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Differentiated human neural stem cells: a new ex vivo model to study HHV-6 infection of the central nervous system

Lidia De Filippis[a], Chiara Foglieni[b], Sara Silva[b], Angelo Vescovi[a,c], Paolo Lusso[b], Mauro S. Malnati[b,*]

[a] *Stem Cell Research Institute and* [b] *Unit of Human Virology, DIBIT, San Raffaele Scientific Institute 20132 Milan, Milan, Italy*
[c] *Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy*

Abstract

Background: HHV-6 is the etiologic agent of *exanthem subitum*, a pediatric illness that may be associated with clinical and laboratory signs of central nervous system involvement. The absence of suitable experimental models has so far hampered the elucidation of the mechanisms of HHV-6-mediated neural cell damage. Recently, the growing knowledge in neurobiology has permitted the establishment of long-term cultures of human neural stem cells (hNSC) that, by virtue of their self-renewal capacity and multipotentiality, provide a valuable tool for the study of neurodegenerative disorders.

Objectives and Study design: We studied the effects of HHV-6 infection in differentiated cultures of hNSC derived from the telencephalic and diencephalic regions of a 13.5 week post conception (pcw) fetal brain. The prototypic HHV-6 strain GS (subgroup A) was used.

Results: hNSC were differentiated *ex vivo* to obtain mixed cultures encompassing astrocytes, neurons and oligodendrocytes. These differentiated hNSC cultures were found to be susceptible to productive HHV-6A infection, resulting in the formation of syncytia associated with phenotypic alterations.

Conclusion: These results demonstrate that hNSC may provide a physiologically relevant model to investigate the pathogenic role of HHV-6 in central nervous system disorders.

Keywords: HHV-6; Human neural stem cells; Astrocytes; Neurons; Neurologic disorders

1. Introduction

Human herpesvirus 6 (HHV-6) is a β-herpesvirus that is virtually ubiquitous in the human population. Two major variants, A and B, have been identified, which differ on the basis of genomic polymorphisms, antigenicity and host-cell tropism (Lusso, 1996). HHV-6B is the causal agent of *exanthem subitum*, a usually benign febrile disease of infancy that, in some instances, may be accompanied by neurological complications and viral invasion of the CNS (Caserta et al., 1994; Yoshikawa and Asano, 2000).

HHV-6 DNA has been consistently detected in brain tissue of normal individuals (Chan et al., 1999), suggesting that HHV-6 may persist as non-pathogenic resident virus in latently infected neural cells (Cuomo et al., 2001). Both HHV-6 variants have been associated with acute neurological disorders, such as meningitis and encephalitis in immunocompetent individuals (Losada et al., 2003; Sloots et al., 1993; Torre et al., 1998, 2005), as well as in pathological specimens of bone marrow-transplanted patients (Rapaport et al., 2002; Wang et al., 1999; Yoshikawa, 2003). In addition, HHV-6 has been proposed as a cofactor for the development of chronic CNS disorders including multiple sclerosis (MS) and chronic fatigue syndrome (Buchwald et al., 1992; Cermelli et al., 2003; Challoner et al., 1995; Soldan et al., 1997). Subsequent studies have pointed to HHV-6A as the most likely variant involved in both of these diseases (Ablashi et al., 2000; Akhyani et al., 2000; Fogdell-Hahn et al., 2005), in agreement with the peculiar neurotropism suggested for HHV-6A (Donati et al., 2005).

The effects of HHV-6 infection on neural cells are still unknown, largely due to the absence of reliable experimental models. Even though HHV-6 infection of human microglia, astrocyte and oligodendrocyte cell lines has been reported (Albright et al., 1998; Donati et al., 2005; Yoshikawa et al., 2002), only one study to date (He et al., 1996) has documented productive infection of cultured primary fetal astrocytes by both HHV-6A (GS) and B (Z-29) strains.

In this paper, we describe the establishment and characterization of a new model for the study of HHV-6

* Corresponding author. Tel.:+39 02 2643 4903; fax: +39 02 2643 4905.
E-mail address: malnati.mauro@hsr.it (M. Malnati).

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neuropathogenesis based on infection of human neural cells (hNSCs) obtained from human fetal brain stem cells. Once differentiated *ex vivo,* these cells give rise to mixed cultures that encompass neurons, astrocytes, and oligodendrocytes. The existence of neural stem cells has been described in the developing and adult CNS of all mammals, including humans. Neural stem cells are intrinsically endowed of extensive self-renewal ability, which allows, under appropriate conditions, their long-term proliferation and expansive growth in culture. By using chemically defined media and mitogenic stimulation with epidermal growth factor (EGF) (Weiss et al., 1996) and/or fibroblast growth factor 2 (FGF2)(Gage et al., 1995; Kilpatrick and Bartlett, 1993; Gritti et al., 1995, 1996, 1999; reviewed in Galli et al., 2003) hNSC can be isolated and extensively propagated *in vitro*: the recent identification of hNSC in the human fetal and adult CNS (Vescovi et al., 1999a; Ostenfeld and Svendsen, 2004; Carpenter et al., 1999; Nunes et al., 2003; Johansson et al., 1999) has promoted these cells as a faithful model to reproduce *in vitro* the fundamental steps of neurogenesis *in vivo*. Another main property of hNSC is multipotentiality which translates into the capacity to give rise to all three major brain cell types (i.e. neurons, astrocytes and oligodendrocytes) (Davis and Temple, 1994; Gritti et al., 1996; Reynolds and Weiss, 1996). Taking advantage of this model, we investigated the ability of the HHV-6A to infect various neural cell types in differentiated hNSC cultures.

2. Materials and methods

2.1. Propagation and differentiation of hNSC

Human neural stem cells originally isolated from the telencefalic and diencephalic regions of a 13.5 pcw fetus were cultured in the presence of 20 ng/ml of human recombinant EGF and 10 ng/ml of FGF-2 (Tebubio, Milan, Italy), in NS-A basal serum-free medium (Euroclone, Irvine, Scotland) containing 2 mM L-glutamine, 0.6% glucose, 9.6 μg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin (sodium salt, grade II; Sigma-Aldrich, St. Louis, MO, USA) (Vescovi et al., 1999b) to induce hNSC differentiation, individual spheres derived from the expansion of homogeneous cultures (Vescovi et al., 2002) were mechanically dissociated and single cells were transferred at a density of 2.5×10^4 cells/cm^2 onto matrigel (Sigma)-coated 4-well chamber slides in the presence of 20 ng/ml FGF-2. After 72 hours the cultures were shifted to NS-A control medium containing 2% fetal bovine serum (FBS) and grown for two weeks obtaining a mixed culture of neural cells containing astrocytes, neurons and few oligodendrocytes. At this stage, the cells were studied for their susceptibility to HHV-6 infection.

2.2. Infection of differentiated hNSC with HHV-6A

HHV-6A (strain GS) was grown in cord blood mononuclear cells (CBMC) characterized by the absence of endogenous HHV-6 or HHV-7 contaminations, as previously described (Santoro et al., 1999). Differentiated hNSC cultures were infected by adding viral stock suspensions at a multiplicity of infection (MOI) of 10 [i.e., 10 half-maximal culture infectious doses (CID_{50}) per cell]. A small amount (100 μL) of the viral input employed in each experiment was subjected to phenol-chloroform DNA purification, as previously described (Locatelli et al., 2000) and the HHV-6 DNA load was quantified by an HHV-6-specific calibrated Real Time PCR assay (Locatelli et al., 2001). After 24 hours, the infectious medium was removed, the cell cultures rinsed once and grown in fresh culture medium. Two hours later, 200 μL of culture supernatant were collected to assess the residual viral DNA contamination. The infected cultures were followed daily by phase contrast microscopy to assess morphological changes. In each experiment, uninfected cultures and cells treated with UV-inactivated virus (16 J/m^2) served as control. Infected cultures were followed for a maximum of two weeks; each experiment was repeated at least twice.

2.3. Immunostaining and confocal microscopy

Two weeks after infection hNSCs were fixed, with 2% paraformaldehyde in Dulbecco's PBS (DPBS), rinsed with DPBS and submitted to indirect immunofluorescence, employing the following primary antibodies: mouse-anti CD46, (clone J4-48 Serotec, Oxford, UK), mouse anti-HHV-6 p41 early/late protein (clone 9A5D12, kind gift of Dr. Balachandran, University of Kansas, Kansas City, KS, USA), rabbit-anti-β-Tubulin III, rabbit-anti-Glial Fibrillary Acidic Protein (Sigma-Aldrich, St. Louis, MO, USA) and mouse-anti-Galactocerebroside C (MAB345, Chemicon, Temecula, CA, USA). After immersion in 1% BSA in DPBS (blocking solution, 30 min, RT), the cells were incubated with a primary Ab opportunely diluted in blocking solution (2 hrs, RT) and revealed either with rabbit-anti-mouse IgG-AlexaFluor 488, or donkey-anti-goat IgG–AlexaFluor 594 (Molecular Probes, Eugene, OR, USA), or swine-anti-rabbit Ig-TRITC (Dako, Carpinteria, CA, USA) in DPBS (30 min, RT). Double staining was performed repeating the same procedure with a different primary Ab and an opportune secondary Ab. 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) nuclear staining (0.2 nM, 20 min, RT) followed.

The slides, rinsed in DPBS and mounted with Fluorsave (Calbiochem, Merck Eurolab Srl, Milan, Italy), were analyzed with a confocal microscope Leica TCS SP2 (Leica Microsystems, Heidelberg, GmbH); 3D free maximum projections obtained from single channel-collected series of images were superimposed using the Adobe PhotoshopCS software.