Human Cytomegalovirus Infection Causes Premature and Abnormal Differentiation of Human Neural Progenitor Cells

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Running title: HCMV Infection Disrupts NPC Differentiation

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Congenital human cytomegalovirus (HCMV) infection is a leading cause of birth defects, largely manifested as central nervous system (CNS) disorders. The principal site of manifestations in the mouse model is the fetal brain's neural progenitor cell (NPC)-rich subventricular zone. Our previous human NPC studies found these cells fully permissive for HCMV and a useful in vitro model system. In continuing work we observed that, under culture conditions favoring maintenance of multipotency, infection caused NPCs to quickly and abnormally differentiate. This phenotypic change required active viral transcription. Whole genome expression analysis found rapid downregulation of genes that maintain multipotency and establish NPCs’ neural identity. q-PCR, Western blotting and immunofluorescence assays confirmed that the mRNA and protein levels of four hallmark NPC proteins (nestin, doublecortin, sex determining homeobox 2 and glial fibrillary acidic protein) were decreased by HCMV infection. The decreases required active viral replication and were due, at least in part, to proteosomal degradation. Our results suggest that HCMV infection causes in utero CNS defects by inducing both premature and abnormal differentiation of NPCs.

INTRODUCTION

Congenital HCMV infection is a leading cause of birth defects, primarily affecting the central nervous system (CNS). Primary infection during pregnancy poses a 30-40% risk of intrauterine transmission, with severe adverse outcomes more likely if the infection is within the first half of gestation (46). Each year approximately 1% of all newborns are congenitally infected with HCMV. Approximately 5-10% of these infants manifest signs of serious neurological defects at birth including deafness, mental retardation, blindness, microencephaly, hydrocephalus and cerebral calcification (2, 4, 65). In addition, 10-15% of congenitally infected infants asymptomatic at birth subsequently develop brain disorders such as sensorineural hearing loss (12, 47, 52). Moreover, accumulating evidence suggests that more subtle changes in human brain development, such as autism and language development may
be related to congenital HCMV infection (68, 76, 77).

Although HCMV can infect a wide range of tissues in vivo (61), the fetal brain is the principal site of the deleterious manifestations of infection. It has been suggested that the severity of the neuropathological changes and clinical outcomes may be associated with the stage of CNS development at which congenital infection occurs, with early gestation infections producing more severe outcomes (3, 46). However, the mechanism of HCMV pathogenesis in the developing CNS remains poorly understood. Studies of HCMV in human subjects have obvious limitations; therefore model systems of both in vitro and in vivo HCMV infection have been devised to provide insights into infection of the developing brain.

Congenital infection studies have been performed principally in the mouse model. Studies in mice revealed that very early embryos were non-permissive to mouse cytomegalovirus (MCMV) infection, as judged by the absence of viral gene expression following blastocyst (25) or zygote injection (71). Mouse embryonic stem (ES) cells were also non-permissive to MCMV infection, however, cells differentiated from these ES cells were susceptible and permissive (37). Mouse multipotent CNS stem cells (neural stem/progenitor cells or NPCs), isolated from the ventricular/peri-ventricular zones of both late stage embryonic mice and from adult mouse brains were permissive for infection. It was reported that MCMV infection inhibited mouse NPC proliferation and differentiation. Neuronal differentiation appeared to be more severely inhibited than glial differentiation (28).

Radial glial cells were the main targets of MCMV during infection in the neonatal (P1-3) mouse (49, 73). These glial cells are thought to be the earliest neural stem cells and play an important role in guiding neuron migration (30). Immunostained brain slice cultures indicated that virus susceptible cells were located in the subventricular zone and cortical marginal regions (areas positive for NPCs) (10, 27). Shinmura et al. (59) found that injection of MCMV into the cerebral ventricles of mouse embryos caused a profound disturbance of neuronal migration and a marked loss of neurons. They proposed that this disturbance might be a cause of microencephaly due to CMV.
infection. These mouse studies found that progenitor cells, as well as glia and neurons, were permissive to CMV infection.

Recent advancements in human NPC isolation and culture (56) allow for the characterization of HCMV infection in this medically relevant human system. Previous studies from our group and others have shown that human NPCs are fully permissive for HCMV infection (11, 35, 38, 43, 44). Our studies found that the timing of viral gene expression and the titers of infectious virions produced in human NPCs were similar, although somewhat protracted, to those of permissive fibroblasts. These studies also showed that astroglia and neurons derived from cultured human NPCs were permissive for infection (35).

In the current study, genome wide expression analysis found downregulation of mRNA levels of several genes important for maintaining NPC multipotency and establishing their neural identity. q-PCR, Western blot and immunofluorescence (IF) analyses performed at various times post infection (pi) on four NPC marker proteins nestin, (NES), doublecortin (DCX), sex-determining homeobox 2 (SOX2) and glial fibrillary acidic protein (GFAP), confirmed array results. Inhibition of HCMV replication delayed protein level declines. Additionally, proteasomal inhibition delayed and decreased NPC marker protein declines. These results suggested that HCMV infection disrupted NPCs' multipotency, and in the absence of exogenous differentiation signals, induced their differentiation toward a non-neuronal lineage, implying a mechanism for the CNS manifestations of HCMV pathogenesis.

MATERIALS AND METHODS

Cell culture The human NPC line, SC27, was obtained postmortem from the brain of a premature neonate (23-24 weeks estimated gestational age). The neonate died of natural causes unrelated to HCMV infection [unpublished results and (56)]. NPCs were cultured as described previously (35). Briefly, NPCs were cultured in DMEM-F12 containing L-glutamine (2mM Glutamax; Gibco/BRL),
penicillin/streptomycin (100U/mL and 100µg/mL), gentamicin (50µg/mL), amphotericin B (Fungizone; Gibco/BRL, 2µg/mL), 10% BIT9500 (5mg/mL bovine serum albumin, 5µg/mL recombinant human insulin, 100 µg/mL human transferrin; Stem Cell Technologies), human basic fibroblast growth factor (bFGF; Prospec, 20ng/mL) and human epithelial growth factor (EGF; Prospec, 20ng/mL). NPCs were cultured as neurospheres by seeding cells into uncoated tissue culture dishes. Under these conditions, NPCs agglomerate into balls of free-floating cells. NPCs were cultured as adherent monolayers by seeding them onto fibronectin-coated dishes. For cell culturing, medium was refreshed by half every other day (d). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

**Virus infection of adherent NPCs** HCMV Towne strain (ATCC#VR977) was used for all infections, and propagated and titrated on a monolayer of human foreskin fibroblasts as described previously (70). Prior to virus infection, NPC monolayer cells were seeded onto poly-D-lysine-coated dishes or uncoated dishes containing poly-D-lysine-coated coverslips. Cells were seeded and allowed to attach overnight, infected at a multiplicity of infection (MOI) of 3 and incubated with virus for 2 h to allow for adsorption. The inoculum was then removed and cells were refed (35). Medium was refreshed by half every other day as necessary. Cells and coverslips were harvested at the indicated times pi. UV-inactivated HCMV was prepared and used as described previously (15).

**NPC neurosphere infection** NPC neurospheres were cultured as described previously (35, 51). To estimate the cell number before infection, an average size neurosphere was dissociated by trypsinization and cells were counted on a hemocytometer. NPC neurospheres were infected at an MOI of ~ 3 with virus or UV- inactivated virus, as described above. Cell and neurosphere morphology changes were monitored at the indicated time points pi. Experiments were performed at least twice.
Drug treatments 9-[(1, 3-dihydroxy-2-propoxy) methyl] guanine (ganciclovir, GCV, 450µM) was used to inhibit virus replication. Mock infection (+ vehicle), mock infection plus GCV, virus infection (+ vehicle), and virus infection plus GCV were included in all experiments. GCV was added at the time of infection and every 24h thereafter; medium was changed by half every other day. Cells and coverslips were harvested at the indicated times pi.

MG132, a proteasome inhibitor, was used to inhibit proteasome activity (78). MG132 was added at a final concentration of 12.5 µM at 24 h pi and incubated for 24 h prior to harvesting at 48 h pi (78); analogously, 12.5 µM MG132 was added at 48 h pi and incubated for 48 h prior to harvesting at 96 h pi.

Live cell morphology changes Cell morphology changes were monitored on an inverted Nikon TMS-F microscope (using a 20X objective). Mock, virus and UV-inactivated virus infections were monitored in parallel. A Nikon COOLPIX 5400 was used to obtain the images. Experiments were performed at least twice and representative images are shown. The magnification for all live cell images presented is x500.

Western blotting Cells were harvested at the indicated times pi by rinsing with phosphate-buffered saline (PBS), trypsinizing, and washing again with ice-cold PBS. Cells were resuspended in ice-cold PBS, counted, and pelleted. Cell pellets were snap frozen in liquid nitrogen and stored at -80°C until the time course was completed. Cell lysates were prepared as described previously (34). Lysates were derived from 1x 10^5 cells for viral proteins and 1.6 x10^5 cells for cellular proteins, with the exception of SOX2, for which 2.4x10^5 cell equivalents were used per lane. Lysates were electrophoresed using SDS-PAGE and transferred to a PROTRAN membrane (Schleicher & Schuell BioScience). All Western blotting experiments were performed twice, with representative images shown.
Immunoprecipitation (IP) for ubiquitinated/sumoylated DCX Modifications of
DCX were examined using IP of cell lysates from mock-infected or virus-infected
NPCs with or without 12.5 μM MG132 treatment. Briefly, 2.3 X 10^6 NPCs were lysed
in 100 μl of denaturing buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1% SDS,
12.5 μM MG132 and 1mM DTT), followed by direct boiling for 10 min to avoid
coop-immunoprecipitation of other modified proteins. The lysates were then diluted
10-fold in 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, 12.5 μM MG132,
100 mM NaF, 0.2 mM sodium orthovanadate, 10 μg/mL leupeptin, and 10 μg/mL
aprotinin, followed by incubation with protein A/G Magbeads (Genscript) for 1h at
4°C on a rotary mixer to preclear the lysates. The precleared lysates were incubated
with anti-DCX Ab (2μg) and allowed to rock gently at 4°C overnight. The next day,
immunocomplexes were recovered by incubation with protein A/G Magbeads for 3h
at 4°C followed by four extensive washes with 1 mL of 250 mM NaCl, 0.1% NP-40,
12.5 μM MG132, 50 mM HEPES, pH 7.0, 5 mM EDTA, and 500 mM dithiothreitol.
After each wash, the tubes were placed on a Dynal Magnetic Particle concentrator.
The proteins were eluted by heating at 95°C for 30 min in 2X Laemmli sample buffer.
Eluted proteins were used for immunoblotting with either anti-ubiquitin (Rabbit
antibody (Ab); Biomol) or anti-Sumo-1 or anti-Sumo-2/3 Abs (both Rabbit; Cell
Signaling).

Dephosphorylation of proteins with shrimp alkaline phosphatase (SAP)
Phosphorylation of DCX was examined using cell lysates from mock-infected or
virus-infected NPCs with or without MG132 treatment. Samples were run in duplicate
on SDS-PAGE and transferred to protran. Prior to blocking, half the membrane with
duplicate samples was cut and separately treated in the presence of 150 units SAP
(Fermentas) while the other half of the membrane was left untreated. The treated
membrane was sealed in a plastic bag to maximize the exposure to SAP and incubated
at 37°C overnight. The next day, both the SAP-treated and untreated membranes were
probed for the presence of the ~ 82 kD DCX band using an anti-phospho-DCX
Immunofluorescent Analysis (IF) The IF protocol used in this study was as previously described (35). Coverslips were mounted in glycerol containing paraphenylene diamine after staining to inhibit photobleaching. Nuclei were counterstained with Hoechst dye. The images were obtained using a Nikon Eclipse E800 fluorescence microscope equipped with a Nikon DXM camera and Metavue software.

Antibodies (Abs) Viral antigens (Ags) were detected after Western blotting with anti-IE1/2 (Ch16.0, IgG1), anti-UL44 (IgG1), and anti-pp65 (IgG1) mAbs (Rumbaugh-Goodwin Institute for Cancer Research, Inc.), and anti-gB (IgG2b) mAb (a kind gift from Dr. Bill Britt). Viral proteins were detected by IF using anti-IE1 (IgG2A; a kind gift from Dr. Britt) or UL44. Cellular proteins were detected by Western blotting with anti-NES (IgG1; Chemicon), anti-DCX and anti-SOX2 (goat polyclonal IgG; Santa Cruz Biotechnology), anti-GFAP (IgG2b; Fitzgerald Industries International, Inc), and anti-actin (clone ACT05; Neomarkers). Secondary Abs used in Western blotting were horseradish peroxidase conjugated sheep anti-mouse or sheep anti-goat Abs (Amersham Bioscience). IF experiments used TRITC-conjugated anti-mouse IgG1 (Southern Biotech) and AlexaFluor 488-conjugated goat anti-mouse IgG2a Abs (Invitrogen).

q-PCR NPCs were cultured, infected (as described above), and harvested at the indicated time points pi. 5x10^5 cells/sample were used for total RNA extraction. Total RNA was extracted from cells by using SV Total RNA Isolation kit (Promega). RNA was reverse transcribed with Superscript II (Invitrogen) or ImProm-II Reverse Transcriptase (Promega). q-PCR was performed on an ABI Prism 7900 (Applied
Biosystems), using Power SYBR Green PCR Master Mix (Applied Biosystems) in 15µl reactions for 40 PCR cycles. Calculations were based on absolute starting quantities, using reactions specific for glucose-6-phosphate dehydrogenase (G6PD) as normalization controls (60). Each experiment consisted of three technical replicates, and their averaged values were used for downstream calculations. Experiments were performed on two separate occasions and their averages are shown in the bar charts. Bars represent the range.

**Molecular cloning** All plasmids used in q-PCR experiments were generated with the pCR8 TOPO cloning kit (Invitrogen). Taq polymerase was used to amplify the insert fragments used as standards in RT-PCR experiments from the cDNA of HCMV infected NPCs. Oligonucleotide sequences are available upon request.

**Whole genome expression analysis** For gene expression analysis, adherent monolayer cells were either mock- or virus-infected. Mock-infected cells were harvested at 12 h pi, virus-infected cells were harvested at 4, 12, and 24 h pi. Virus and mock infections were performed in duplicate, with each individual sample harvested separately, thereby acting as biological replicates for analysis. RNA was extracted using the SV Total RNA Isolation kit (Promega). First and second strand cDNA was synthesized using SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) and poly (T)-nucleotide primers containing a sequence recognized by T7 RNA polymerase. The resulting cDNA was used as a template to generate biotin-tagged cRNA from an *in vitro* transcription reaction, using the BioArray High-Yield RNA Transcript Labeling Kit (T7) (Enzo Diagnostics). 15µg of the resulting biotin-tagged cRNA was fragmented to strands of 35-200 bases following prescribed protocols (Affymetrix). Subsequently, 10 µg of this fragmented target cRNA was hybridized (at 45°C, rotating for 16 hours in an Affymetrix GeneChip Hybridization Oven 640) to probe sets present on an Affymetrix HG-U133 2.0 PLUS
array. The GeneChip arrays were washed and then stained (SAPE, streptavidin-phycoerythrin) on an Affymetrix Fluidics Station 450, followed by scanning on an Affymetrix GeneChip 3000 Scanner 7G.

### Whole genome expression data analysis:

Raw fluorescence data was normalized using MAS5 software. All features classified as absent in all samples by the MAS5 algorithm were omitted from further analysis. A second present/absent call was calculated by MAS5 for each feature based on their individual p-values. The present/absent calls for the biological replicates were combined, requiring both replicates to be present for the feature to be considered present. The signal strengths (SSs) of the 2 biological replicates were averaged. A threshold signal strength (TSS) was determined for the mock, 4h pi, 12 h pi and 24h pi samples. The TSS was set at the absolute SS value at which 5% of the absent features exceeded this number (mock=220, 4h=222, 12h=234 and 24h=236).

Gene regulation was classified as ON, OFF or Fold-Regulated (see Supplemental Table I). Genes classified as turned ON by HCMV were absent and below the mock-TSS (m-TSS) in the mock sample and present and above the timepoint-TSS (tp-TSS) in the virus infected sample. Genes classified as turned OFF by HCMV were present and above the m-TSS in the mock sample and absent and below the tp-TSS in the virus infected sample.

Fold-Regulated genes were those present in both the mock and viral samples, and for which both features were above their respective TSSs. All fold regulation comparisons were mock/virus samples. To further narrow the Fold-Regulated group the distribution of the features meeting the above parameters was examined and a fold regulation significance factor was determined for each timepoint. The significance factor was set to the value along the distribution curve where the curve flattened to a gradual asymptotic slope (see Fig. 2A). The significance factors used were 4h=3-fold, 12h=6-fold and 24h=8-fold. Additionally, genes were also considered Fold-Regulated if they were present in both mock and viral samples, but below their respective TSSs.
if the mock/virus sample comparison exceeded the significance factor when their 
appropriate TSS value was substituted. The Fold-Regulated value reported in the table 
for these genes was either mock/tp-TSS or m-TSS/virus.

Any gene represented by more than one feature after the above culling, was 
included in Supplementary Table I with the highest regulated feature (since oligos of 
features with lower regulation might be located in untranscribed regions or in 
alternatively spliced exons). A gene represented by one (or more) feature(s) turned 
ON or OFF, but which was also Fold-Regulated was included in the Fold-Regulated 
category. Classification of gene product function used the latest annotation 
information from the Affymetrix website.

Data Deposition Original MAS5 data for the whole genome expression analysis has 
been deposited in the GEO database, accession # GSE19345.

RESULTS

HCMV infection alters NPC attachment and migration behaviors. Standard 
culturing protocols call for NPCs to be grown either as free-floating neurospheres 
over uncoated surfaces (Fig. 1A), or as monolayers on fibronectin-coated surfaces 
(not shown). When transferred to fibronectin-coated plates, neurospheres attached to 
the coated surface and subsequently cells spread onto the substrate (Fig. 1B) (14, 51, 
56). In our previous studies, we observed that when NPC neurospheres were glially 
differentiated by replacement of mitogens (EGF and bFGF) with fetal bovine serum, 
they attached tightly to uncoated surfaces and cells began to spread across the surface 
within 2 h ((35) and unpublished). We also observed that NPCs cultured as 
monolayers reverted to neurospheres when transferred to uncoated surfaces in the 
absence of a differentiating stimulus. This reversion to neurospheres illustrates the 
propensity of these cells to resume their normal behavior patterns in the absence of 
outside influences.

During our current studies we observed an effect corollary to the attachment
behavior noted above, but much more intriguing. In parallel with other ongoing NPC monolayer infections, NPC neurospheres in uncoated dishes were infected with HCMV. These neurospheres were grown under conditions designed to maintain multipotency, however, following HCMV infection they spontaneously attached to the uncoated plates, suggesting that differentiation had been initiated. Between 4 and 12 h pi, individual cells began migrating from the spheres (Fig. 1C). Cells withdrew back into spheres at 18 h pi and exhibited the normal cell rounding phenotype of permissively infected cells (8). By 96 h pi these infected NPCs began to spread again. During the second spread, the NPCs’ morphology resembled that of HCMV infected fibroblasts at late (L) times pi (8). All infected NPC neurospheres were disassociated by 120h pi, consistent with the observation of others (72). UV-inactivated virus infections of neurospheres did not lead to attachment or migration (Fig. 1D). These experiments found that HCMV infection altered NPC neurosphere behavior in culture and that de novo viral protein expression was necessary to induce the altered attachment characteristics. Further, the HCMV-infected NPCs’ attachment behavior was similar to that of NPCs under glial differentiation conditions (35)(and unpublished), which suggested a change in gene expression profiles.

HCMV-induced alterations of NPC neurosphere attachment and migration were associated with a loss of multipotency. The similarities in attachment characteristics between the glial differentiated NPCs and the infected neurospheres prompted us to perform whole genome expression analysis of infected NPCs, paying particular attention to self-renewal and differentiation characteristics. A minor obstacle presented itself in our investigation of the HCMV infected NPC neurosphere attachment behavior. The multilayer structure of the free-floating neurospheres renders a simultaneous infection of all the component cells impossible. Unfortunately, asynchronous infections confound all timing-dependent results. We reasoned that the altered attachment behavior had been initiated solely by the accessible cells, since no other environmental changes were made. Therefore, we used monolayer cultures for
all further experiments. NPCs were infected at an MOI of 3 and harvested at 4, 12 and
24 h pi for mRNA analysis. These data sets were compared to mock-infected NPCs
harvested at 12 h pi. Although not as prevalent as at later timepoints, significant
changes in mRNA levels began to occur as early as 4 h pi, as outlined below and
depicted in Figure 2, Table I and Supplementary Table I.

In NPCs we found HCMV infection caused changes in the regulation of 721
genes; 477 were downregulated and 244 were upregulated at 4, 12 or 24 h pi (see
Supplemental Table I). The functions of 492 of these genes are currently known and,
of these, 322 were downregulated and 170 upregulated (percentages noted below
represent fractions of only genes of known function). Functional grouping revealed
genes whose products are involved in cell cycle control, cell signaling and metabolism.
Ion channels, secreted proteins, cell surface receptors, transcription factors and
structural proteins were also represented in the array analysis. Representative genes
from each of these classes are given in Table I and their distribution among different
functional groups is shown in Figure 2B. It is known that cellular transit through the
G1/S checkpoint is essential for the optimal replication of HCMV (as reviewed in
(54)), therefore it was not surprising that many cell cycle control proteins were
upregulated (or turned on) by infection of NPCs. These included the genes cyclin E,
E2F1 and 2 and cdt1, which were also influenced by infection in permissive
fibroblasts (62). The cell cycle genes downregulated by infection in NPCs included
the cell signaling protein NEDD9, which is involved in neural crest cell migration (1).

The gene expression analysis revealed a number of upregulated cell surface
receptors and signaling genes involved in immune functions or stress responses
including CD55, which accelerates the decay of complement proteins and prevents
immune system damage to the host cells (41). A more significant number of genes
were downregulated. A noticeable number of glycosyl transferases and other adhesion
molecule modifying enzymes localizing to the Golgi apparatus (ST6GALNAC 4 and
5, ST8SIA3 and ST8SIA4, GCNT1) were downregulated. In addition, seven different
collagens (including COL1A1 and A2 and COL6A1) and five adhesion genes
PCDH10, NRP1 and 2, NFASC and NLGN4X) had significantly reduced mRNA levels in virus-infected cells. These changes to the cells’ secretion patterns, many of which began as early as 4 h pi, may have contributed to the altered adhesion characteristics of HCMV-infected NPC neurospheres seen at this early timepoint.

Interestingly, ~17% of all known genes were neuron-related. We found neuron-related genes among every class of genes present. These were particularly prevalent in the receptor category, with 52% (22/42) of these genes being neuron-related. Remarkably, 86% of these neuron-related receptor genes (19/22) were downregulated and included the glutamate receptors GRIA1, 2 and 3 and the neural adhesion molecules NRP1 and 2 and NRXN3. Additional neuron-related genes that were downregulated included peripheral myelin protein 2 (PMP2), and neuron-specific ion channel subunits CACNB2, CACNG4 and CACNG5, as well as the transcription factors ATXN3, RUNX4 and SOX4, a pan-neuronal gene activator that acts during neurogenesis and neuronal maturation (5). These results suggest that HCMV-infected NPCs have a diminished capacity to differentiate in a neuronal direction.

The most striking result from the gene expression analysis was the regulation of genes involved in the maintenance of the self-renewing, multipotent state of the NPC. There were large changes for several important genes including: 1) the transcription factor ID4, whose downregulation is associated with “precocious” differentiation (36); 2) the structural gene GFAP and the cell signaling related gene DCX, both of which are present in most human NPC cultures (20, 40, 45, 56, 64, 69); 3) SOX2, a transcription factor associated with the maintenance of stem cell identity and which was recently shown to be essential for reprogramming of terminally differentiated fibroblasts to induced pluripotency (45, 64, 69); 4) the microRNA MIR21, whose loss is associated with the subsequent loss of SOX2 (29); 5) and FABP7, a fatty acid transporter which, with the intermediate filament protein nestin (NES), is associated with neural stem cells and the developing brain (24). The large downregulation in numerous critical progenitor cell markers indicated the infected NPCs’ loss of...
multipotency via differentiation. Given the large number of neuronal genes
downregulated, this differentiation appeared to exclude a neuronal lineage.

Multipotency gene expression was downregulated at the mRNA level and
decreased at the protein level by HCMV infection. Because gene expression
analysis had shown that the important NPC genes DCX, SOX2 and GFAP were
downregulated, their expression changes at the mRNA and protein level were further
investigated throughout the timecourse of infection. Additionally, nestin (NES), a
protein specific for NPCs under our culture conditions (56), was included in the
experiments (even though NES regulation did not meet the gene expression criteria, it
was downregulated approximately three fold). q-PCR analysis of these four genes
showed all four genes’ expression levels to be decreased by at least 10-fold at 24 h pi,
with DCX and GFAP levels decreased by more than 100-fold (Fig. 3A). mRNA
expression levels remained low throughout the duration of the experiment for all four
genes. The protein levels of NES, DCX, SOX2 and GFAP were not decreased at early
times pi (Fig. 3B), however, beginning at 48 h pi, steady state protein levels began to
decline in virus-infected cells. Each of the proteins displayed different profiles (Fig.
3C). The most rapid decrease in steady-state protein levels observed was for the NPC
transcription factor SOX2, which was noticeably decreased at 48 h pi and
undetectable from 72 h pi on. GFAP was reduced at 48 h pi and became undetectable
by 120 h pi. Interestingly, the faster migrating form of the protein disappeared more
rapidly. DCX levels declined at 72 h pi and were undetectable from 96 h pi on. NES
levels were markedly decreased at 96 and 120 h pi and undetectable at 144 h pi. These
experiments found that HCMV infection significantly inhibited NPC marker mRNA
synthesis and decreased the steady state levels of these marker proteins, thereby
compromising the multipotency and limiting the differentiation capacity of these
developmentally critical cells.

Downregulation of NPC multipotency genes was delayed when virus replication
was inhibited. To narrow the list of viral factor(s) responsible for the downregulation of NPC markers during HCMV infection, ganciclovir (GCV) was used to inhibit virus replication. GCV selectively and competitively inhibits the viral DNA polymerase, terminating viral replication and preventing Late (L) protein synthesis (16). The following viral proteins were used as markers of the temporal progression of infection to monitor GCV’s effects: IE72 (IE1) and IE86 (IE2), the most prominent proteins of the immediate early (IE) stage; UL44, a representative of early (E) proteins and of the development of replication centers; and two structural proteins, the tegument protein pp65 and the glycoprotein gB, were employed to mark the transition to the L stage of HCMV infection. The stages and development of viral replication centers in NPCs were described previously (35). Briefly, at 48 and 72 h pi, a mixture of multiple-small foci (indicating pre-replication sites), bi-polar foci and single large foci (both indicative of advanced replication) were observed. The majority of cells developed single large replication foci by 96 h pi.

In NPCs, GCV inhibited the establishment and development of viral replication centers similar to its effect on virus-infected fibroblasts (Fig. 4A and B) (48). All cells were positive for IE1 in both untreated cells and GCV treated cells by 48 h pi (Fig. 4A). Indirect immunofluorescence (IF) determined that untreated cells developed replication foci by 48 h pi. In a small percentage of GCV-treated cells multiple small foci were seen by 96 h pi, but in the large majority of these treated cells distinct UL44 foci did not form through 96 h pi (Fig. 4A). In Figure 4A, the brightness levels of UL44 were increased in the far right panels to show that even in overexposed conditions, no foci were observed in the GCV-treated cells. Steady state protein level expression of IE1/IE2, UL44, pp65 and gB expression were determined by Western blotting (Fig. 4B). GCV significantly inhibited the expression of IE2 and UL44, pp65 and gB were completely blocked and were undetectable in GCV-treated cells during the 144 h timecourse. IF and Western blotting analysis determined that GCV blocked viral protein expression between the IE and E stages in NPCs and, judging from the absence of replication centers, inhibited viral replication as expected.
The protein levels of the NPC marker proteins NES, DCX, SOX2 and GFAP were
determined in GCV-treated and untreated cells at various times pi (Fig. 4C). As was
observed, in untreated infected cells (Fig. 3), the steady state levels of all four proteins
declined by 96 h pi when compared to uninfected controls. However, in GCV-treated
infected cells, this decline was much less dramatic. In GCV-treated infected cells,
three of the four marker proteins were detectable until 144 h pi, past the times when
any of these proteins were detectable in untreated infected cells. We found that active
viral replication in HCMV-infected NPCs contributed significantly to the decrease of
NPC marker protein steady state levels.

q-PCR was used to investigate the changes in NPC marker mRNA levels after
GCV treatment (Fig. 5). The gene expression data in Figure 3 had revealed mRNA
level changes (virus/mock) occurred by 24 h pi, therefore samples from virus-infected
(v), virus-infected and GCV-treated (GCV), and UV-inactivated virus-infected (UV)
cells were harvested at 12, 24, and 48 h pi and compared to mock-infected (m) cells.
The last time point was included to account for any delay caused by GCV treatment.
Although nearly all of the virus-infected cells (+ or -GCV) stained positive for IE1 by
12 h pi, at this timepoint no significant changes in the NPC markers could be
measured in any comparison (v/m, GCV/m, and UV/m). However, at 24 and 48 h pi,
all four NPC markers in the v/m comparison were downregulated to levels similar to
those in Figure 3 (Fig. 5, darkest shade of each color). GCV-treatment of
HCMV-infected cells delayed and decreased the decline seen in mRNA levels of all
four genes analyzed (Fig. 5, middle shade of each color). For example, GFAP was
downregulated 34-fold at 24 h pi and 180-fold at 48 h pi in virus-infected cells, but
less than 3-fold at 24 h pi and approximately 10-fold at 48 h pi in GCV-treated
infected cells. Similar results were obtained for SOX2, DCX and NES. Overall the
expression levels of the measured genes were approximately 10-fold lower at 24 and
48 h pi in virus-infected cells versus GCV-treated infected cells. No differences were
observed in mRNA (Fig. 5, lightest shade of each color) and protein (data not shown)
levels for the analyzed genes between cells infected with UV-inactivated virus

compared to mock-infected cells at any time point. These data indicated that de novo viral transcription and protein synthesis coupled with active replication were required to induce the complete observed reduction in NPC marker proteins.

Proteasomal inhibition slowed the decline in steady state levels of the NPC marker proteins. Since modification to the ubiquitin-proteasomal machinery has been noted in HCMV-infected cells (reviewed in (54)), mock- and virus-infected cells were treated with MG132 to determine if proteasomal degradation was involved in the decline of steady state levels of the NPC markers. SOX2 was reduced to near the detection limit at 48 h pi and NES, DCX, and GFAP were reduced by 96 h pi (Fig. 3), therefore MG132-treated cells and controls were harvested at these time points. The literature varies on the concentrations of MG132 used to fully inhibit proteasomal activity, from a low of 0.1 µM to a high of 50 µM (17, 26). We chose an intermediate level of 12.5 µM MG132 for our studies. Kaspari and colleagues showed that in high MOI infection, MG132, at concentrations above 0.5 µM, did not inhibit IE1/IE2 expression, but did inhibit viral replication and concatamer cleavage (26). Importantly, these cells were continuously treated with MG132 for 72 h. To limit the inhibitory effects of MG132 on HCMV infection, while achieving MG132 proteasomal inhibition, treatment was begun at 24 h pi (and harvested at 48 h pi) or at 48 h pi (and harvested at 96 h pi). Under these conditions, IE1 and IE2 expression was not inhibited by MG132 at 48 h pi or 96 h pi (Fig. 6A). However, there was at least partial inhibition of early protein synthesis (as measured by UL44) and complete inhibition of late protein synthesis (as measured by gB). We concluded that, as seen previously, our incubation conditions for MG132 also partially inhibited viral replication.

NES protein levels were unaffected at 48 h pi under any conditions. However, at 96 h pi, the HCMV-induced decrease in NES protein levels was inhibited by the addition of MG132 (Fig. 6B). At 48 and 96 h pi, DCX protein levels were slightly higher under MG132 treatment in both mock and viral samples. In addition, at both 48 and 96 h pi, all MG132 treated mock- and virus-infected cells showed a slower
migrating protein band of ~ 82kD. GFAP protein levels were unchanged at 48 h pi, but were slightly elevated in both mock and viral MG132-treated cells at 96 h pi. At both 48 h pi and 96 h pi, SOX2 levels were unchanged after MG132 treatment in mock-infected cells, indicating the proteasome was not generally involved in SOX2 degradation under normal cellular conditions. However, from undetectable levels in untreated infected cells, SOX2 protein levels increased significantly in MG132-treated infected cells, indicating that proteasomal degradation was involved in the decline observed during infection.

The more slowly migrating form of DCX, which appeared only after proteasomal inhibition, was investigated to discern whether this was a sumo- or ubiquitin-modified form of the protein. The DCX Western blots were repeated using shorter and longer exposures to discern differences. Also, MG132 was added to the sample buffer during lysate preparation to inhibit any latent proteolytic activity. The shorter exposure in Figure 6C shows a large increase in steady state levels of DCX in both mock and viral samples treated with the drug. Longer exposure with MG132 in the lysate revealed a laddered effect above the main DCX band, indicating modification of the protein. This effect was more prominent in the viral samples. DCX was immunoprecipitated (IP) from the lysates, and run on a Western blot (Fig. 6D). These blots were probed with anti-ubiquitin or -sumo Abs. Only very minor modifications occurred in the mock MG132-treated samples, whereas the virus-infected cells had significant modification of both ubiquitin and sumo moieties on the DCX protein. Combined with the observed effects of MG132 treatment (Fig. 6B), these IP results indicate that the decreased steady-state levels of the NPC marker proteins (particularly in DCX, NES and SOX2) were due, not only to transcriptional downregulation of their respective genes, but also, at least in part, to proteasomal degradation.

DISCUSSION

Normal brain structure and function are developmentally regulated by four different, but related factors (7): 1) the proliferation of self-renewing NPCs, 2) the
differentiation of NPCs to mature neurons and glia, 3) the proper migration of these neurons and glia (33), and 4) the formation of proper synaptic connections. In utero HCMV infection can cause significant damage to the developing fetal brain. Our current studies find that HCMV infection in vitro can perturb at least the first two factors regulating fetal brain development.

In previous studies we observed that, after changing the composition of the culture medium to induce differentiation to glia, NPCs attached to uncoated surfaces (35). During the current study infected neurospheres attached to uncoated surfaces. Subsequently, individual cells migrated away from the spheres. This occurred without alterations to the composition of the culture medium, suggesting that HCMV infection induced premature differentiation. UV-irradiated virus infections showed that de novo viral protein expression was necessary to induce this cellular behavioral change. These results prompted our investigation of gene expression profiles to gain insight into possible causes of this differentiation-like behavior.

The most remarkable changes in gene expression we observed were the downregulation of the mRNAs for several NPC marker proteins including SOX4, FABP7, ID4, MIR21, DCX, NES, SOX2 and GFAP. These proteins all play critical roles in neural differentiation and/or maintenance of the multipotent state of the NPC. q-PCR and Western blotting of DCX, NES, SOX2 and GFAP confirmed the downregulation of mRNA and the decreases in protein levels of these four NPC markers, respectively.

The four marker proteins we have investigated in depth are well characterized. DCX plays an important role in neuron migration signaling during brain development and it is a marker of early migratory neuroblasts. DCX haploinsufficiency can lead to varying degrees of mental retardation. The extent of retardation is linked to the quantity of arrested neurons in the white matter (3, 19, 63). Loss of DCX from the infected NPCs suggests an abnormally differentiated state.

NES encodes an intermediate filament forming protein that is downregulated during NPC differentiation (32, 57). NES is associated with cytoplasmic trafficking in
NPCs and plays an important role in the distribution and organization of critical cellular factors regulating cell proliferation and differentiation (39, 53). Additionally, NES is located at 1q23 (an area that has been shown to be selectively targeted by HCMV) (42). Therefore, the downregulation of NES (and perhaps other neural genes) may be related to the selective effect of HCMV at 1q23.

SOX2 is a transcription factor involved in embryonic development and stem cell self-renewal (6, 13, 66, 67). The importance of SOX2 has been highlighted in recent literature by its involvement in reprogramming terminally differentiated cells into iPSCs (45, 64, 69). Loss-of-function mutations in SOX2 can cause improper eye and brain development (22, 55, 74, 79). SOX2 downregulation in uninfected NPCs is associated with, and necessary for, both glial and neural differentiation. We previously found that complete downregulation of SOX2 was protracted after differentiation in vitro, taking up to three weeks (35). In the absence of forced neural differentiation, HCMV infection of NPCs rapidly downregulated SOX2, with decreases observable after 4 h pi in whole genome expression analysis. This suggests that an abnormal and premature differentiation was induced. Moreover, the down-regulation of SOX2 mRNA was accompanied by that of the microRNA MIR21, regulation of which is related to SOX2's during differentiation (29).

During development, GFAP is first expressed in differentiating pluripotent stem cells and marks their differentiation toward a neural precursor lineage (21, 31). GFAP is also used as a marker for differentiation of NPCs to an astroglial lineage. NPCs differentiated to astroglia displayed strong GFAP positivity. HCMV infection did not affect GFAP expression in these differentiated astroglia (35) (and unpublished data). This was in sharp contrast to our observation in undifferentiated NPCs, where as early as 24 h pi, GFAP mRNA levels decreased 100-fold with protein declines beginning at 48 h pi. This suggested that GFAP's function and/or regulation was considerably different in NPCs than in astroglia, which has been suggested by others (80). In our experiments, changes to attachment and spreading characteristics observed during infection of neurospheres paralleled those seen during astroglial differentiation.
However, GFAP levels in infected NPCs were markedly decreased rather than increased as in differentiated astroglia, further suggesting abnormal differentiation. The downregulation of other neuronal markers observed in the whole genome expression experiments also indicated these infected cells were not neuronal in nature. Thus, although premature differentiation appeared to result from HCMV infection, the resultant cells were neither astroglia nor neurons. Loss of both ID4 and SOX4 also supports the conclusion that differentiation was abnormal.

MCMV infection has been shown to inhibit CNS stem cell differentiation, with neuronal differentiation being more severely inhibited than glial differentiation (28). Odeberg’s group produced similar results in human cells (43, 44). These investigators attempted to differentiate NPCs using standard culture medium manipulations after the onset of HCMV infection. They found that infection appeared to inhibit differentiation of NPCs down either neuronal or glial pathways, as measured by the absence of GFAP+ (glial marker) cells or β tubulin+ (neuronal marker) cells 7 d pi. In contrast, we found rapid downregulation of GFAP, as well as other markers of multipotency and multiple neuronal lineage markers, in the absence of culture medium changes. Given the results we observed, we suspect that the infected NPCs in the earlier studies were no longer multipotent and were unable to be differentiated to neurons or glia by any means. Our results find that HCMV infection, rather than blocking an enforced differentiation trajectory, causes premature differentiation into an abnormal state.

In an attempt to narrow the field of viral factors involved in the downregulation of the NPC markers, GCV was used to inhibit HCMV replication in NPCs. IE1, and low levels of IE2 and UL44 were detectable in GCV treated cells, but no E-L or L protein expression was observed. GCV treatment of infected cells delayed and reduced the downregulation/decreases of NPC marker mRNA and protein levels. Importantly, UV-inactivated virus infections did not alter the expression of NPC markers at the mRNA or protein level (data not shown), indicating that de novo viral protein expression was necessary for downregulation/decreases to occur. The GCV
results suggested that the altered fate of infected NPCs required a full complement of viral IE and E proteins and, that perhaps, active viral replication also played a role in these changes. mRNA downregulation occurred more rapidly than protein declines and coincided with IE gene expression, making IE gene products likely culprits. In fact, data gathered during a sub-optimal infection in which IE mRNA expression was drastically delayed, produced dramatically delayed and decreased changes in NPC marker mRNA levels (data not shown). Chambers and colleagues found the following genes expressed at the IE stage: IE1, IE2, IRS1, TRS1, UL36, UL37, UL69 and UL110 (9). Particularly important for gene regulation are the transcription factors IE1, IE2, TRS1, IRS1, and the tegument protein UL69. IE1 has been shown to interact with several cellular proteins (50) and IE2 is known to interact with several cellular transcription factors and histone deacetylases (75) and (reviewed in (54)). Our future analyses will concentrate on these viral proteins, which seem likely to be responsible for downregulation of NPC markers at the mRNA level.

We also investigated one of the probable mechanisms behind the decreases in NPC markers at the protein level, virus-induced proteosomal degradation. Incubation of cells with MG132, a proteasome inhibitor, partially inhibited the decreases in marker proteins. Although MG132 did not affect viral IE protein expression, viral E and L protein expression was inhibited by treatment with the drug (26). Thus, in order for the complete decline in NPC marker protein levels observed without drug treatment to occur, it appeared a full complement of viral proteins may have been required. Earlier studies showed that NES was ubiquitinated and degraded via the proteasomal pathway (53). Our IP experiments found that DCX was both ubiquitinated and sumoylated, with only minor modification in mock-infected cells. Others have shown viral infection induces unusual sumoylation of the cellular protein Daxx (23). These results suggest that HCMV may induce proteasomal degradation of host cellular factors to create a suitable environment for virus propagation, resulting in alterations in NPC behavior.

Interestingly, in our DCX Westerns we saw a slower migrating form at ~82 kD,
which prompted us to perform the IPs shown in Figure 6D. Although there were sumo
and ubiquitin modifications on the virus+MG samples, these modifications could not
explain the presence of this slower migrating form in the mock- and virus-treated
samples. A more thorough analysis of the literature revealed that the addition of
MG132 to cells could cause the irregular phosphorylation of certain substrates (58).
In addition, when researching additional DCX Abs, it became apparent that DCX can
become phosphorylated after certain stimuli and that one of the phosphorylated forms
migrates at approximately 82 kD (18). We explored the possibility that this
MG132-induced additional band might be a byproduct of phosphorylation by treating
our lysates with shrimp alkaline phosphatase (SAP) to look for removal of this 82 kD
band. Figure 6E shows that this was indeed the case.

In conclusion, we found that HCMV infection caused NPCs to prematurely and
abnormally differentiate. This process appears to be mediated by the rapid
downregulation of genes that maintain neural progenitor multipotency and of genes
establishing these cells’ neural identity. Analysis of gene expression data also
indicated an inhibition of their neuronal differentiation. Our results indicated that
HCMV infection quickly induced NPC differentiation and powerfully disrupted the
infected cell’s fate.

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Figure 1: HCMV infection caused NPC neurospheres to attach and spread on uncoated surfaces, emulating differentiation. Neurospheres were cultured and infected as described in Materials and Methods. (A) Uninfected neurospheres in uncoated dishes. (B) Normal cell migration 24 h post plating (hpp) onto fibronectin-coated dishes. (C) Timecourse of morphology changes induced by HCMV infection of neurospheres. (D) Infection using UV-irradiated virus did not induce attachment of neurospheres. Magnification for all live cell images =500x.

Figure 2: HCMV infection altered mRNA expression levels of multiple cellular genes. Whole genome expression analyses were performed 4, 12, and 24 hours pi as described in Materials and Methods. A) Numbers of significant genes were plotted versus fold regulation to determine cutoffs for each timepoint as described in Materials and Methods. B) Genes of known function determined to be significantly up- or down-regulated at 4, 12 or 24 hours pi were categorized into functional groupings. The genes in the neural-related category are included among the eight other functional groups, in addition to being separated for emphasis. TS= transcription factors.

Figure 3: NPC marker proteins were downregulated at the mRNA level and decreased at the protein level by HCMV infection. Monolayer NPCs were infected with HCMV and harvested at the indicated times pi for analysis of either mRNA or protein levels. (A) q-PCR analysis of NES, DCX, SOX2 and GFAP mRNA levels from 0-144 h pi. Log₁₀ ratios of viral/mock are given. Calculations were based on absolute starting quantities, using reactions specific for glucose-6-phosphate dehydrogenase (G6PD) as normalization controls. The averages of two experiments are shown in the bar charts. Bars represent the range. (B) NPC marker protein steady state levels at early times pi. (C) NPC marker protein steady state levels at late times pi. Actin is used as a loading control in B and C. M= mock infected; V= virus-infected in all figures.
Figure 4: Decreases in the NPC markers at the protein level were delayed by GCV treatment. Monolayer NPCs were infected with HCMV, either with or without GCV, as described in Materials and Methods. Cells were analyzed at the indicated times pi. (A) Cells on poly-D-lysine coated coverslips were analyzed at the indicated time points, fixed, and stained for IE1 expression and UL44 foci formation and development. Infection without GCV is shown in the left panels, infection with GCV in the right panels. In the far right panels, brightness levels of UL44 were increased/adjusted to show that even after overexposure, no foci were present in GCV-treated cells. At 72 and 96 hpi, UL44 exposure time of untreated cells (0.15 sec) was half that of GCV-treated (unadjusted) cells (0.3 sec) due to increasingly higher levels of UL44 in the untreated cells (as can be observed in panel B). Nuclei were counterstained with Hoechst (H). Scale bar=5 μm. (B) Viral protein steady state levels were assessed in the presence and absence of GCV. Viral IE (IE1/IE2), E (UL44), E-L (pp65) and L (gB) antigens were assessed. (C) Steady state levels of NPC marker proteins were determined in the same timecourse. Actin was used as a loading control in B and C.

Figure 5: Down regulation of the NPC markers at the mRNA level was delayed by GCV treatment. mRNA levels of NPC markers were determined by q-PCR at the indicated times pi as in Figure 3. Each of the four proteins is represented by three shades of one color. The darkest shade of each color is the untreated viral/mock ratio. The middle shade is GCV-treated virus/mock ratio. The lightest shade is the UV-inactivated virus/mock ratio.

Figure 6: Treatment of cells with MG132 partially blocked NPC marker protein degradation. Monolayer NPCs were mock- or virus-infected, treated with MG132 (12.5 μM) or untreated as described in Materials and Methods, and harvested at the indicated times pi. (A) Viral protein steady state levels. (B) NPC marker protein
steady state levels. (C) DCX steady state levels at 48 h pi. Two exposure lengths for
the same blot are shown. (D) IP using anti-DCX Ab, then Western blotting using
anti-ubiquitin or sumo-1 Abs. (E) Duplicate samples were run on a single blot,
transferred to protran and then half the blot was treated with SAP as described in
Materials and Methods prior to probing with anti-phospho-DCX Ab. Blots were then
probed for actin for loading and staining controls. In panels C and D and E, MG132
was added to the lysis and IP buffers.

Table I: Infection of NPCs affected expression of cellular genes in numerous
functional groupings. Representative genes from each of the functional groupings are
listed. At each time pi, whether or not each gene is turned off (OFF) or on (ON) or
down- or up-regulated (DOWN or UP, respectively) is indicated. Note that NES was
included in this table due to its importance as an NPC marker. Although
downregulated (by threefold) it did not meet the stringent threshold requirements of
the microarray analysis and is therefore not included in Supplemental Table I. Its entry,
and those of the SOX2 gene at 4 and 24 h pi (2.5 fold and 6.5 fold, respectively), and
MIRN21 at 12 h pi (5 fold) are marked with asterisks.
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