 24 hours, the fraction of apoptotic nuclei in CT-like (**top**) and ST-like cultures (**bottom**) was determined by TUNEL analysis. Depicted is the mean $\pm$ SD of three independent experiments (the same as depicted in Figure 2). Experimental groups within each panel labeled with different letters (a, b, or c) are statistically different ($P < 0.05$).

ment of both culture types with UV-inactivated and filtered HCMV preparations did not significantly affect nuclei numbers at 24 hours compared to control cultures ($P > 0.05$). TNF- α /INF- γ -treated CT-like cultures lost ~60% of nuclei but ST-like cultures lost fewer than 20%.

Apoptosis of trophoblasts remaining in culture after 6 and 24 hours was measured as the fraction of nuclei having double-stranded DNA nicks as visualized by TUNEL analysis. Six hours after virus exposure, for a given culture type, all groups contained the same fraction of TUNEL-positive nuclei ($P > 0.05$) (average $\pm$ SD for CT-like cultures, $3.95 \pm 0.66\%$; for ST-like cultures, $1.5 \pm 0.46\%$). However, 24 hours after virus exposure both HCMV infected CT- and ST-like cultures showed a significantly increased apoptosis frequency compared to control cultures (from 3.3 to 8.3% for CT-like cultures and 0.94 to 3.5% for ST-like cells, $P < 0.05$) (Figure 3). Treatment of both culture types with UV-inactivated and filtered HCMV preparations did not significantly increase the TUNEL frequency at 24 hours compared to control cultures ($P > 0.05$). Both TNF- α /INF- γ -treated cultures had a significantly increased frequency of TUNEL-positive nuclei compared to control cultures (from 3.3% to 14.6% for CT-like cultures and from 0.94 to 2.1% for ST-like cultures, $P < 0.05$, Figure 3).

We asked whether cell loss and apoptosis occurred during HCMV infection of other epithelial cells that can be

infected by HCMV: HeLa, a well known cervical carcinoma cell line, and CaCo, a colon carcinoma cell line.³⁶ We found that HCMV infection (2% IE-positive nuclei for HeLa and 7.6% for CaCo at a MOI of 10 in 24 hours) did not increase cell loss or apoptosis relative to mock-infected controls (data not shown).

Taken together, these observations strongly suggest that trophoblasts are lost by accelerated apoptosis induced by HCMV infection. However, cell loss at 24 hours in HCMV-infected cultures (~50%) is consistently much higher than the frequency of apoptosis (8.3% and 3.5% for CT- and ST-like cells, respectively).

Because of the large difference between cell loss and apoptosis frequencies, we asked whether cells that were simultaneously infected and undergoing apoptosis might be preferentially lost in culture. Nonadherent cells were collected from HCMV-infected ST and CT-like cultures 24 hours after HCMV challenge and evaluated for apoptosis by TUNEL analysis and for HCMV infection by IE immunofluorescence. Nonadherent cells from CT-like cultures were $98.3 \pm 0.58\%$ TUNEL-positive and $6.1 \pm 0.81\%$ IE-positive whereas nonadherent cells from ST-like cultures were $96.1 \pm 1\%$ TUNEL-positive and $17.2 \pm 7.0\%$ IE-positive. Thus, almost all cells lost from the cultures were undergoing apoptosis and <20% were HCMV infected.

HCMV Kills Only Uninfected Cells in Culture

The above results suggested that most of the cell loss in HCMV-challenged cultures was in the uninfected population. This suggestion is supported by the data in Figures 1 and 2 showing that 24 hours after virus challenge CT- and ST-like cultures lose the same fraction of nuclei even though the infection frequency in CT-like cultures is 10-fold higher than in ST-like cultures. To spatially characterize the relationship between infected and dying cells, we performed two-color immunofluorescence analysis of HCMV-IE expression (red in Figure 4A) and apoptotic nuclei (by TUNEL analysis, green in Figure 4A) on cells remaining adherent in the cultures. Concurrent nuclear DNA nicking and IE expression would result in nuclei with a combined yellow color (see Figure 4B, CT panel, for a very rare example). One day after exposure to virus, there were green and red, but almost no yellow, nuclei in both CT- and ST-like cultures. Thus, HCMV IE- and TUNEL-stained nuclei were in mutually exclusive populations in the adherent population. The same result was found at day 5 of infection (data not shown). Thus, for at least 5 days after HCMV infection, only uninfected trophoblasts in the adherent cultures are undergoing apoptosis.

HCMV-Induced Trophoblast Cell Loss Is Mediated by TNF- α .

The above observations that infection increases trophoblast apoptosis but only in uninfected cells suggests that HCMV-induced apoptosis is mediated by a soluble factor released by infected cells. The most likely (and only

Figure 4. Relationship of trophoblasts expressing nuclear HCMV-IE antigen and those undergoing apoptosis during infection by HCMV (**A**) and transfection (**B**) by HCMV IE genes. CT-like and ST-like trophoblasts were challenged with HCMV strain AD169 at a MOI of 10 (**A**) or transfected with HCMV IE1-72 or IE2-86 expression plasmids (**B**) as described in Materials and Methods. After 24 hours, cultures were fixed, stained for nuclear DNA nicking (TUNEL), and immunostained for nuclear expression of HCMV-IE antigen and visualized by fluorescence (IE with Alexa Fluor 546, red; TUNEL with Alexa Fluor 488, green) as described in Materials and Methods. Double-labeling for IE proteins and TUNEL gives a yellow color (see **arrow** in CT, **B**). This experiment was performed three times with cells from two different placentas with the same results.

known) death-inducing factor for primary villous trophoblasts is the cytokine $\text{TNF-}\alpha$.^{27,28} We therefore first asked whether HCMV infection induced production of biologically active TNF in the cultures. We found that HCMV infection increased TNF activity in culture supernatants of both CT- and ST-like cultures. One day after infection, the supernatant levels of biologically active TNF in CT-like cultures were 9.8 ± 2.1 pg/ml compared to 4.7 ± 1.6 pg/ml in uninfected control cultures whereas levels increased in ST-like cultures from undetectable (<0.5 pg/ml) to 11 ± 2.2 pg/ml ($n = 3$ independent experiments). Thus, HCMV infection increased supernatant TNF levels in CT-like cultures twofold with similar levels of accumulation induced in ST-like cultures, which without infection produced no detectable TNF.

These results suggested that HCMV-stimulated trophoblast apoptosis might be mediated by $\text{TNF-}\alpha$. This suggestion was tested by carrying out the cell loss and apoptosis experiments depicted in Figures 2 and 3 in the presence of excess neutralizing antibody to $\text{TNF-}\alpha$. We first determined that antibody treatment does not inhibit infection: 24 hours after HCMV challenge $15 \pm 0.35\%$ of nuclei were IE-positive in antibody-treated ST-like cul-

tures compared to $16 \pm 0.71\%$ in controls and $32.2 \pm 2.9\%$ in antibody-treated CT-like cultures compared to $31.2 \pm 3.8\%$ in controls. We then asked whether the antibody inhibited infection-induced culture damage: both HCMV-induced cell loss (Figure 5A) and apoptosis (Figure 5B) for both CT- and ST-like cultures were completely inhibited by $\text{TNF-}\alpha$ antibody. These observations argue that HCMV-induced trophoblast loss and apoptosis is mediated by infection-induced release of $\text{TNF-}\alpha$.

Concomitant treatment with the growth factor EGF also completely inhibits $\text{TNF-}\alpha$ -induced apoptosis of placental trophoblasts.^{27,37} As predicted of a $\text{TNF-}\alpha$ -driven trophoblast apoptosis process, EGF completely inhibits CMV-induced trophoblast apoptosis (Figure 6).

HCMV-Induced Trophoblast Cell Loss and Apoptosis Is Mediated by Viral IE1 and IE2 Genes

The kinetics of HCMV-induced trophoblast loss and apoptosis suggest a very early event in viral replication to be responsible. The earliest event, virus coat protein

Figure 5. The effect of neutralizing antibody to TNF- α on HCMV-induced trophoblast cell loss (A) and apoptosis (B). CT-like (open bars) and ST-like (filled bars) cultures were challenged with HCMV strain AD169 at a MOI of 10 and cultured for 24 hours in the presence and absence of 20 μ g/ml of TNF- α antibody as described in Materials and Methods. Nuclei remaining in culture and percent apoptotic nuclei were quantitated as detailed in Materials and Methods and in the legends to Figures 2 and 3. Depicted are the averages $\pm$ SD of pooled results from two independent experiments, each containing two replicate wells. Experimental groups within each panel and cell type labeled with different letters (a or b for CT, α or β for ST) are significantly ($P < 0.05$) different.

interactions with trophoblast plasma membranes, seems unlikely because UV-inactivated virus preparations fail to induce significant cell loss and apoptosis (Figures 2 and 3). We therefore asked whether transcription of viral immediate early (IE) genes might alone induce trophoblast damage.

Parallel cultures of CT- and ST-like cells were individually transfected with mammalian expression plasmids carrying IE1-72, IE2-55, and IE2-86 genes driven by the CMV IE promoter⁴ or the empty vector plasmid or infected with HCMV at an MOI of 10, then cultured for 24 hours. At this time, the frequencies of IE-positive nuclei in IE2-55-, IE1-72-, and IE2-86-transfected CT-like cultures were $24.6 \pm 5.9\%$, $25.2 \pm 4.5\%$, and $22.3 \pm 1.8\%$, respectively, and for ST-like cultures $20.1 \pm 0.6\%$, $22.5 \pm 2.2\%$, and $20.8 \pm 7.4\%$, respectively. These levels are comparable to CT expression levels at day 1 after infection with HCMV (see Figure 1). Cell loss and apoptosis were monitored as described above. The results show that transfection with empty plasmid does not decrease cell number or increase the apoptosis frequency for either culture type but that transfection with IE1-72 and IE2-86 genes resulted in a significant loss of cells (Figure

Figure 6. The effect of EGF on apoptosis induced by HCMV infection and by transfected HCMV IE genes in CT-like and ST-like cultures. CT-like and ST-like cultures were prepared and challenged with HCMV, or transfected with HCMV-IE1-72 or IE2-86 expression plasmids. Apoptosis was assessed by TUNEL analysis 24 hours after challenge as described in Materials and Methods and the legends to Figures 2 and 3 and 7. Depicted are the averages $\pm$ SD of triplicate samples from three independent experiments using cells from two different placentas. Experimental groups within cell type labeled with different letters (a or b for CT, α or β for ST) are significantly ($P < 0.05$) different.

7A) and increase in apoptosis frequencies (Figure 7B) relative to the empty plasmid-transfected control. Even though all three IE genes were expressed at similar frequencies (see above), their effects on cell loss and apoptosis vary in the order IE1-72 > IE2-86 > IE2-55, with the latter showing elevated, but not statistically significant, effects ($n = 3$ independent experiments).

The relationship of IE1-72 and IE2-86 expression and apoptosis was also examined by two-color immunofluorescence (for IE proteins and apoptosis, TUNEL) 24 hours after transfection (Figure 4B, note that Figure 4, A and B, were from different experiments with differing numbers of cell in the culture). Even with IE protein expression (red) and TUNEL expression (green) near 20%, overlapping expression (yellow) was never observed, suggesting paracrine death. As with HCMV-infected trophoblasts, IE gene transfection increased TNF- α supernatant levels (from 1.4 ± 0.06 to an average of 2.9 ± 0.02 pg/ml for CT-like cultures and from below detection to an average of 1.3 ± 0.18 pg/ml for ST-like cultures). Despite these very low supernatant levels of TNF- α after transfection, both CT-like and ST-like cell loss and apoptosis induced by IE genes were inhibited by neutralizing TNF- α antibody (Figure 7, A and B). EGF also completely inhibited IE1-72- (Figure 6) and IE2-86-induced trophoblast apoptosis (data not shown), further supporting the conclusion that CMV-IE gene-induced apoptosis of neighboring cells is mediated by TNF- α .

Discussion

Placental infections by HCMV are accompanied by villous inflammations (villitis), *in utero* transmission of the infection from mother to fetus, and low birth weight babies.^{38,39} Villitis associated with placental infections is characterized by regional loss of the trophoblast lining of

Figure 7. The effect of neutralizing antibody to TNF- α on IE gene-transfected trophoblast-induced cell loss (**top**) and apoptosis (**bottom**). CT- (open bars) and ST-like (filled bars) cultures were infected with HCMV strain AD169 at a MOI of 10 or transfected with empty vector, IE2-55-, IE1-72-, and IE2-86-expressing plasmids and incubated with or without anti-TNF- α antibody as indicated. Nuclei remaining and percent TUNEL-positive nuclei were determined after 24 hours of culture, as described in Materials and Methods. Depicted are the averages $\pm$ SD for two independent experiments with cells from different placentas. Experimental groups within each panel and cell type labeled with different letters (a, b, or c for CT; α , β , or γ for ST) are significantly ($P < 0.05$) different.

the villous placenta. Although villous trophoblast infection is seen *in vivo*^{15,20,22} and primary villous trophoblasts can be infected in culture^{23,24} the crucial relationship between trophoblast infection and infection-related loss of the villous trophoblast barrier has never been investigated. We here show an $\sim 50\%$ loss of cells from cultures of primary villous trophoblasts in the first 24 hours after HCMV challenge. This loss occurs before progeny virus release (which occurs only after a week of culture²⁴), suggesting that cell loss by progeny virus-induced cytolysis is not likely. On the other hand, our findings that cell loss is accompanied by parallel increases in apoptosis, that virtually all lost cells are undergoing apoptosis, and that the anti-apoptotic agents TNF- α antibody and EGF also inhibit infection-induced cell loss strongly argue that infection-induced apoptosis causes the loss. Further analysis of the coincidence of HCMV-IE expression and double-stranded DNA nicking in nuclei of cultured cells revealed that only uninfected cells undergo apoptosis

while attached to the tissue culture dish. Thus, HCMV infection of villous trophoblast populations has two separate effects: it induces paracrine apoptosis of uninfected cells and it prevents autocrine apoptosis of infected cells. These results argue that a local HCMV infection of the placental trophoblast does not directly lead to its death but rather to a massive and rapid loss of neighboring trophoblasts. We suggest that this secondary loss of trophoblasts after infection strongly contributes to HCMV-related placental villitis.

Our finding that HCMV infection did not increase cell loss and apoptosis in HeLa and CaCo cells suggests that paracrine killing by infected cells is not a general phenomenon of all epithelial cells. However, in the absence of cycloheximide CaCo cells, but not HeLa cells, undergo apoptosis induced by the combination of TNF- α and IFN- γ (data not shown). In addition, our line of HeLa was readily infected by HCMV strain AD169 whereas earlier reports indicate the opposite.⁴⁰ Thus, a conclusive answer requires a more comprehensive investigation with various primary epithelial cells.

Our data showing 96 to 98% of nonadherent cells to be TUNEL-positive after HCMV infection with 6 to 17% being HCMV-IE antigen-positive contrasts with the strict segregation of TUNEL-positive and infected cells in the adherent population. We suggest that the massive loss of cells from cultures in the first 24 hours after HCMV challenge may slough areas of the culture containing both apoptotic and infected nonapoptotic cells. Because trophoblasts, like other epithelial cells, may undergo anoikis after disruption of adhesion,⁴¹ nonapoptotic infected cells in the sloughed population may undergo apoptosis secondary to loss of adhesion.

Our data shows that HCMV-induced apoptosis and cell loss occur within 24 hours of infection and is thus an early event in the virus life cycle. The earliest event in herpesvirus host-cell interaction is induction of an IFN-like response induced by interaction of virus coat proteins with the plasma membrane and results in up-regulation of, among other genes, NF- κ B, a nuclear factor known to regulate TNF- α transcription.^{4,42-44} The finding in this study that neutralizing antibody to TNF- α completely inhibits infection-induced apoptosis of uninfected trophoblasts shows TNF- α is required for HCMV-induced paracrine killing. However, UV-inactivated virus does not alone stimulate appreciable TNF- α production, apoptosis, or cell loss suggesting that virus binding and internalization are not sufficient for the magnitude of death observed and that viral gene transcription and translation are required.

HCMV IE gene expression is the most rapid viral transcriptional event, with gene products appearing in primary trophoblasts within 24 hours (see Figure 1). There are several HCMV IE genes, the most common of which map to the ie2/ie2 region of the viral genome and are translated as IE1-72, IE2-86, and IE2-55 proteins. These gene products promote expression of subunits of the host cell nuclear transcription factor NF- κ B, which in turn mediates both TNF- α transcription and resistance to TNF- α -induced apoptosis.^{4,45} Our present results show that expression of IE1-72 and IE2-86 alone mimic the ability of

whole virus to stimulate TNF- α -mediated paracrine killing in the first 24 hours after infection. This report confirms earlier observations in other cell types of a dual role for HCMV IE genes in up-regulation of TNF- α transcription and protection against TNF- α -stimulated apoptosis in infected cells.¹⁰ However, this is the first report of a dual role for HCMV IE function in primary placental trophoblasts and, more generally, to the consequences of damage to uninfected neighboring cells in any tissue. This is also the first study of the expression and function of individual HCMV IE genes in primary trophoblasts and the first to document meaningful (>1%) levels of transfection of any plasmid into these cells.

Placental trophoblasts are resistant to induced cell death, including attack by decidual immune effector cells⁴⁶ and Fas ligand.⁴⁷ However, primary villous trophoblasts readily undergo TNF- α -stimulated apoptosis.^{27,28} Interestingly, the steady state levels of TNF- α in culture supernatants, although increased by HCMV challenge to ~10 pg/ml, are three orders of magnitude lower than levels required for equivalent killing by exogenous TNF- α (10 ng/ml of exogenous TNF- α). Because the effects of HCMV-induced TNF- α can be inhibited by antibody, its interaction with uninfected trophoblasts must be public (available to outside intervention). These observations are similar to findings that TNF- α antibody could inhibit trophoblast apoptosis by adherent monocytes even though supernatant TNF- α levels were low.³⁷ However, this latter study also showed that the cytotoxic effects were local and did not extend further than ~5 mm from sites of monocyte adhesion. Taken together, these studies argue that HCMV infection leads to rather high local accumulations of TNF- α that can induce apoptosis of nearby cells not protected by HCMV IE expression.

TNF- α is a centrally important cytokine in placental development.⁴⁸ It is produced in the villous placenta⁴⁹ and both p55 and p75 receptors are found on the trophoblast *in vivo*⁵⁰ and on cultured villous trophoblasts.⁵¹ The role of TNF- α in placental development is not necessarily limited to apoptosis and may depend on a micro-environment that regulates apoptosis and allows other functions of activated p55 receptors.⁵² Indeed, the cytotoxic effects of TNF- α on primary trophoblasts can be inhibited by concomitant presence of EGF.²⁷ Our observation that EGF inhibits HCMV-induced trophoblast loss and apoptosis further supports the conclusion that HCMV-induced trophoblast damage is mediated by TNF- α and suggests that damaging effects of HCMV on the villous trophoblast may also be subject to microenvironmental regulation.

Villous trophoblasts exist in two morphologically and biochemically identifiable differentiation states: the ST, a mature and extended syncytium that is the functional lining of the villous placenta and CTs, immature progenitor cells to the ST.¹⁷ In this study we examined culture approximations of these states. After plating highly purified villous trophoblasts, adhering for 4 hours, washing, and incubating overnight, the resulting CT-like cultures consist of >90% mononucleated cells that do not express syncytial differentiation markers such as placental lactogen, chorionic gonadotropin,³³ and placental alka-

line phosphatase.³⁰ These same cells when cultured 6 days continuously in the presence of EGF are >80% syncytialized, express hPL⁵³ and hCG (M Garcia-Lloret and L Guilbert, unpublished results), have microvilli⁵⁴ and are termed ST-like. HCMV infects CT-like cultures to a greater degree than it infects ST-like cultures (at 24 hours, 24.5% for CT *versus* 3.2% for ST, Figure 1). However, the fraction of nuclei lost 24 hours after HCMV infection in each cell type was similar (~50%) as were TNF- α supernatant levels (between 10 and 11 pg/ml). The similarity in CT-like and ST-like culture supernatant TNF- α levels and nuclei losses after HCMV infection is in accord with TNF- α mediating HCMV-induced cell loss in the cultures. The higher ratio of supernatant TNF- α to IE-positive nuclei in infected ST- than CT-like cultures indicates that either the former secrete more TNF- α per infected cell than the latter or have a lower capacity for removing the cytokine. Very importantly, the very low levels of TNF- α in supernatants of uninfected ST-like cells suggests that the functional outer ST layer facing maternal blood does not release TNF- α until activated, in this case by virus infection.

The ability of HCMV infection to induce paracrine killing of trophoblasts is a consequence of the rather slow virus replication cycle in these cells. HCMV IE gene products appear in primary trophoblasts within 24 hours of challenge (see Figure 1) but infectious progeny virus is not released until several days after infection.²⁴ Thus, TNF- α secretion induced by IE gene expression precedes by days neighboring cell infection that could inhibit TNF- α -induced apoptosis. Thus, a localized infection of the ST (the first step in a hematogenous infection of the placenta) could result in enhanced apoptosis of near-by unprotected (uninfected) ST and underlying CT. This would be predicted to both increase ST aging into syncytial knots and compromise the renewal capacity of the local trophoblast, either through CT apoptosis (this study) or infection-modified inhibition of CT-ST fusion (under investigation). These outcomes are in accord with placental pathologies associated with IUGR: a rat model is characterized by excessive TNF- α expression⁵⁵ and human IUGR placentas are characterized by excessive villous trophoblast apoptosis²⁶ and enhanced syncytial knot formation.⁵⁶

The present studies also have implications for mechanisms of hematogenous transmission of HCMV from mother to fetus across the placenta. An intuitive model of HCMV passage would have an infected villous trophoblast releasing progeny virus basally into fetal tissue. However, observations that productively infected villous trophoblasts release less than 5% of progeny virus in conventional cultures²⁴ do not support such an infection and release model. Further, the little progeny virus released from polarized ST-like membrane cultures is >99% apical (toward maternal, not fetal, circulation,⁵⁷). Thus, an infection and basal release mechanism of placental passage of HCMV is not likely. However, the above-mentioned hypothesis that ST infection ultimately leads to regional loss of the trophoblast barrier presents a condition that would allow access of infected maternal leukocytes into the villous stroma. That *in utero* transmis-

sion of HCMV might be secondary to virus-induced trophoblast damage is in accord with correlations between vertical transmission of HCMV and placental villitis⁵⁸ and between placental infections by HCMV and IUGR.³⁹

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