The Effect of Cellular Differentiation on HSV-1 Infection of Oligodendrocytic Cells

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Abstract

Herpes simplex type 1 (HSV-1) is a neurotropic virus that infects many types of cells. Previous studies have demonstrated that oligodendrocytic cells are highly susceptible to HSV-1 infection. Here we analysed HSV-1 infection of a human oligodendrocytic cell line, HOG, and oligodendrocyte precursor cells (OPCs) cultured under growth or differentiation conditions. In addition to cell susceptibility, the role of the major cell receptors for viral entry was assessed. Our results revealed that OPCs and HOG cells cultured under differentiation conditions became more susceptible to HSV-1. On the other hand, viral infection induced morphological changes corresponding to differentiated cells, suggesting that HSV-1 might be inducing cell differentiation. We also observed colocalization of HVEM and nectin-1 with viral particles, suggesting that these two major HSV-1 receptors are functional in HOG cells. Finally, electron microscopy assays indicated that HSV-1 may be also entering OLs by macropinocytosis depending on their differentiation stage. In addition, vesicles containing intracellular enveloped virions observed in differentiated cells point to an endocytic mechanism of virus entry. All these data are indicative of diverse entry pathways dependent on the maturation stage of OLs.

Introduction

Several infectious agents, ranging from mycobacteria to retroviruses, have been proposed to be associated with demyelinating diseases such as Multiple Sclerosis (MS), in which oligodendrocytes (OLs), the myelin-forming cells in the central nervous system (CNS), may be the initial target for the pathogenic onset [1,2,3]. Of all studied organisms, members of the viral family Herpesviridae are among the most promising candidates [3,4,5,6,7,8]. In addition to other herpesviruses (for example Epstein-Barr virus or human herpesvirus 6), herpes simplex virus type 1 (HSV-1), has been linked to the possible aetiology or development of several neurodegenerative diseases and virus-induced demyelination [9,10,11,12]. Previous reports have shown that a human oligodendrocyte-derived cell line is highly susceptible to HSV-1 [13], and that the virus may play a role in triggering MS relapses during clinical acute attacks of MS, at least in the most frequent clinical presentation of the disease, the relapsing-remitting form [14]. Besides neurodegenerative diseases, HSV-1 may also be involved in cognitive alterations in bipolar or schizophrenia dysfunctions [15].

Herpesviruses usually infect their hosts for life, after the initial infection of epithelial cells, the virions spread to neurons and establish latent infections in sensory ganglia [16]. In some cases, the virus spreads into the CNS to cause encephalitis or meningitis [17]. HSV-1 entry into a diverse range of cell types has been described [18]. The entry of HSV into various cell types follows a complex process [19,20].

The initial attachment of HSV-1 to the cell surface is mediated by glycoproteins B (gB) and C (gC). This interaction with heparan sulfate proteoglycans (HSPGs) enables the binding of viral gD to one of its receptors on the host cell surface. This binding triggers conformational changes in gD that allow the activation of gH/gL, which in turn activate the fusion effector gB [21,22]. Cellular proteins binding to HSV gB have also been identified but their roles in the entry process or in cell tropism remains unsolved [23,24,25]. Molecules derived from three structurally different groups have so far been described as gD receptors in the host, Herpes Virus Entry Mediator (HVEM), a member of the tumor necrosis factor receptor family, nectin-1 and —2 from the immunoglobulin superfamily and distinctive sites in heparan sulfate (HS) generated by a specific 3-O-sulfotransferase (3-O-ST) [26,27,28,29]. Nectin-1 and HVEM appear to be the principal gD-binding entry receptors although they bind distinct regions of the gD ligand [20]. They are coexpressed in many cells and used by the majority of tested clinical strains of HSV-1, as well...
as HSV-2 [30]. HVEM expression has been found in liver, kidney, lymphoid tissues, lung and in several cell lines. Nectin-1 is the main, although not exclusive, HSV receptor on epithelial and neuronal cells, whereas nectin-2 use seems to be limited to only few viral mutant strains [27,30,31,32,33]. It is worth noting that nectin-1 is an adhesion molecule present at adherent junctions in polarized cells, such as epithelial and neurons cells, and in cell-cell contact in some cultured cells [34]. 3-O-ST HS can be used as an entry receptor for HSV-1 but not HSV-2 in multiple cell lines like neuronal or endothelial cells [27,35]. Although in all cases, binding of gD to a specific receptor is required during HSV entry, membrane fusion can take place directly at the cell surface or, in some cases, following virus endocytosis. Why the virus chooses one or another pathway is largely unknown. However, studies with cell cultures of different origin –SY5Y, HeLa or Vero cell lines– suggest that nectin-1-mediated internalization may direct HSV to the endocytic pathway, possibly with the cooperation of integrins [36,37,38].

Finally, binding of HSV-1 to its cellular receptor –or receptors– seems to be sufficient for the induction of intracellular signalling even in the absence of subsequent virion entry [39]. Differential expression of cellular genes associated with NF-kB, Jak/Stat or p13K/Akt pathways has been observed by means of microarray studies, highlighting the effect of HSV-1 glycoproteins, particularly gD, on this process [39,40].

Oligodendrocyte precursor cells (OPCs) give rise to oligodendrocytes during embryonic and postnatal development as well as in the adult CNS and can be differentiated in vitro into mature myelin-forming OLs [41,42,43,44]. In vitro, OLs are characterized by a complex arborisation of cell processes and in vivo, these processes terminate in flat membranous sheets –rich in myelin proteins and lipids– that spiral wrap around and insulate neuron axons [45]. In the present report, we characterize HSV-1 infection of a human oligodendrogial cell line, HOG, and OPCs in primary cell culture. Cells were cultured in growth or differentiation media, their differential susceptibility to viral infection was determined and the role of the major cell receptors for viral entry was investigated.

**Materials and Methods**

**Antibodies and Reagents**

Anti nectin-1 monoclonal antibody CK41 and anti-HVEM polyclonal antibody R140 have been described previously [46,47]. Horseradish peroxidase-conjugated secondary anti-IgG antibodies were purchased from Millipore (Billerica, MA, USA). Anti-green fluorescent protein GFP rabbit polyclonal serum A6455, Alexa 488- , Alexa 647- and Alexa 594-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR, USA). DNA size marker was from Invitrogen. Polyclonal rabbit anti-HSV-1 antibody was from DAKO. Monoclonal mouse anti-PLP MAB388 antibody was from Millipore. Anti-nectin-1 mouse monoclonal antibody CK6 was from Santa Cruz Biotechnology. Anti-HVEM mouse monoclonal antibody, low-glucose DMEM, fetal bovine serum (FBS), human insulin, triiodothyronine (T3), apo-transferrin, sodium selenite, putrescine, dihydrocycl AMP (dbcAMP), carboxymethylcellulose sodium salt (CMC) medium-viscosity and protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mowiol was from Calbiochem (Merck Chemicals, Germany). HS4C3 antibody was a kind gift of Dr. R. Longnecker, (Northwestern Medical School, Chicago, USA).

**Cells and Virus**

The HOG cell line, established from a surgically removed human oligodendroglioma [48] was kindly provided by Dr. A. T. Campagnoni (University of California, UCLA, USA). Cells were cultured on Petri dishes in growth medium (GM) containing low-glucose DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/mL) and streptomycin (50 μg/mL) at 37 °C in an atmosphere of 5% CO2. To induce differentiation, cells were cultured in serum-free differentiation medium (DM) containing low-glucose DMEM supplemented with antibiotics and 50 μg/ml apo-transferrin, 0.5 mg/1 insulin, 30 nM triiodothyronine (T3), 30 nM sodium selenite and 16.1 mg/l putrescine. Cells cultured in this medium were also treated with 0.5 mM dbcAMP and IBMX at a final concentration of 0.5 mM.

OPCs from postnatal P0 mice were generated as described [42,49,50] in the facilities of Hospital Nacional de Parapléjicos (Toledo, Spain). All animal experiments were carried out in accordance with Spanish (RD233/88) and European (2010/63/EU) regulations, and they were approved by the Animal Review Board at the Hospital Nacional de Parapléjicos (SAPA001). To differentiate OPCs, cells were maintained in differentiation medium [42] for 3 days. Cells cultured in that same medium for 24 h were considered as undifferentiated control.

K26GFp was a kind gift of Dr. Desai (Johns Hopkins University, Baltimore, USA). It was obtained by fusing GFP to the HSV-1 capsid protein VP26 [51]. The R120vGF, EGFP recombinant virus was propagated in E5 cells, a Vero cell line expressing the ICP4 protein of HSV-1 [52]. K26GFp and wild type HSV-1 (F strain, DNA genome sequence GenBank GU734771) viruses were propagated and titrated on Vero cells. GFP-MAL2/MAL-diHcRed/HOG cells are HOG cells stably transfected with GFP-MAL2, a construct encoding a chimera consisting of GFP fused to the amino-terminal end of MAL2, and with MAL-diHcRed, a dimeric red fluorescent protein [53].

**Viral Infections**

For viral infection assays, 1.2x106 HOG cells growing in 25 cm² flasks were mock-infected or infected with the corresponding virus. During viral adsorption, cells were maintained in DMEM with antibiotics and 5% FCS. Subsequently, cultures were rinsed and cultured in its corresponding medium. Viral titer was quantified by an endpoint dilution assay determining the TCID50 in Vero cells, considering the final dilution that shows cytopathic effect and using the Reed and Muench method.

For plaque assay, confluent monolayers of cells plated in 6-well tissue culture dishes were infected with serial dilutions of HSV-1. After viral adsorption, cells were washed and overlaid with CMC. The CMC solution was prepared in distilled water at 2% (w/v) and stirred at room temperature for one hour. CMC overlay (1% final concentration) was prepared by mixing equal volumes of CMC 2% and 2x concentrated GM or DM. Two millilitres of CMC overlay were added to each well. Plates were incubated at 37 °C in a humidified 5% CO2 incubator for 48 hours. The CMC overlay was then aspirated, cells were washed with PBS and fixed in 4% paraformaldehyde for 20 min. Plaques were visualized by staining with crystal violet.

**Construction and Characterization of R120vGF Recombinant HSV-1 Virus**

The recombinant R120vGF virus was obtained by transfecting plasmid DNA of pH14GF digested with EcoRI and HindIII into E5 cells, infected with HSV-1 mutant strain d120 deficient in...
Recombinant virus was plaque-purified five times in E5 cells. The nant progeny was selected by using EGFP expression as a marker. ICP4 [52], using lipofectamine 2000 (Invitrogen). The recombi-
cells was isolated by QIAamp DNA Micro Kit (QIAGEN). p41HS and GFP-AS primers (Fig. 1E, lane I). DNA from infected
this had been replaced by the EGFP chimeric gene amplified by
and HTK6R primers (Fig. 1E, lane A) but not from the UL41
(Fig. 1E, lanes B and F) and DNA of HSV-1 strain F (Fig. 1E, lanes
9cassette by the SphI-EcoRV
13 to 527 from ATG of UL41) and 658 bp for the chimeric
TAGGTGAGGGTTGGTCAG-3 for the chimeric EGFP
gene of recombinant virus. PCR products were analyzed by 1%
arose gel electrophoresis, and the specificity of the amplification
products was confirmed by DNA sizes of 479 bp for the HSV-1
TK (nt 102 to 581 of coding TK sequence), 540 for the UL41
(Fig. 1E, lanes C and G). Using DNA of the R120vGF virus as template, specific
fragments could be amplified from the TK gene by using HTK6D
and HTK6R primers (Fig. 1E, lane A) but not from the UL41
gene by using HL41S and HL41AS primers (Fig. 1E, lane E), since
this had been replaced by the EGFP chimeric gene amplified by
p41HS and GFP-AS primers (Fig. 1E, lane I). DNA from infected
cells was isolated by QIAamp DNA Micro Kit (QIAGEN).

Antibody Blocking Assay
HOG cells cultured in a 24-well tissue culture dish were washed with free-serum DMEM and incubated with 10 µl of antibodies
(1:10 dilution) to block their corresponding receptor: R140 to block HVEM and CK41 to block nectin-1. Incubation with both
antibodies simultaneously was also performed. Following incubation at 4°C for 1 h, an equal volume of K26GFP diluted in free
serum medium was added to cells at an m.o.i. of 1. Virus was incubated at 4°C for 1 h. After viral adsorption, cells were washed with
PBS, incubated for 20 h with their respective media containing blocking antibodies and processed for flow cytometry. Cells not blocked
with primary antibody were used as controls.

Detection of Heparan Sulfates
To visualize HSPGs, we cultured HOG cells in GM or DM. After 24 hours, cells were washed with free-serum DMEM and incubated for 20 minutes at 4°C with WGA-594 (5 µg/ml). Then, cells were washed twice in PBS, fixed in 4% paraformaldehyde for 20 min and washed in PBS. Finally, cells were incubated with TO-
PRO-3 to stain nuclei. To detect 3OS-HS we used HS4C3 antibody. HOG cells were cultured in GM or DM. After 24 hours, cells were fixed in 4% paraformaldehyde for 20 min, washed in PBS and permeabilized with 0.2% Triton X-100. After that, cells were blocked with 3% bovine serum albumin in PBS for 30 min and incubated with HS4C3 antibody (diluted 1:10 in blocking
solution) for 1 hr at room temperature. Both incubations were performed in the presence of 0.5 M NaCl to avoid unspecific
crossreaction of the antibody.

Immunoblot Analysis
Samples were subjected to SDS-PAGE in 10% acrylamide gels under reducing conditions and transferred to Immobilon-P
membranes (Millipore). After blocking with 5% non-fat dry milk, 0.05% Tween 20 in PBS, blots were incubated for 1 h at room
temperature with primary antibodies. After several washes with 0.05% Tween 20 in PBS, blots were incubated for 1 h with
secondary antibodies coupled to horseradish peroxidase, washed extensively, and developed using an enhanced chemiluminescence
Western blotting kit (ECL, Amersham, Little Chalfont, UK).

Real-time Quantitative RT-PCR Assay
Real-time quantitative RT-PCR assay was performed as previously described [54]. Briefly, total RNA from triplicate
samples of HOG cells infected with HSV-1 cultured in 60-mm dishes under growth or differentiation conditions was extracted using
RNasey Qiagen Mini kit (Qiagen, Valencia, CA, USA). RNA integrity was evaluated on Agilent 2100 Bioanalyzer (Agilent
Technologies, Santa Clara, CA) and quantification of RNA was carried out in a Nanodrop ND-1000 spectrophotometer (Thermo
Fisher Scientific). All the samples showed 260/280 ratio values around 2, which correspond to pure RNA. RNA Integrity
Number (RIN) values were between 9.3 and 10, corresponding to RNA samples with high integrity. Genomic DNA contamina-
tion was assessed by amplification of representative samples without reverse transcriptase (RT). RT reactions were performed using
the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems PN 4390712) following manufacturer’s instructions.

Primer sequences (5’-3’) were as follows: for nectin-1, ACTCGCTCTCGGCTTGAC and CCATACATG-GAGTCGTTCACC; for HVEM, ATCCGTG-
TAGCTGGTTGCC and GGAAGGTGAGATAAGCAGACCA. We used the NormFinder algorithm to identified 18S as the most
suitable genes for the normalization due to its high stability.

Immunofluorescence Microscopy
Cells grown on glass coverslips were fixed in 4% paraformal-
dehyde for 20 min and rinsed with PBS. Cells were then
permeabilized with 0.2% Triton X-100, rinsed and incubated
for 30 min with 3% bovine serum albumin in PBS. For double and
triple-labeled immunofluorescence analysis, cells were incubated
for 1 h at room temperature with the appropriate primary
antibodies, cells were then rinsed several times and incubated at
room temperature for 30 min with the relevant fluorescent
secondary antibodies. Controls to assess labeling specificity
included omission of the primary antibodies. After thorough
washing, coverslips were mounted in Mowiol. Images were
obtained using an LSM510 META system (Carl Zeiss) coupled
to an inverted Axiovert 200 microscope. Processing of confocal
images and colocalization analysis was made by FIJI-ImageJ
software.

Flow Cytometry Analysis
To perform FACS analysis, HOG cells were dissociated in 0.05% trypsin/0.1% EDTA (Invitrogen) for 1 minute at room
temperature, then washed and fixed in 4% paraformaldehyde for
15 minutes and, finally, rinsed and resuspended in PBS. Cells were
analyzed using a FACScan (BD Biosciences).

Electron Microscopy
HOG cells cultured at 37°C in GM or DM were mock-infected or
infected with HSV-1 at an m.o.i. of 50. At different time points
post-infection, cells were fixed in 4% paraformaldehyde in 0.1 M
sodium phosphate buffer, pH 7.4, at 37°C for 2 hours. Then, they
were washed in PBS containing 20 mM glycine and processed by
freeze substitution as previously described [13]. Samples were
examined with a JEM 1010 transmission EM (Jeol, Tokyo, Japan).
Effect of Differentiation on HSV1 Infection of OLs

A

GM

DM

B

Positive cells (%)

GM

DM

C

Infection:

GM

DM

D

Titer (TCID50/ml)

GM

DM

E

Kbp

L

A

B

C

D

E

F

G

H

I

J

K

M

F

Infection:

GM

DM

25 kDa

anti-GFP

G

Titer (TCID50/ml)

UND

DIF

H

Infection:

UND

DIF

anti-HSV-1
Results

Culturing HOG Cells in Differentiation Medium Increases Susceptibility to HSV-1

The susceptibility of a human oligodendrocyte-derived cell line was previously assessed in our laboratory [13]. Here, we analyze the effect of oligodendrocyte differentiation on HSV-1 infection. HOG cells were cultured in GM or DM and infected with HSV-1. Plaque assay showed a significantly larger number of plaques in DM compared to cells cultured in GM when cells are infected with the same viral dose (Figure 1A). Similar results were obtained by flow cytometry analysis of HOG cells infected at an m.o.i. of 0.5 with GFP-tagged HSV-1 K26GFP. As shown in Figure 1B, a significant increase in the number of GFP-expressing cells 24 hours after infection was confirmed in cells cultured in DM compared to GM cultures. Immunoblotting assay also showed an increase in viral protein detection in cells cultured in DM compared to those cultured in GM (Figure 1C). To avoid differences in the number of cells in GM and DM cultures, we took into account the growth rate of GM and DM cells, so that at the time of infection, the number of cells in both cultures were the same. Although at 20 h.p.i. the number of cells did not vary significantly, to control the amount of protein loaded, we performed the experiment either loading equal number of cells or equal amount of protein, obtaining similar results in both cases.

Finally, HOG cells were cultured in GM or DM and infected at an m.o.i. of 0.1 with HSV-1. Progeny virus was titrated to determine the 50% tissue culture infective dose (TCID50)/ml. After 20 h p.i., viral yield in DM-cultured HOG cells was significantly higher compared to cells cultured in GM (Figure 1D). To investigate whether the increment in viral yield was due, at least in part, to an increase in viral entry, we carried out the infection of HOG cells using R120vGF, an EGFP-expressing recombinant HSV-1 lacking ICP4. Figure 1E shows PCR amplification of the genes encoding thymidine kinase (TK), virion host shutoff (vhs, UL41) and chimeric EGFP from R120vGF DNA (lanes A, E and I respectively); parental HSV-1 strain d120 DNA (lanes B, F and J respectively) and HSV-1 strain F DNA (lanes C, G and K respectively). Negative PCR controls without DNA are also shown (lanes D, H and M). After entry into cells, R120vGF expresses EGFP and immediate early proteins, but is unable to complete the viral cycle due to the absence of ICP4. Using this tool, we can measure GFP signal and immediate early protein production to estimate whether HSV entry is altered in HOG cells cultured under differentiation conditions. This novel viral construction allows to estimate HSV-1 entry determining either GFP fluorescence –by flow cytometry or immunofluorescence– or by immunoblot, providing new methods to the study of HSV-1 entry into cells. HOG cells cultured in GM and DM were infected with R120vGF at an m.o.i. of 0.1. After 24 h.p.i., equal amounts of protein were subjected to SDS-PAGE and analyzed by immunoblotting with anti-GFP antibody. As in the previous experiments, an increase in viral signal was observed in HOG cells cultured in DM (Figure 1F), suggesting that differentiation is affecting viral entry. As indicated above, we observed an increase in the number of plaques in HOG cells cultured in DM compared to cells cultured in GM. However, the average size of plaques corresponding to cells cultured in DM was also increased, suggesting that other factors –apart from viral entry– might also be involved.

To extend the results obtained with HOG cells to primary cultures, we studied HSV-1 infection in mouse OPCs. Primary OPCs cultured in differentiation medium for 24 h (undifferentiated) or 3 days (to allow spontaneous differentiation) were infected at an m.o.i. of 1 with HSV-1, and the viral productivity was titrated 20 h.p.i determining the TCID50/ml. Viral yield in differentiated cells was significantly higher compared to undifferentiated cells (Figure 1G). Also, immunoblotting assay showed an increase in viral protein detection in differentiated OPCs cultured for 3 days compared to undifferentiated cells cultured for 24 hours (Figure 1H).

HSV-1 Infection Induces Differentiation in HOG Cells

Once it was established that culturing HOG cells in differentiation medium increased infection by HSV-1, we decided to ascertain whether viral infection was also able to induce changes corresponding to a more advanced differentiation stage. For this purpose, HOG cells grown on glass coverslips were cultured in GM or DM and subsequently mock-infected or infected at an m.o.i. of 0.5 with K26GFP for 20 h. As previously observed [55], we detected an increase of proteolipid protein (PLP) levels in HOG cells cultured in DM (Figure 2A). Interestingly, an increase in PLP levels was also detected in cells cultured in GM and infected with K26GFP (Figure 2A). Surprisingly, PLP increased not only in infected cells, but also in non-infected cells. It is possible that contact with non-infectious particles or infected cells may be sufficient to trigger a response that induces differentiation of non-infected cells. Alternatively, factors secreted by infected cells might induce differentiation of non-infected cells. Further experiments will be needed to test these two possibilities. In addition, myelin-like sheets and other morphological features corresponding to differentiated cells were also observed in infected cells cultured in GM (Figure 2B). Finally, GFP-MAL2/MAL6-HcRed/HOG cells [53] grown on glass coverslips were cultured in GM or DM and thereafter mock-infected or infected at an m.o.i. of 0.5 with HSV-1. Cells cultured in GM, exhibited myelin-like sheets enriched in...
expression of HSV-1 receptors in HOG cells into MAL-positive vesicles during viral egress. However, further studies will be necessary to demonstrate this hypothesis.

Expression of HSV-1 Receptors in HOG Cells

To investigate whether the major cell receptors for HSV-1 play a role in the increase of susceptibility of differentiated OLs to the infection, we monitored expression of HVEM, nectin-1 and 3-OS HS along the process of differentiation. We first analysed expression of HSPG, which act as an attachment factor for HSV gC and gB, by immunofluorescence assay. We incubated HOG cells in GM or DM with wheat germ agglutinin (WGA), a lectin that binds to N-acetylglucosamine, coupled to Alexa-594. HSPG was highly expressed on the surface of HOG cells and no significant changes were observed during differentiation (Figure 3A and B). In contrast, the detection of the specifically modified 3-OS-HS, which acts as a receptor for gD, with monoclonal antibody HS4C3 was negative in HOG cell line under growth and differentiation conditions (data not shown).

When we performed similar immunofluorescence assays with antibodies against nectin-1 (CK41) and HVEM (R140), slight changes in these two HSV-1 receptors took place between growth and differentiation conditions. This assay was performed incubating live cells with the antibodies in serum-free DMEM for 20 minutes at 4°C. After that, cells were fixed and processed for immunofluorescence analysis as described in the materials and methods section. Nectin-1 detection was slightly decreased in HOG cells cultured in DM whereas HVEM expression increased (Figure 4A and D). Although immunofluorescence provides important information on the location of receptors and allows a rough comparison of expression levels, it is not a robust quantitative measure. To address the quantitative effect, immunoblot analysis was performed with anti-nectin-1 (CK6) or HVEM (mouse monoclonal) antibodies and confirmed these immunofluorescence results (Figure 4B and E). We loaded either equal number of cells or equal amount of protein, obtaining similar results in both cases. Finally, to determine whether HVEM and nectin-1 expression was modified following cell differentiation, we quantified the mRNA using RT-qPCR in cells cultured either in GM or DM. Quantitative RT-qPCR confirmed an increase of HVEM and a slight decrease in nectin-1 expression in HOG cells cultured under differentiation conditions when compared to GM cultured cells (Figure 4C and F).

Cell Receptors Involved in HSV-1 Entry into HOG Cells

Once the presence of nectin-1 and HVEM in the HOG cell surface was established, we analysed the role of these two receptors during the HSV-1 infection. We first carried out an antibody blocking assay. HOG cells incubated with anti-nectin-1 (CK41) or anti-HVEM (R140) or both antibodies simultaneously, were infected with K26GFP. After 20 h p.i., cells were fixed and processed for GFP flow cytometry as a measure of infection. Although we detected a slight blocking effect, especially with HVEM antibodies, neither anti-nectin-1 nor anti-HVEM antibody treatment efficiently blocked HSV-1 entry into HOG cells cultured in GM or in DM as compared to controls without blocking antibodies. Nevertheless, incubation with both antibodies simultaneously induced a more significant blocking effect (Figure 5A). On the other hand, analysis of HOG cells infected with K26GFP at 4°C for 1h and processed for confocal indirect immunofluorescence analysis with anti-HVEM polyclonal and anti-nectin-1 monoclonal antibodies 5 minutes after the shift to 37°C, showed partial colocalization of viral particles with nectin-1 and HVEM (Figure 5B). These data suggest that both HVEM and nectin-1 are functional as HSV receptors in oligodendrocytic cells and that HVEM may play a bigger role when these cells differentiate.

Study of Viral Entry by Electron Microscopy

It has been proposed that HSV-1 entry can proceed by macropinocytosis/endocytosis in a cell-type dependent manner, regardless of which receptor in used [36,56,57,58]. To determine whether endocytosis is involved in HSV entry into HOG cells, we used direct observation by electron microscopy. HOG cells cultured in GM or DM were mock-infected or infected with HSV-1 at an m.o.i. of 50. At 5, 10, 20 and 30 minutes p.i., cells were fixed and processed for observation (see material and methods). In cells cultured in DM for 20 minutes, membrane protrusions similar to planar lamellipodia (Figure 6A and B) and circular ruffles (Figure 6C) were observed in the vicinity of virions. These structures have been described as a part of the process of viral entry by macropinocytosis in different cells [58]. Furthermore, enveloped virions were detected in vesicles at 30 min post infection (Figure 6D), which is indicative of virus endocytosis. Altogether, these data suggest that macropinocytosis may be involved in HSV-1 entry into differentiated HOG cells. Finally, intracellular unenveloped virions were observed in cells cultured both in GM (Figure 6E) and DM (F) 5 minutes p.i., suggesting that membrane fusion had occurred. Thus, this pathway does not seem to be altered during differentiation.

Discussion

HSV-1 can infect a diverse range of cell types and tissues including, neurons, retinal or conjunctival epithelial cells the as well as oral and genital mucosa [31,32,59,60]. Previous studies have shown that OLs are highly susceptible to HSV-1 infection [13] and glial specific cellular proteins such as myelin-associated glycoprotein, MAG, have been implicated in viral entry [25]. In the present work, the effect of oligodendroglial cell differentiation on HSV-1 infection has been investigated. HOG cells exhibit characteristics related to immature OLs such as GalC and CNPase expression. Culturing of HOG cells in DM induces some differentiation hallmarks, such as proliferation of processes and the increase in MBP and MOG expression. Nevertheless it is not possible to find significant quantitative differences in GalC and CNPase between HOG cells grown in GM versus DM [61]. In a previous study [53] we observed the presence of myelin-like membrane sheets –structures previously described in primary and mixed cultures [62,63,64]– in HOG cells cultured in DM. We also detected an increment in PLP expression during differentiation of these HOG cells and accumulations of PLP in myelin-like sheets [55]. Moreover, these myelin-like sheets contained vesicles enriched in the apical molecule CD59 and MAL, a major myelin protein [53]. In summary, HOG cells cultured in DM acquire a more differentiated phenotype characterized by morphological features –elongation of processes and emergence of myelin-like sheets–, biochemical changes –an increase in MBP, MOG and PLP– accumulation of myelin proteins –such as PLP and MAL– in myelin-like sheets. These characteristics make HOG cells an appropriate model to study changes in HSV-1 infection between cells with different developmental stages.
In this study, our results show that culturing HOG cells in DM or maintaining OPCs in differentiation culture conditions for 3 days enhanced HSV-1 infection. To ascertain possible factors involved in this increased susceptibility, we first monitored expression of the best characterized HSV-1 receptors (i.e., HVEM, nectin-1 and 3-OS-HS) along the process of HOG cell differentiation. Immunofluorescence microscopy revealed that expression of the attachment HSPGs remained elevated and unchanged throughout differentiation. In contrast, the specifically sulfated 3-OS HS was not detected in HOG cells using antibody HS4C3.

Figure 2. Effect of HSV-1 infection on cell differentiation of HOG cells. Cells cultured in GM or DM were mock-infected or infected at an m.o.i of 0.5 with K26GFP for 20 h. Then, cells were fixed and processed for confocal immunofluorescence analysis with an anti-PLP monoclonal antibody detected with an Alexa Fluor 555 secondary antibody. PLP signal is increased in mock-infected cells cultured in DM and in infected cells cultured in GM (A). In addition, membrane processes and myelin-like sheets (arrows) can be noticed in cells cultured in GM infected with HSV-1 or K26GFP (B). C. GFP–MAL2/MAL–diHcRed/HOG cells cultured in GM or DM were mock-infected or infected at an m.o.i of 0.5 with HSV-1 for 20 h. Cells were then fixed and processed for confocal immunofluorescence analysis with an anti-HSV-1 polyclonal antibody and an Alexa Fluor 647 secondary antibody. Myelin-like sheets enriched in exogenous MAL (arrow) can be observed in infected cells cultured in GM. All images correspond to the projection of the planes obtained by confocal microscopy. In panel A nuclei were stained with TO-PRO-3. DIC: Differential Interference Contrast. To make the cells more visible, DIC contrast of the whole images has been adjusted.

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Furthermore, by means of immunofluorescence microscopy, immunoblot analysis and RT-qPCR, we have detected an increase of HVEM and a slight decrease of nectin-1 in HOG cells cultured in DM in comparison to GM treated cells. Previous works have
Effect of Differentiation on HSV1 Infection of OLs

A  NECTIN-1  NECTIN-1/TO-PRO-3

B  GM  DM

C  Relative nectin-1 mRNA levels (arbitrary units)

D  HVEM  HVEM/TO-PRO-3

E  GM  DM

F  Relative HVEM mRNA levels (arbitrary units)


Figure 4. Expression of nectin-1 and HVEM in HOG cells. A. Cells cultured in GM or DM were fixed and processed for confocal immunofluorescence analysis with CK41 anti-nectin-1 antibody. Primary antibody was detected using an Alexa Fluor 555 secondary antibody. Images correspond to the projection of the planes obtained by confocal microscopy. B. HOG cells were subjected to SDS–PAGE and analyzed by immunoblotting with an anti-nectin-1 polyclonal antibody. Immunoblot assays showed a slight decrease of nectin-1 in cells cultured in DM. C. RTqPCR quantification of relative nectin-1 mRNA expression levels in HOG cells cultured in GM or DM showed a decrease in nectin-1 expression in differentiated cells compared to cells cultured in growth conditions. D. Cells cultured in GM or DM were fixed and processed for confocal immunofluorescence analysis with R140 anti-HVEM antibody. Primary antibody was detected using Alexa Fluor 555 secondary antibody. Images correspond to the projection of the planes obtained by confocal microscopy. E. HOG cells were subjected to SDS–PAGE and analyzed by immunoblotting with an anti-HVEM polyclonal antibody. Immunoblot assays showed an increase of HVEM in cells cultured in DM. C. RTqPCR quantification of relative HVEM mRNA expression levels in HOG cells cultured in GM or DM showed a significant increase in HVEM expression in differentiated cells compared to cells cultured in growth conditions. DIC: Differential Interference Contrast. doi:10.1371/journal.pone.0089141.g004

Figure 5. Role of HVEM and nectin-1 on viral entry in HOG cells. A. Antibody blocking assay was performed incubating HOG cells with R140 anti-HVEM or CK41 anti-nectin-1 or both antibodies simultaneously at 4 °C for 1 h. Cells were then infected with an equal volume of K26GFP diluted in free serum medium at an m.o.i of 1 for 1 h. After viral adsorption, cells were washed with PBS, incubated for 20 h with their respective media containing blocking antibodies and processed for flow cytometry. Controls correspond to cells not blocked with primary antibody. The percentage of infection in differentiated cells blocked with both antibodies simultaneously is lower than control without blocking antibodies (C). B. HOG cells cultured in DM and infected at an m.o.i. of 1 with K26GFP were fixed and processed for confocal indirect immunofluorescence analysis with R140 anti-HVEM and CK41 anti-nectin-1 monoclonal antibodies. Panels correspond to three confocal slices of 0.8 μm. Arrows point to colocalization of virus with receptors. DIC: Differential Interference Contrast. doi:10.1371/journal.pone.0089141.g005
demonstrated that nectin-1 has a major role in HSV-1 entry into neurons [33,65]. In our oligodendroglial model, the expression of this receptor in HOG cells is rather low, but we observed some colocalization with viral particles. HVEM expression in HOG cells was higher than nectin-1, and, again, we observed colocalization of HVEM with viral particles. These results suggest that both nectin-1 and HVEM are functioning as HSV-1 receptors in HOG cells. Accordingly, blocking with either anti-nectin-1 or anti-HVEM antibodies did not induce a significant decrease in viral infection since preventing the use of one receptor may lead the virus to take advantage of the other more extensively. In addition, blocking with both antibodies simultaneously induced a decrease in viral infection in differentiated cells, supporting the functional role of these receptors in viral entry into HOG cells depending on the differentiation stage. The fact that the combined effect is greater in differentiated HOG cells, where HVEM is more highly expressed, suggest that a basal level of nectin-1 activity is present in all cells. However in differentiated cells the anti-HVEM antibody-alone and in combination with anti-nectin-1 is more potent, thereby highlighting the predominant role of HVEM in differentiated cells. It is noticeable that under our experimental conditions, anti-HVEM and anti-nectin-1 antibodies together did not completely block entry. In other systems, 3-OS HS is the major receptor for HSV-1 [66]. Because nectin-1 and HVEM are not the

Figure 6. Study of viral entry by electron microscopy. HOG cells cultured in GM or DM were mock-infected or infected with HSV-1 at an m.o.i. of 50. At 5, 10, 20 and 30 minutes p.i., cells were fixed, washed and processed by freeze substitution. In cells cultured in DM membrane protrusions similar to planar lamellipodia (A and B) and circular ruffles (C) were observed at 20 minutes p.i., suggesting viral entry by macropinocytosis. Vesicles containing intracellular enveloped virions (D) (arrows) were also observed in cells cultured in DM at 30 minutes p.i., suggesting entry by endocytosis. Intracellular unenveloped virions were observed in cells cultured in GM and DM at 5 minutes p.i. (E and F), suggesting entry by fusion. Samples were examined with a JEM 1010 transmission EM (Jeol, Tokyo, Japan). N = nucleus. M = plasma membrane.
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in viral infection in differentiated cells, supporting the functional role of these receptors in viral entry into HOG cells depending on the differentiation stage. The fact that the combined effect is greater in differentiated HOG cells, where HVEM is more highly expressed, suggest that a basal level of nectin-1 activity is present in all cells. However in differentiated cells the anti-HVEM antibody-alone and in combination with anti-nectin-1 is more potent, thereby highlighting the predominant role of HVEM in differentiated cells. It is noticeable that under our experimental conditions, anti-HVEM and anti-nectin-1 antibodies together did not completely block entry. In other systems, 3-OS HS is the major receptor for HSV-1 [66]. Because nectin-1 and HVEM are not the
only receptors for HSV, we attempted to address the role of 3-OH- HS as an entry receptor in these cells. In preliminary experiments, we were unable to specifically detect 3-OH-HS in HOG cells using antibody HS4C3. However, these data do not allow us to rule out a role for 3-OH-HS in HSV-1 entry into oligodendrocytes. Such an activity could partly account for the residual entry observed in the presence of antibodies blocking nectin-1 and HVEM. 3-OH- HS are generated by six isoforms of HS 3-O-STs. At the moment, there are no available data about the set of 3-O-STs expressed in human oligodendrocytes. Expression of 2-O-ST, the enzyme responsible for 2-O-sulfation, is downregulated during maturation of OLs, although an increase in 2-O-HS has been observed after injury to the adult rat brain [67]. 3-O-STs are often co-expressed in various combinations. While the isoforms 3-O-ST-3, −5 and −6 are most commonly expressed, isoforms 3- O-ST-2 and −4 were undetectable in other cell lines examined [35]. This complexity warrants further experiments to determine the expression pattern of 3-O-STs during oligodendrocyte differentiation and determine whether 3-OH-HS plays a role in HSV entry in these cells.

MAG is a cell-surface molecule expressed in myelin sheath [68,69]. MAG is involved in myelin maintenance and in myelin-axon interactions, acting as an inhibitor of axonal regeneration [70,71]. It has been reported that MAG is associated with HSV-1 gB, suggesting that it is involved in HSV infection of neural tissues [25]. In our cellular model, preliminary studies by RTqPCR revealed a negligible expression of this myelin protein in HOG cells even in differentiation culture conditions (data not shown), thus suggesting that the role of this protein in viral entry into HOG cells is very limited at best.

HSV-1 can enter different cell types using different pathways: fusion at a neutral pH, low-pH-dependent endocytosis and low-pH-independent endocytosis [56,57,72]. We used electron microscopy to define the entry pathways of HSV-1 into OLs. This approach is useful to directly observe virions at various stages of entry. However, interpretation of EM snapshots needs to be related with functional data to validate the fact that observed virions reflect a functional entry pathway. In cells cultured in DM membrane protrusions similar to planar lamellipodia and circular ruffles [73] were observed, suggesting that HSV-1 may be entering OLs by macropinocytosis (depending on their differentiation stage). Similar cellular protrusions have been associated to HSV-1 during entry by phagocytosis-like uptake involving re-arrangement of actin cytoskeleton and trafficking of the viral particles in phagosome-like vesicles. This pH-dependent and clathrin-independent viral entry is characterized by the presence of cell surface protrusions and clustering of gD receptors in large vesicles [58]. In addition, vesicles containing intracellular enveloped virions were also observed in cells cultured in DM, suggesting entry by endocytosis. Further studies will be necessary to define the role of pH in this pathway.

Finally, viruses like HBV, HPV and HIV have been shown to induce cell differentiation [74,75,76,77]. Therefore, we wanted to ascertain not only whether differentiation triggered changes in HOG cell susceptibility to HSV-1, but also whether HSV-1 was able to drive changes in cell morphology compatible with cell differentiation. After infection, immunofluorescence microscopy revealed an increase of PLP in cells infected with HSV-1 cultured in growth conditions. Also, morphological changes corresponding to differentiated cells were also observed in infected cells cultured in GM. Moreover, HSV-1 infected MAL-expressing HOG cells cultured in GM exhibited myelin-like sheets enriched in exogenous MAL. Altogether, these observations indicate that HSV-1 infection can induce the formation of structures corresponding to more differentiated stages of oligodendrocytes. Unexpectedly, partial colocalization between HSV-1 and exogenous MAL appears most prominently in vesicles located at the end of the processes. This raises the possibility that MAL-positive vesicles may be involved in delivering viral particles towards the end of the processes and subsequently, outside the cells. However, further studies will be necessary to specifically address the potential role of MAL in HSV-1 egress.

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Author Contributions

Conceived and designed the experiments: RB-M ET JAL. Performed the experiments: RB-M AJC LAD BG BM. Analysed the data: RB-M ET CK. Contributed reagents/materials/analysis tools: ET CK FDC. The paper: RB-M CK JAL.

References

1. Kakalacheva K, Munz C, Lunemann JD (2011) Viral triggers of multiple sclerosis. Biochim Biophys Acta 1812: 132–140.
2. Ji Q, Perchellet A, Goverman JM (2010) Viral infection triggers central nervous system autoimmunity via activation of CD8+ T cells expressing dual TCRs. Nat Immunol 11: 629–634.
3. Swanborg RH, Whittem-Hudson JA, Hudson AP (2003) Infectious agents and multiple sclerosis—Are Chlamydia pneumoniae and human herpes virus 6 involved? J Neuroimmunol 136: 1–8.
4. Giraudon P, Bernard A (2009) Chronic viral infections of the central nervous system: Aspects specific to multiple sclerosis. Rev Neurol (Paris) 165: 789–795.
5. Sotelo J (2007) On the viral hypothesis of multiple sclerosis: participation of varicella-zoster virus. J Neurovirol 13: 113–116.
6. simulator T (2007) On the viral hypothesis of multiple sclerosis: participation of varicella-zoster virus. J Neurovirol 13: 113–116.
7. Christensen T (2005) Association of human endogenous retroviruses with multiple sclerosis and possible interactions with herpes viruses. Rev Med Virol 15: 179–211.
8. Alvarez-Lafuente R, Garcia-Monjo M, Rojas A, Heras V, Dominguez-Mozo MI, Barcelone M, et al. (2006) Herpesviruses and multiple sclerosis. J Med Virol 80: 60–63.
9. Rizzo R, Gentili V, Casetta I, Caselli E, De Gennaro R, et al. (2013) Altered natural killer cells’ response to herpes virus infection in multiple sclerosis involves KIR/CD152 expression. J Neuroimmunol 251: 55–64.
10. Repici R, Oboros K, Wengel V, Groen F, Kuis K, et al. (2006) Detection of human endogenous retrovirus W protein expression by herpes simplex virus type 1: implications for multiple sclerosis. J Neurovirol 12: 65–71.
11. Pietropaolo V, Fioret D, Musichet M, Anzivino E, Santini M, et al. (2005) Detection of human herpesviruses and polyomaviruses DNA in a group of patients with relapsing-remitting multiple sclerosis. New Microbiol 28: 199–203.
12. Sanders VJ, Wadell AE, Felisan SL, Li X, Conrad AJ, et al. (1996) Herpes simplex virus in postmortem multiple sclerosis brain tissue. Arch Neurol 53: 125–133.
13. Bello-Morales R, Fedetz M, Aicena A, Tabares E, Lopez-Guerrero JA (2005) High susceptibility of a human oligodendroglial cell line to herpes simplex type 1 infection. J Neurovirol 11: 190–196.
14. Ferrante P, Mancuso R, Pagani E, Guerrini FR, Calvo MG, et al. (2000) Molecular evidences for a role of HSV-1 in multiple sclerosis clinical acute attack. J Neurovirol 6 Suppl 2: S109–114.
33. Simpson SA, Manchak MD, Hager EJ, Krummenacher C, Whitbeck JC, et al. (2000) HVEM receptor and its ligand, nectin-1, mediate herpes simplex virus entry into cells in vitro. J Infect Dis 182: 1619–1626.

34. Warner MS, Geraghty RJ, Cohen GH, Eisenberg RJ, Spear PG (1998) Entry of alpha-herpesviruses mediated by poliovirus receptor-related protein 1 (PVR). J Virol 72: 7404–7417.

35. Shukla D, Lin J, Blacklock P, Shwartz NW, Bai X, et al. (1999) A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus type-1 entry. Mol Cell Biol 19: 11042–11051.

36. Suenaga T, Arii J, Suenaga T, Oyama M, Kozuka-Hata H, et al. (2010) Non-cell-cell adhesion. J Cell Sci 123: 3713–3722.

37. Suenaga T, Arii J, Suenaga T, Oyama M, Kozuka-Hata H, et al. (2010) Non-cell-cell adhesion. J Cell Sci 123: 3713–3722.

38. Suenaga T, Arii J, Suenaga T, Oyama M, Kozuka-Hata H, et al. (2010) Non-cell-cell adhesion. J Cell Sci 123: 3713–3722.

39. Suenaga T, Arii J, Suenaga T, Oyama M, Kozuka-Hata H, et al. (2010) Non-cell-cell adhesion. J Cell Sci 123: 3713–3722.

40. Cheshenko N, Trepanier JB, Stefanidou M, Buckley N, Gonzalez P, et al. (2013) HVEM and nectin-1 leads to the induction of intracellular signalling in the absence of virus entry. PLoS One 8: e59650.

41. Cheshenko N, Trepanier JB, Stefanidou M, Buckley N, Gonzalez P, et al. (2013) HVEM and nectin-1 leads to the induction of intracellular signalling in the absence of virus entry. PLoS One 8: e59650.

42. Cheshenko N, Trepanier JB, Stefanidou M, Buckley N, Gonzalez P, et al. (2013) HVEM and nectin-1 leads to the induction of intracellular signalling in the absence of virus entry. PLoS One 8: e59650.

43. Cheshenko N, Trepanier JB, Stefanidou M, Buckley N, Gonzalez P, et al. (2013) HVEM and nectin-1 leads to the induction of intracellular signalling in the absence of virus entry. PLoS One 8: e59650.
74. Sanderson CM, Smith GL (1999) Cell motility and cell morphology: how some viruses take control. Expert Rev Mol Med 1999: 1–16.
75. Lara-Pezzi E, Serrador JM, Montoya MC, Zamora D, Yanez-Mo M, et al. (2001) The hepatitis B virus X protein (HBx) induces a migratory phenotype in a CD44-dependent manner: possible role of HBx in invasion and metastasis. Hepatology 33: 1270–1281.
76. Yankaskas JR, Hazleip JE, Conrad M, Koval D, Lazarowski E, et al. (1993) Papilloma virus immortalized trachéal epithelial cells retain a well-differentiated phenotype. Am J Physiol 264: C1219–1230.
77. Levy DN, Fernandes LS, Williams WV, Weiner DB (1993) Induction of cell differentiation by human immunodeficiency virus 1 vpr. Cell 72: 541–550.