Original Article

Characterization of JC Polyomavirus Derived from COS-IMRb Cells

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SUMMARY: JC polyomavirus (JCPyV) causes progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the central nervous system affecting immunocompromised patients. The study of PML-type JCPyV in vitro has been limited owing to the inefficient propagation of the virus in cultured cells. In this study, we carried out long-term culture of COS-7 cells (designated as COS-IMRb cells) transfected with PML-type M1-IMRb, an adapted viral DNA with a rearranged non-coding control region (NCCR). The JCPyV derived from COS-IMRb cells were characterized by analyzing the viral replication, amount of virus by hemagglutination (HA), production of viral protein 1 (VP1), and structure of the NCCR. HA assays indicated the presence of high amounts of PML-type JCPyV in COS-IMRb cells. Immunostaining showed only a small population of JCPyV carrying COS-IMRb cells to be VP1-positive. Sequencing analysis of the NCCR of JCPyV after long-term culture revealed that the NCCR of M1-IMRb was conserved in COS-IMRb cells without any point mutation. The JCPyV genomic DNA derived from a clone of COS-IMRb-3 cells was detected, via Southern blotting, as a single band of approximately 5.1 kbp without deletion. These findings suggest the potential of using COS-IMRb-3 cells as a useful tool for screening anti-JCPyV drugs.

INTRODUCTION

JC polyomavirus (JCPyV) causes progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the central nervous system affecting immunocompromised patients (1). Patients with acquired immunodeficiency syndrome (AIDS) are associated with a higher incidence of PML than patients suffering from other immunosuppressive diseases (2). Given the restricted in vitro propagation of JCPyV, cultured cells that support efficient propagation of JCPyV have not been widely reported. The DNA

sequence of the non-coding control region (NCCR) is categorized based on the two types of JCPyV, designated as the archetype and PML-type. The latter originated from the rearrangement of the archetype sequence (3). Several studies have reported efficient propagation of archetype JCPyV in COS-7 cells, a monkey kidney cell line immortalized by a replication-defective simian virus 40 (SV40) (4–6). In contrast, a study using COS-7 cells transfected with PML-type JCPyV DNA (Mad-1) revealed low production of PML-type JCPyV for several weeks after transfection (4). Persistent infection of the human neuroblastoma cell line IMR-32 with JCPyV strain Mad-1 by serial passages resulted in the generation of adapted viruses consisting three species (M1-IMRa, M1-IMRb, and M1-IMRc) with rearranged NCCR (7,8). The rearranged NCCR was required for the efficient growth of JCPyV in IMR-32 cells, and the highest level of viral propagation among cells transfected with one of three adapted clones was reported in cells transfected with M1-IMRb (8).

In this study, we established COS-7 cells (designated as COS-IMRb cells) generating PML-type JCPyV (M1-IMRb) and characterized their viral replication by analyzing the viral genome. Viral quantification using hemagglutination (HA) and production of viral protein 1

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(VP1) antigen in COS-IMRb cells were also performed. We found that the NCCR of M1-IMRb was conserved during the propagation of JCPyV in a clone of COS-IMRb cells. Moreover, JCPyV genomic DNA derived from COS-IMRb-3 cells was detected, via Southern blotting, as a single band of size approximately 5.1 kbp without deletion.

MATERIALS AND METHODS

Cell lines and plasmids: COS-7 cells and IMR-32 cells were obtained from DS-Pharma Biomedical Co., Ltd. (Osaka, Japan). COS-7 cells are simian virus 40 (SV40)-transformed simian cells that constitutively express the SV40 T antigen (9). IMR-32 cells are a human neuroblastoma cell line (10). COS-7 cells and IMR-32 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. JCPyV M1-IMRb was directly cloned from an adapted virus to IMR-32 cells (8). The M1-IMRb plasmid contained a single copy of the entire JCPyV at the *EcoRI* site of the plasmid vector, pUC19. The M1-IMRb plasmid (8) was kindly provided by Dr. Yogo (The University of Tokyo).

Transfection: COS-7 cells were cultured in 6-well plates and were allowed to reach approximately 70% confluence. JCPyV DNA (1 µg), excised from the M1-IMRb plasmid with *EcoRI*, was transfected using FuGENE 6 transfection reagent (Roche, Indianapolis, IN, USA), as described previously (11). After replacement of the medium 24 h post-transfection, cells were continuously cultured in maintenance medium with transfer at split ratios of 1:3.

DNA replication assay using quantitative PCR: DNA replication assay using quantitative polymerase chain reaction (Q-PCR) was performed to quantify the amount of replicated JCPyV DNA, as described previously (12,13). JCPyV DNA (1 µg), excised from a M1-IMRb plasmid with *EcoRI*, was transfected into both COS-7 and IMR-32 cells using the FuGENE 6 transfection reagent (Roche). After 48 h and 72 h of transfection, the cellular and viral DNAs were extracted from cell cultures using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). The extracted DNA (1.0 µg) was digested using *DpnI* in a volume of 30 µL at 37°C overnight to remove the input JCPyV DNA, because *DpnI* specifically digests the methylated sites on DNA derived from *Escherichia coli*. After heat inactivation of *DpnI* at 80°C for 20 min, we used the digested DNA (160 ng) as a template for Q-PCR. Since the target sequence of Q-PCR contains two *DpnI* sites, the replicated JCPyV DNA (*DpnI*-resistant) in COS-IMRb cells could be amplified with high specificity (13).

Q-PCR analysis was performed using ABI PRISM 7900HI (Applied Biosystems, Foster City, CA, USA). To exclude the influence rendered by the differences in transfection efficiency between COS-7 and IMR-32 cells, Q-PCR was carried out to quantify total JCPyV DNA levels in transfected cells. Total JCPyV DNA involves both input (*DpnI*-sensitive) and replicated (*DpnI*-resistant) DNA. DNA replication was measured by subtracting the input JCPyV DNA from total JCPyV DNA. The transfection efficiency in each sample was

normalized with reference to the amount of input DNA. Data were represented in terms of copy numbers per cell. Experiments were performed using three independent cultures.

HA assay: Confluent cells in 75 cm² flasks were washed twice with phosphate-buffered saline (PBS), resuspended in 500 µL of 1 mM Tris-HCl buffer (pH 7.5) containing 0.2% bovine serum albumin (BSA), and frozen and thawed three times. The lysates were treated with 0.05 mg neuraminidase (Type V; Sigma Chemical Co., St. Louis, MO, USA) at 37°C overnight, and then incubated at 56°C for 30 min. Thereafter, the lysates were centrifuged at 1,500 rpm for 10 min at 4°C and the supernatants were collected. The resultant supernatants were serially diluted with PBS containing 0.2 % BSA and assayed for HA using human group O erythrocytes (KOHJIN BIO CO., Saitama, Japan) as described previously (14). HA assays were performed using three independent cultures.

Immunocytochemical staining: Immunocytochemical staining was performed as described previously (15). COS-IMRb cells after 47 days of culture were plated on a Lab-Tek II Chamber Slide (Thermo Fisher Scientific, Rochester, NY, USA). The culture medium was removed 3 days later, washed twice with PBS, and then fixed in cold acetone at -20°C. The JCPyV VP1 antigen was reacted with an anti-human JCPyV VP1 antibody (Abcam, Cambridge, MA, USA), followed by labeling with a Histofine SAB-PO (MULTI) Kit (Nichirei, Tokyo, Japan) and staining using a Peroxidase Stain DAB kit (Nacalai Tesque, Kyoto, Japan). COS-7 cells were used as a negative control.

Sequencing of the JCPyV NCCR: The JCPyV NCCR was amplified by PCR using two primers, JRR-5 and JRR-6, as described previously (16). The purified PCR products were sequenced using 3.2 pmol of JRR-5 and JRR-6. All NCCR sequences obtained from the three PCR products were compared with the M1-IMRb sequence (GenBank: D17436.1). Sequence alignments were performed using ClustalW on the DNA Data Bank of Japan (DDBJ) website.

Southern blotting: The JCPyV genome was detected by Southern blotting using a digoxigenin-labeled JCPyV probe, as previously described (6). Viral DNA (1 µg) was digested with *BamHI*. The DNA fragments separated by electrophoresis were transferred onto a nylon membrane (Roche) and hybridized with digoxigenin-labeled JCV M1-IMRb DNA. JCPyV DNA was detected using a DIG Luminescent Detection Kit (Roche). The DNA Molecular Weight Marker III (Roche) was used as a size marker.

Statistical analysis: The statistical differences between the groups were determined using Student's *t*-test.

RESULTS

Replication of JCPyV in COS-7 cells and IMR-32 cells: As a DNA replication assay, Q-PCR analysis combined with *DpnI* treatment was carried out to quantify the amount of replicated JCPyV DNA. At 48 h and 72 h post-transfection, total DNA was extracted from the cells. In order to quantify the amount of JCPyV DNA, Q-PCR was performed using three samples. The

Fig. 1. Q-PCR analysis of replicated JCPyV DNA. The replicated JCPyV DNA treated with *DpnI* was quantified using Q-PCR from 3 independent COS-7 and IMR-32 cultures at 48 h and 72 h post-transfection. Data are shown as the mean $\pm$ standard deviation. The amount of the input JCPyV DNAs was subtracted that of replicated DNAs from that of total viral DNAs to measure DNA replication. The transfection efficiency in each sample was normalized with reference to the amount of input DNAs.

Fig. 2. HA titers in COS-IMRb cells. Virus production was quantified in three samples of COS-7 cells and COS-IMRb cells using HA assays. COS-7 cells, used as a negative control, did not exhibit any detectable HA (< 2). 33D, 33 days post-transfection; 40D, 40 days post-transfection; 50D, 50 days post-transfection; 61D, 61 days post-transfection. Data are shown as the mean $\pm$ standard deviation.

Fig. 3. Immunocytochemical staining of VP1 in COS-IMRb cells. JCPyV VP1 antigen was detected using the anti-JCPyV VP1 antibody. The photomicrograph is representative of VP1 antigen (brown) in the nuclei of giant cells in COS-IMRb cells (a), and no signal in COS-7 cells (b) (Magnification $\times 200$).

input JCPyV DNA in transfected cells was quantified to exclude the influence of differences in the transfection efficiency between COS-7 and IMR-32 cells. The amounts of replicated JCPyV DNA from COS-7 cells transfected with M1-IMRb at 48 h and 72 h were 8.1-fold and 8.9-fold ($P < 0.01$) greater than that from IMR-32 cells transfected with M1-IMRb, respectively (Fig. 1). These results suggested that COS-7 cells supported more efficient replication of M1-IMRb than IMR-32 cells.

Propagation of JCPyV in COS-IMRb cells: The HA titers of the lysates derived from COS-IMRb cells at 33, 40, 50, and 61 days post-transfection were 213 ± 73 , 314 ± 147 , 298 ± 195 , and 426 ± 147 HA units, respectively (Fig. 2). The HA titers of non-transfected COS-7 cells were below 2 HA units. These findings

indicate that COS-IMRb cells can efficiently propagate over a long culture period.

Detection of JCPyV VP1 in COS-IMRb cells: The production of JCPyV VP1 in COS-IMRb cells was examined using immunocytochemical staining at 50 days post-transfection. VP1 antigen was detected in the enlarged nuclei of COS-IMRb cells (Fig. 3-a). Immunostaining showed that VP1-positive cells were a small population of COS-IMRb cells. In contrast, JCPyV-positive cells were not detected in the non-transfected COS-7 cells (Fig. 3-b). These findings indicated that COS-IMRb cells represent a JCPyV carrier cell culture.

Sequence analysis of the JCPyV NCCR in COS-IMRb cells: DNA fragments containing the JCPyV NCCR were amplified from the viral DNA of COS-

Fig. 4. PCR analyses of the NCCR of three COS-IMRb clones. The JCPyV NCCR was amplified DNA derived from COS-IMRb cells at 61 days post-transfection using PCR. PCR products were detected by electrophoresis on 1.0% agarose gel. The size (437 bp) of three PCR products was identical to that of M1-IMRb as a positive control. Lane 1, 100 bp DNA ladder size marker; Lane 2, COS-7 cells as a negative control; Lane 3, COS-IMRb clone 1; Lane 4, COS-IMRb clone 2; Lane 5, COS-IMRb clone 3; Lane 6, M1-IMRb plasmid as a positive control.

IMRb cells at 61 days post-transfection using PCR. PCR products were detected by electrophoresis on a 1.0% agarose gel. The sizes (437 base pairs [bp]) of the three PCR products were identical to that of M1-IMRb (Fig. 4). Moreover, the three PCR products also exhibited sequence similarity with that of M1-IMRb (data not shown). These findings indicated that the JCPyV derived from COS-IMRb cells conserved the NCCR of M1-IMRb.

Characterization of the JCPyV in COS-IMRb-3 cells: COS-IMRb-1, 2, and 3 cells were obtained using three independent cultures of COS-7 cells transfected with M1-IMRb by blind passage at split ratios of 1:3. They were used for long-term culture to establish a carrier culture producing high titers of JCPyV. As a comparative experiment, the HA titers of lysates derived from COS-IMRb-1, 2, and 3 cells after 76 days from transfection were measured to be $1,024 \pm 0$, $1,024 \pm 0$, and $2,048 \pm 0$ HA units, respectively (Fig. 5). COS-IMRb-3 cells showed the highest HA activity among the three clones. The COS-IMRb-3 cells were then utilized for Southern blotting. A previous report demonstrated that the detection of JCPyV was based on Southern blotting of the total cellular DNA (13). In general, JCPyV is a cell-associated virus. Since the culture medium supernatant contains smaller amounts of DNA, detection of intact and deleted viral DNA necessitates large volumes of culture medium providing supernatant enriched with DNA. Viral DNA was extracted from COS-IMRb-3 cells and 1 μ g of the same was digested with *Bam*HI. The DNA fragments were separated by electrophoresis and transferred onto a nylon membrane to detect JCPyV DNA in COS-IMRb cells by Southern blotting, using a digoxigenin-labeled JCPyV probe. We detected a single band of approximately 5.1 kbp without deletion (Fig. 6). The size of the 5.1 kbp fragment was identical to that of the complete JCPyV M1-IMRb. The results of Southern blotting showed that no defective interfering (DI) particles were produced by the COS-IMRb-3 cells.

Fig. 5. COS-IMRb-3 cells showed the highest HA activity among three clones. Three clones of COS-IMRb cells (designated COS-IMRb-1,2,3 cells) were subjected to long term culture as a comparative experiment. The HA titers of lysates derived from each clone were determined at 76 days post-transfection described in the text. Data are shown as the mean $\pm$ standard deviation.

Fig. 6. Detection of the JCPyV genome in COS-IMR-3 cells. Low-molecular-weight DNA from COS-IMRb-3 cells at 76 days was digested with *Bam* HI. DNA fragments were separated by electrophoresis and transferred onto a nylon membrane. JCPyV DNA derived from COS-IMRb cells were detected by Southern blotting using a digoxigenin-labeled JCPyV probe. The detected band was the size of approximately 5.1 kbp (arrow). Lane 1, DNA molecular weight marker III, DIG-labeled; Lane 2, COS-7 cells; Lane 3, COS-IMRb-3 cells.

DISCUSSION

Given the limited propagation of JCPyV in cultured cells, little is known about the mechanism by which PML occurs. It has been reported that IMR-32 cells are permissive for PML-type JCPyV and the JCPyV

replicated in IMR-32 cells that contained three rearranged NCCR (M1-IMRa, M1-IMRb, and M1-IMRc) (7,8). These rearranged NCCRs were required for efficient replication of JCPyV within the IMR-32 cells (8). When COS-7 cells were transfected with Mad-1 DNA, the parental viral DNA of M1-IMRb exhibited weak propagation of JCPyV (4). Q-PCR analysis combined with *DpnI* treatment indicated that the amount of replicated JCPyV DNA in COS-7 cells transfected with M1-IMRb was significantly greater than that in transfected IMR-32 cells at 72 h post-transfection. COS-7 cells are known to constitutively express SV40 T antigen, which stimulates the replication of JCPyV DNA (4–6). This finding may be attributed to the expression of SV40 T antigen in COS-7 cells transfected with M1-IMRb. However, multiple host factors participate in JCPyV replication in COS-7 cells. In the future, we aim to isolate the additional host factors participating in JCPyV replication using COS-7 cells.

In this study, we carried out long-term culture of COS-7 cells transfected with M1-IMRb DNA (COS-IMRb cells). The HA titers in COS-IMRb cells were 213 HA units at 33 days and reached a maximum value of 426 HA units at 61 days post-transfection. Thus, the viral copy number seemed to gradually increase after one month of culture. A previous study indicated that the HA titer of COS-7 cells transfected with Mad-1 DNA was 32 HA units at 28 days post-transfection (4). Another study demonstrated that the HA titer of IMR-32 cells transfected with M1-IMRb DNA was 256 HA units at 28 days post-transfection (8). The HA titers of COS-IMRb cells were greater than those of COS-7 cells transfected with Mad-1 DNA and similar to those of IMR-32 cells transfected with M1-IMRb DNA after approximately 30 days of culture. IMR-32 cells transfected with M1-IMRb DNA showed significant cytopathic effects (CPEs). In contrast, no apparent CPE changes were observed in COS-IMRb cells. We previously established JCI cells, which are a carrier cell culture persistently producing PML-type JCPyV by infection with IMR-32-adapted JCPyV (17). Immunostaining in the present study showed that VP1-positive cells were a small population in COS-IMRb cells, similar to the results from the JCI cell study. The JCPyV produced in JCI cells had an alteration in the same NCCR (designated CR-JCI), which was generated from the NCCR of M1-IMRa by deletion of a 28 bp sequence (17). It was shown that CR-JCI was less efficient in inducing viral propagation than the NCCR of IMR-32-adapted JCPyV (17). Thus, the structure of CR-JCI may be important for maintaining the carrier cell culture. Alteration of the NCCR may occur in COS-IMRb cells as well as JCI cells during the long-term culture period. Thus, the NCCR of JCPyV in COS-IMRb cells was sequenced. Unlike JCI cells, the JCPyV produced in COS-IMRb cells maintained the NCCR of M1-IMRb without point mutations. IMR-32 cells, but not COS-7 cells, appeared to allow NCCR rearrangement. The efficiency of JCPyV replication in IMR-32 cells was dependent on the structure of the NCCR. Conversely, SV40 T antigen promoted JCPyV replication in COS-7 cells without NCCR rearrangement. COS-IMRb-3 cells showed the highest

HA activity among the three different cultures. The possibility that the persistent infection of COS-IMRb-3 cells was partly supported by DI particles cannot be excluded. Thus, Southern blotting was performed to assess the characteristics of the viral genome in COS-IMRb-3 cells. Southern blotting revealed JCPyV DNA derived from COS-IMR-3 cells as a single band of approximately 5.1 kbp without deletion. In a previous study, the viral genome derived from JCI cells showed significant variation in the size of the clones, suggesting that JCI cells are likely to produce a significant amount of DI particles (17). In another study, DI particles were found to be involved in maintaining persistent infection with SV40 related to JCPyV (18). COS-7 cells express SV40 T antigen, a strong stimulator of JCPyV replication. T antigen of SV40 and JCPyV possesses DNA binding and helicase activity, which promotes viral replication by arresting the cell cycle at the G2 phase as a result of induction of Ataxia-telangiectasia-mutated (ATM)- and ATM- and Rad3-related (ATR)-mediated G2 checkpoint pathways (19). G2 arrest in IMR-32 cells expressing SV40 T antigen was greater than that in cells expressing JCPyV T antigen (19). In contrast, human embryonic kidney (HEK) cells, which do not express SV40 T antigen, supported the replication of JCPyV (20). It has been shown that the HEK-adapted JCPyV contains two defective mutants (21) and an NCCR rearrangement (22), suggesting that replication may occur via complementation (21). As JCI cells do not express SV40 T antigen, they produce a significant amount of DI particles. Therefore, there is a possibility that the persistent infection of JCI cells was partly supported by DI particles as well as HEK-adapted JCPyV. It is possible that the SV40 T antigen is one of the key factors that support the establishment of persistent infection in COS-IMRb-3 cells where no DI particles are produced.

These findings indicate that COS-IMRb-3 cells are more suitable than the culture system using JCI cells for the stable production of JCPyV used in the screening of anti-JCPyV drugs.

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Conflict of interest None to declare.

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