Published in final edited form as:

J Virol Methods. 2009 July ; 159(1): 122–126. doi:10.1016/j.jviromet.2009.02.023.

Generation and Characterization of JCV Permissive Hybrid Cell Lines

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Abstract

JC virus (JCV) is a human neurotropic polyomavirus whose replication in the central nervous system induces the fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML). JCV particles have been detected primarily in oligodendrocytes and astrocytes of the brains of patients with PML and in the laboratory its propagation is limited to primary cultures of human fetal glial cells. In this short communication, the development of a new cell culture system is described through the fusion of primary human fetal astrocytes with the human glioblastoma cell line, U-87MG. The new hybrid cell line obtained from this fusion has the capacity to support efficiently expression of JCV and replication of viral DNA in vitro up to 16 passages. This cell line can serve as a reliable culture system to study the biology of JCV host cell interaction, determine the mechanisms involved in cell type specific replication of JCV, and provide a convenient cell culture system for high throughput screening of anti-viral agents.

Keywords

JCV infection; replication; permissive cells; cell to cell fusion; hybrid cells

The lack of a convenient and reliable cell culture system has significantly hampered the ability to study the mechanisms involved in the life cycle of the human polyomavirus, JC virus (JCV), the causative agent of the demyelinating disease, progressive multifocal leukoencephalopathy (PML) (Khalili et al., 2003; Major et al., 1995). Replication of JCV in vitro takes place on an average of three or more weeks in culture (Radhakrishnan et al., 2003). As such, propagation of wild type and mutant viral stocks is a labor intensive and time consuming undertaking. In comparison, the life cycle of the highly related simian virus 40 (SV40) is significantly shorter with death of host cells evident in as little as 24 h post infection. The availability of several kidney epithelial cell lines including CV-1, for cultivation and study of SV40 have helped to decipher the mechanisms involved in gene regulation, viral replication, and virus-host interaction. Unlike SV40, knowledge about the pathways involved in the life cycle of JCV remains more limited, mainly due to the absence of a continuous well characterized cell line that permits efficient replication of JCV. In the laboratory, replication of JCV has been limited to primary cultures of human fetal glial cells and thus far no small animal model that supports viral replication has been identified (for review, see Khalili et al, 2003). The difficulties associated with obtaining human fetal brain tissues for the preparation of primary cultures,

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which are costly, labor intensive, and can vary in purity from preparation to preparation, have prompted several investigators to develop cell lines including SVG and POJ that support the JCV infection cycle (Mandl et al., 1987; Major et al., 1985; Frye et al., 1997). However, the utility of these lines for studying initial events that stimulate viral gene expression and replication is limited as the culture systems are transformed with either SV40 (SVG-A) or JCV (POJ) genomes and constitutively express the T-antigens of these viruses. Thus, the constitutive presence of T-antigen in these cells bypasses the immediate early events in the JCV infection cycle including activation of the early promoter and expression of T-antigen. To overcome this issue, an alternative strategy of cell fusion was employed between permissive primary human fetal astrocytes and the non-permissive human glioblastoma cell line, U-87MG and subsequently several hybrid cell lines were developed to study JCV life cycle.

To create hybrid cell lines, a PEG-mediated cell fusion method with negative selection by HPRT between primary human fetal astrocytes (PHFA) and HPRT deficient U-87MG cells was employed. The efficiency of fusion was evaluated by the appearance of bi-nucleated cells that had a distinct flattened morphology two hours after cell fusion (Figure 1). At twenty-four hours, the morphology of the cells was more elongated and spindle-shaped, and the hybrid clones retained this morphology thereafter (Figure 1). The clones were plated in selective media (HAT-medium) to confirm that they were deficient in HPRT activity (data not shown). Two clones, named HC-7 and HC-15, were selected for further study from a total of thirty-two.

Examination by Western blot analysis of whole cell extracts of the astrocyte marker protein, glial fibrillary acidic protein (GFAP) revealed that, unlike PHFA, which expresses GFAP, neither of these proteins were detected in extracts from U-87MG cells, or in the hybrid cells, HC-7 and HC-15 (Figure 2, Panel A). To compare the growth properties and DNA content of the hybrid cells with the parental cells flow cytometry assay was performed. By determining the cell cycle profile of the clones, the distribution of the cells at G0/G1, S, and G2/M phases and determined the percentage of apoptotic cells was determined. As shown in Figure 2 (Panel B), a modest decline in the cell distribution at G0/G1 of the hybrid clones compared to their parental cells was noted. At the G2/M stage, the profile of the hybrid clones appear to be closer to PHFA than U-87MG (HPRT-) cells. Furthermore, a larger number of apoptotic cells was noted in clonal cells than those in the parental cells as was observed, apoptosis was detected in HC-15 (19.1%) versus PHFA (10.4%) and U-87MG (9.7%). These observations showed a subtle variation in the cell cycle distribution of the U-87MG and PHFA cells upon their fusion. However, no significant differences in the doubling times of the parental and the hybrid cells were observed, which ranged between 27 and 30 hours (data not shown).

To examine the infectivity of these cells with JCV, hybrid cells at passage six after fusion, along with PHFA and U-87MG parental cells were infected with JCV Mad-1 at a multiplicity of infection (MOI) of one. At 7 and 14 days post-infection, cells were harvested and examined for replication of viral DNA and expression of viral proteins by Southern and Western blot analyses, respectively. As expected viral DNA and the early and late viral proteins were detected in infected PHFA but not U-87MG cells (Figure 3, compare lanes 1-3 and 4-6). Viral DNA and proteins were detected in both HC-7 and HC-15 cells at days 7 and 14 after infection (lanes 8 and 9, and 11 and 12). While the levels of viral gene expression and production of T-antigen and VP1 in HC-7 and HC-15 were comparable to those from PHFA, the level of viral DNA replication in HC-15 was noticeably higher than in the PHFA and HC-7 cells. Expression of the unrelated cellular protein, Grb2, served as a control for equal protein loading of the Western blot. Further studies are required to understand the molecular events involved in high efficiency of JCV DNA replication in HC-15 cells.

Immunocytochemical staining of viral proteins in JCV infected cells was determined at day 8 post infection. As seen in Figure 4, consistent with previous observations (Radhakrishnan et

al, 2003), T-antigen and VP1 were detected predominantly in the nucleus of PHFA and HC-7 and HC-15. Further, the late auxiliary protein, agnoprotein, which is critical for viral replication (Akan et al., 2006; Khalili et al., 2005; Sariyer et al., 2006) displayed a mostly perinuclear appearance in both PHFA and the hybrid cells. These observations indicate that similar to primary human fetal astrocytic cultures, the hybrid cells have the capacity to efficiently support viral gene expression and replication.

To test the longevity of the hybrid cells in supporting JCV replication, HC-15 cells at various passages ranging from 6 to 26 were infected with JCV and replication of viral DNA and expression of the viral late protein were determined at day 8 post-infection. As shown in Figure 5, replication of JCV DNA and expression of VP1 was detected in passage 6, 11 and 16, but not 26, suggesting that the hybrid cells lose their ability to support JCV replication after repeated passaging in culture, i.e. >16 passages. Similar results were obtained with HC-7 cells (data not shown). To rule out the involvement of receptors in the loss of permissiveness of high passage hybrid clones, in the next series of experiments, cells were transfected with JCV DNA and replication of the DNA and expression of viral late proteins was determined. As shown in Figure 5C, consistent with the findings from Figure 5A, the replication of the viral genome in the cells at passage 16 is barely detectable and completely abrogated by passage 26, supporting the notion that the loss of the replication of the viral genome in high passage hybrid lines is not due to the loss of receptors or any other barriers imposed during the viral entry process. In addition, results from Western blotting showed complete disappearance of viral late protein expression by passage 26. Altogether, these results show that fusion of PHFA and U-87MG produces cell lines that are permissive for JCV for several passages, at least up to 16.

After attachment of JC virus to the cell membrane and endocytosis, viral DNA enters the nucleus of the infected cells where it can be stimulated by specific transcription factors to produce the early gene product, T-antigen. T-antigen then recruits cellular proteins to stimulate viral DNA replication and late gene expression that results in the production of progeny capsids. In addition, several studies have ascribed a critical role for Agnoprotein in successful virus production (Johannessen et al., 2008; Matoba et al., 2008; Sariyer et al., 2006, 2008). The major distinction between the more ubiquitous SV40 and JCV rests on the tissue specificity of JCV gene expression, which is more restricted. In addition, SV40 and JCV exhibit a high degree of species specificity with viral replication restricted to the natural host, i.e. simian and human.

One of the main drawbacks of hybridoma cell technology is the instability of the genome of hybrid clones (Duelli et al., 2005, 2007; Ganem et al., 2007; Hernandez et al., 1996). It is possible that the gradual loss of JCV replication at the higher passages of the hybrid lines reflects genomic instability of the hybrid clones. It should be noted that JCV proteins, T-antigen and Agnoprotein have been shown to interfere with DNA repair machinery and to induce chromosomal instability (White and Khalili, 2005; Khalili et al., 2003, 2005). Thus, the sensitivity of the hybrid clones to chromosomal damage may be enhanced upon JCV infection, leading to the loss of genes upon repeated passaging that are responsible for expression of specific factors that potentiate the JCV genome. Another possibility for restricted JCV replication at higher passages, though not mutually exclusive, may come from the activation of cell-type specific inhibitors of viral gene expression. This possibility is consistent with earlier fusion studies in which mouse fibroblasts were fused with transformed hamster glial cells and the expression of JCV large T-antigen was lost in the hybrid cells (Beggs et al., 1988; 1990). These observations suggest that expression of negative regulatory protein(s) in the hybrid cells can suppress JCV gene expression and replication. Further studies are required to understand the mechanism for extinction of JCV in the hybrid cells.

In addition to glial origin cells including primary human fetal astrocytes and the T-antigen transformed glial origin cell lines POJ and SVG described above, some strains of JCV have

been able to grow in non-glial cell culture systems (Khalili et al., 2003). These include primary cultures of schwann cells and neuroblastoma cell lines, as well as kidney epithelial cells (Akatani et al., 1994; Assouline and Major 1991; Hara et al., 1998). In addition, JCV has been shown to replicate in hemotopoietic progenitor cells, B lymphocytes, and tonsillar stromal cells with varying efficiency (Monaco et al., 1996). Of note, JCV infection does not appear to be restricted at the level of binding and cell entry (Ashok and Atwood, 2006). The cell lines capable of supporting JCV infection have all been of human origin, with a few notable exceptions being of simian origin (Hara et al., 1998). These findings are consistent with the high degree of species specificity seen with the polyomavirus family and also with the natural history of JCV infection in humans.

As an alternative to primary cultures of human fetal astrocytes, hybrid human cell lines were produced by fusing primary human fetal astrocytes with the human malignant glioblastoma cell line, U-87MG by negative selection using HPRT. Several JCV-permissive hybrid cell lines were identified and two of these clones were examined in detail with respect to hybrid properties and viral propagation. Both hybrid cell lines and PHFA cells were infected and compared with respect to viral transcription and replication. It was demonstrated that the newly-generated hybrid cell lines support JCV replication and viral protein production at multiple passages. The hybrid lines supported JCV replication and propagation at early passages but lost that ability at late passages, suggesting that these cells may provide a system for identifying positive and negative regulators of viral transcription and replication. In addition, these newly-generated hybrid cell lines may serve as good tissue culture model system for JCV replication and propagation and may help us develop more effective strategies to prevent JCV infection in glial cells.

Acknowledgments

Thank you to past and present members of the Department of Neuroscience and Center for Neurovirology for sharing of ideas and reagents and Cynthia Schriver for preparation of the manuscript. This work was supported by grants awarded by NIH to KK.

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Figure 1. Morphological features of parental (U-87MG-HPRT-deficient and PHFA) and hybrid clones (HC-7 and HC-15) before and after the cell fusion process

Phase contrast images of hypoxanthine phosphoribosyl transferase (HPRT)-deficient U-87MG cell line (U-87MG-HPRT-deficient) and PHFA before PEG-mediated fusion (upper panels). The hypoxanthine phosphoribosyl transferase (HPRT)-deficient U-87MG cell line was created from the parental U-87MG cell line by negative selection with 8-azaguanine (Sigma, #A5284) as described previously (Amano et al., 1974). HPRT-deficient U-87MG cells were isolated by selection with 8-azaguanine (Sigma, #A5284) and used as a fusion partner with PHFA cultures which were prepared and maintained as described previously (Radhakrishnan et al., 2003). Polyethylene glycol (PEG) mediated fusion between U-87MG HPRT-deficient and PHFA cells

was performed by combining equal numbers of U-87MG HPRT-deficient and PHFA cells in serum free medium containing 50% PEG 1500 (PEG 1500, Roche) (Beggs et al., 1988). PEG mediated fusion resulted in double nucleated cells 2h after fusion while at 24h after fusion, most of the cells showed a single nucleated phenotype. Fused cells were grown in HAT selection medium for 72h and clonal cell lines were isolated by limiting dilution. Isolated clones HC-1 through HC-32 were further analyzed. Phase contrast images of representative hybrid clones HC-7 and HC-15 at passage 6 show a phenotype distinct from their parental cells (bottom panels).

Figure 2. Marker protein expression and flow cytometric analyses of hybrid clones

(A) Western blot analysis of GFAP expression in parental and hybrid cells. Whole-cell extracts were prepared from U-87MG-HPRT-deficient, PHFA, HC-7, and HC-15 cells, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Expression of the cellular protein, GFAP, was determined using an anti-GFAP antibody. Grb2 was probed as a loading control. (B) Flow cytometric analysis of the parental (U-87MG HPRT-deficient and PHFA) and hybrid clones (HC-7 and HC-15). Cells were fixed in 70% ethanol, stained with Guava Cell Cycle Reagent (Guava Technologies), and DNA content determined with the Guava Easycycle Mini machine according to the manufacturer's instructions (Guava Cell Cycle Reagent, Guava Technologies).

Figure 3. JCV replication and gene expression in hybrid clones (HC-7 and HC-15) and their parental cells

Southern blot analysis of viral DNA isolated from infected cells (U-87MG HPRT-deficient, PHFA, HC-7 and HC-15 cells) (upper panel). Cells were infected with Mad-1 strain of JC virus and maintained in DMEM supplemented with 2% FBS and antibiotics at 37°C until collected for protein extraction or DNA isolation. Transfection/infections were performed as described previously (Sariyer et al., 2006). Low molecular weight viral DNA was purified from cells infected with the Mad-1 strain of JC virus at the indicated time points post-infection using a QIAprep-spin miniprep kit as described previously (Ziegler et al., 2004 and Sariyer et al., 2008). Purified low molecular weight DNA was digested with DpnI/BamHI and resolved by 1% agarose gel electrophoresis. Replicated viral genome was analyzed by Southern blotting using the entire Mad1 genome radiolabeled with [³²P]α-dCTP by Klenow reaction as probe as described previously (Sariyer et al., 2008). In parallel, Western blot analysis was performed on whole cell extracts prepared from U-87MG-HPRT-deficient, PHFA, HC-7 and HC-15 cells uninfected or infected with JC virus. Expression of JCV proteins was detected with antibodies recognizing large T-antigen (pAb416, Oncogene Research Products), and VP1 (pAb597, kindly provided by Walter Atwood). DNA or proteins isolated from uninfected cells served as negative controls (i.e., 0 days). Western blotting with anti-Grb2 antibody (Santa Cruz, C-19) demonstrates the equal loading of the samples (bottom panel).

Figure 4. Immunocytochemistry for viral proteins in hybrid clones and parental cells infected with JCV

U-87MG HPRT-deficient, PHFA and the hybrid clones HC-7 and HC-15 were infected with Mad-1 strain of JCV, seeded onto chamber slides, and fixed with ice-cold acetone 8 days after infection. Immunocytochemistry to detect viral proteins was performed as described previously (Safak et al., 2002; Sariyer et al., 2006) with anti-large T-antigen (pAb416) and anti-VP1 (pAb597) monoclonal antibodies and anti-agnoprotein polyclonal antibody (Del Valle et al, 2002) for 8 h. Cells were then washed with PBS, incubated with FITC-conjugated secondary antibodies for 2 h, then mounted with aqueous mounting medium and examined by indirect immunofluorescence microscopy. Results revealed expression of T-antigen, agnoprotein and VP1 in PHFA, HC-7, and HC-15 cells but not in U-87MG cells.

Figure 5. JCV replication is restricted at later passages of the hybrid clones

Different passage numbers of the hybrid clone HC-15 (P6, P11, P16 and P26) were infected with the Mad-1 strain of JCV and viral DNA was isolated by QIAprep-spin miniprep kit at 8 days post infection. A. Purified low molecular weight DNA was digested with DpnI/BamHI and analyzed by Southern blotting to detect replicated viral DNA. In lane 1, 2 ng of Mad-1 genome digested with BamHI was loaded as a positive control. In lane 2, a DNA sample isolated from uninfected cells was processed as in lanes 3-6 and loaded as a negative control. B. Whole cell extracts were prepared and analyzed for VP1 expression by Western blotting using anti-VP1 antibody. Reprobing of the blot with an anti-Grb2 antibody demonstrated the equal loading of the samples. In lane 1 protein samples prepared from uninfected cells was

loaded as a negative control. **C.** Hybrid clones transfected with JCV Mad-1 genome (10 µg/flask) using lypofectamine (GibcoBRL). Eight days posttransfection, viral DNA was isolated and analyzed by Southern blotting as described for Panel A. **D.** In parallel, whole cell protein extracts were prepared and analyzed by Western blotting as described for Panel B. Trxn: Transfection, Infx: Infection.