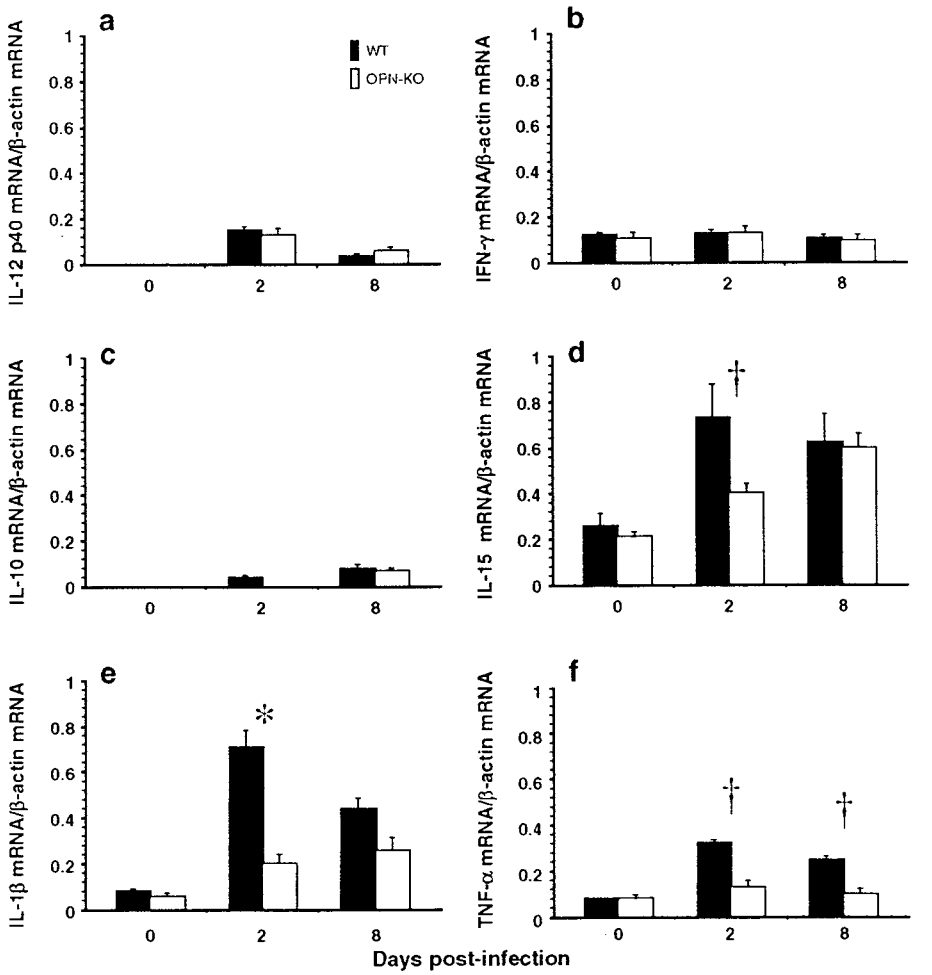

FIG. 4. Comparison of mRNA expressions for IL-12 p40 (a), IFN-γ (b), IL-10 (c), IL-15 (d), IL-1β (e), and TNF-α (f) in WT and OPN-KO mice on day 0 (before infection) and on days 2 and 8 after RV infection (1×10^6 PFU). The mRNAs for IL-12 p40, IFN-γ, IL-10, IL-15, IL-1β, and TNF-α were determined by semi-quantitative RT-PCR. Data are standardized by quantification of β-actin mRNA used as an internal control. Bars represent the mean ± SEM of the results obtained with 10 mice per group in two independent experiments using 5 mice per group. Statistically significant differences between WT and OPN-KO mice (*$p < 0.001$; †$p < 0.05$) are indicated.

Immunohistochemical study of OPN in the striated border, goblet cells, and monocytes/macrophages in the small intestines of WT mice showed OPN-positive reactions on day 2 post-infection (Fig. 2d), although no OPN-positive reaction was observed on day 0 (before infection) (Fig. 2b). On day 8, the striated border showed a slightly positive reaction, although most goblet cells showed a negative reaction (Fig. 2f). In contrast, OPN-KO mice showed negative reactions for OPN (Fig. 2h and j). The same results were obtained in OPN mRNA in small intestinal cell analysis. In WT mice, the expression of OPN mRNA in small intestinal cells was not observed on day 0 (before infection) and then increased 48 h post-infection, but was not detected on day 8 (Fig. 3).

Concerning the role of OPN in immune responses, it has been described that OPN polarizes the Th-1-related cytokine response and contributes to protective immunity against pathogens, such as herpes simplex virus type 1 and *L. monocytogenes* (2,18). OPN is also found to play an active role in adhesion and to induce the migration of macrophages in immune reactions (2,18,21); however, there was no difference in the expressions of mRNA for Th-1-related cytokines, IL-12 p40, and IFN-γ in the small intestine between OPN-KO and WT mice infected with RV (Fig. 4a and b). This appears to be found in both the murine model and human cases. Experiments using IFN-γ-gene- or IFN-γ-receptor-gene-deficient suckling mice demonstrated that IFN does not seem to be a major inhibitor of RV diarrhea (1,27). In human cases, children with typical RV infection do not exhibit significant serum levels of IFN-γ during the acute stage (3).

Expression of IL-10 mRNA in small intestinal cells showed a similar pattern to that of mRNA for Th-1-related cytokines (Fig. 4c). In the analysis of macrophage-related cytokines, mRNA expression of IL-15, IL-1β, and TNF-α in small intestinal cells of WT mice was increased after infection, peaking on day 2, while mRNA expression was generally higher on day 8 in OPN-KO mice (Fig. 4d–f). Expressions of mRNA for IL-15, IL-1β, and TNF-α in WT mice on day 2 post-infection were significantly higher than in OPN-KO mice (Fig. 4d–f).

On day 8, the expression levels of mRNAs were not significantly different between OPN-KO and WT mice, except TNF-α mRNA. These results are supported by previous studies, which demonstrated the rapid production of these cytokines from macrophages after infection, and these cytokines were involved in important non-cellular mechanisms in the immune response to viral infections (4,9). IL-15 is specifically responsible for the upregulation of NK cell activity against viral infections (9). IL-1β also plays a critical role in the inhibition of RV entry into epithelial cells, blocks RV replication, and upregulates NK cells (4,8). Taken together, these cytokines are thought to play a major role in recovery from RV diarrhea, and macrophages and NK cells may act in the innate response to RV infection.

IL-15, IL-1β, and TNF-α are secreted from activated macrophages (17,21,28). In this study, pathological examination showed infiltration of monocytes/macrophages and lymphocytes into the inherent mucosal layer of the small intestine of WT mice on day 2 post-infection (Fig. 2c). These infiltrated cells and the striated border of the small intestine showed the expression of OPN. One study indicated that RV infection of a specific cell was not required to induce OPN, although RV infection induced the production of OPN in intestinal epithelial cells (25). Thus, the expression of OPN seemed to be a secondary stimulation by factors other than RV antigen. Recent studies demonstrated that IL-1 and TNF-α are important factors in OPN expression (17,28). The ability of IL-1-receptor antagonist treatment to inhibit the induction of OPN production clearly demonstrated a role of IL-1 in the upregulation of OPN, which may play a positive feedback role in macrophage activation (28). Similarly, TNF-α induced OPN from macrophages (17). NK cells also induced OPN mRNA expression during macrophage activation (21). Thus, the expressed OPN by cytokine stimulation may recruit macrophages and T cells to the virus-infected site, and may stimulate those cells.

OPN has been found in breast milk as well as intestinal epithelial cells (21,22). OPN in the intestinal lumens of suckling mice consists of OPN in breast milk and from intestinal epithelial cells. OPN, moreover, is recognized by $\alpha_v\beta_3$ and $\alpha_4\beta_1$ integrins as ligands of the cell adhesion domain (5,26). These integrins are also known as receptors for RV entry into cells (11,12). These pieces of evidence suggest that OPN may inhibit RV attachment or entry into cells; however, the role of OPN in the inhibition of RV attachment or entry into cells via a receptor remains unresolved.

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Disclosure Statement

No conflicting financial interests exist.

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ORIGINAL ARTICLE

Inhibitory effect of serotonin antagonists on JC virus propagation in a carrier culture of human neuroblastoma cells

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ABSTRACT

Human polyomavirus, JCV, causes fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML). It has been shown that $5HT_{2A}R$ acts as a cellular receptor for JCV on human glial cells. In the current study, we examined the inhibitory effects of $5HT_{2A}R$ antagonists, ketanserin and ritanserin, both on JCV infection and on propagation by using human neuroblastoma cells IMR-32 and JCI, which continuously produce JCV. Transcriptional analysis revealed that $5HT_{2A}R$ was constitutively expressed in JCI cells. Treatments with $5HT_{2A}R$ antagonists led to a significant reduction in the titers of progeny viruses and the population of infected JCI cells. In addition, the amount of JCV genomic DNA was decreased in JCI cells in the presence of $5HT_{2A}R$ antagonists. These results indicate that $5HT_{2A}R$ antagonists have an inhibitory effect on JCV infection and reproduction, and JCI cells are applicable to an experimental model for pharmacological evaluation of antiviral agents against JCV.

Key words $5HT_{2A}R$ antagonist, JC virus, JCI cell.

JC polyomavirus is a causative agent of a demyelinating disease in the central nervous system, which is known as PML. PML occurs predominantly in immunosuppressed patients, such as those with AIDS and advanced malignancies (1). Several reports have suggested that active antiretroviral therapy against HIV using protease inhibitors is effective in improving the survival of AIDS-related PML patients (2, 3); however, previous studies have failed to confirm the sufficient efficacy of treatments with two antiviral agents against JCV, cytosine arabinoside and cidofovir, in individuals with PML (4, 5).

Several studies have demonstrated that JCV uses $5HT_{2A}R$, which is a serotonin receptor, to infect glial cells and oligodendrocyte progenitor cells and that JCV infection of target cells is impaired in the presence of $5HT_{2A}R$ antagonists, ketanserin and ritanserin (6–8). Thus, these compounds have attracted much interest as potential therapeutic drugs for PML.

In the previous study, we developed a system for long-term culture of human neuroblastoma cells infected with JCV (JCI cells) (9). Considering that JCI cells can be easily passaged in conventional culture medium and that they support the continuous production of high-titer JCV without virus inoculation (9), a carrier culture system of JCI cells may be applicable to an experimental model for pharmacological evaluation of antiviral agents against

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List of Abbreviations: BKV, BK virus; CIAP, calf intestine alkaline phosphatase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutination; HAU, hemagglutination unit; $5HT_{2A}R$, 5-hydroxytryptamine 2A receptor; JCV, JC virus; nt, nucleotide; PML, progressive multifocal leukoencephalopathy.

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JCV. To assess this possibility, we analyzed the effects of $5HT_{2A}R$ antagonists, ketanserin and ritanserin, on JCV propagation in JCI cells.

MATERIALS AND METHODS

Drugs

$5HT_{2A}R$ antagonists, ketanserin and ritanserin, were obtained from Sigma-Aldrich (St Louis, MO, USA) and dissolved in DMSO (Wako Pure Chemical Industries, Osaka, Japan).

Cell culture

JCI cells were obtained by infecting human neuroblastoma IMR-32 cells (10) with the Mad-1 strain of JCV, as described previously (9). JCI cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (9).

Detection of $5HT_{2A}R$ expression in JCI cells by RT-PCR

Total RNAs were extracted from JCI cells using TRIZOL (Invitrogen, Carlsbad, CA, USA), and 1 μg RNA was subjected to cDNA synthesis using SuperScriptIII RNase H-Reverse Transcriptase and oligo(dT)$_{12-18}$ primer (Invitrogen). DNA fragments of the *5HT$_{2A}$R* gene were amplified using a pair of primers termed $5HT_{2A}R$-1 in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The primer sequences and RT-PCR conditions have been described elsewhere (11). Alternatively, RT-PCR was carried out using primers specific to a housekeeping gene, *GAPDH*, as an internal control. The amplified products were separated using 2% agarose gel electrophoresis and photographed.

Infection inhibition experiments

IMR-32 cells were grown to 70% confluency in a six-well plate and pretreated for 24 hr at 37°C in DMEM containing 2% FBS in the absence or presence of ketanserin or ritanserin at final concentrations of 0.1 μM, 1.0 μM and 10 μM. At 24 hr post-treatment, the cells were infected with JCV derived from JCI cells (512 HAU/well) for 1 hr at 37 °C. At 1 day post-infection, cells were harvested and washed twice with PBS. Cellular and viral DNAs were extracted from four or five cultures of the cells using a QIAamp DNA Mini kit (Qiagen). JCV DNAs were then quantitated by real-time PCR according to the protocol described below.

Treatment of JCI cells with $5HT_{2A}R$ antagonists

JCI cells were plated in a 25 cm^2 flask and incubated in culture medium in the absence or presence of ketanserin or ritanserin at final concentrations of 10 μM and 100 μM. Four days after drug treatment, the cells were harvested, passaged at a split ratio of 1:4, and further incubated in fresh culture medium supplemented with each compound. The cells were passaged for 36 days according to this protocol.

Hemagglutination assay

As JCV capsids have the property of agglutinating human type O erythrocytes, and the HA assay has traditionally been used to determine virus titer (12), the cells, which had been untreated or treated with drugs in a 25 cm^2 flask, were resuspended in 1 ml of 1 mM Tris-HCl (pH 7.5) containing 0.2% BSA and subjected to freeze–thaw cycles. The cell lysates were treated with 50 μg/ml neuraminidase (Type V; Sigma-Aldrich) at 37°C overnight, incubated at 56°C for 30 min, and centrifuged at 200 $\times g$ for 10 min at 4°C. The supernatant was serially diluted with 50 μl PBS containing 0.2% BSA in a 96-well round microplate (Costar, Cambridge, MA, USA). Following incubation at 37°C for 1 hr, the sample was mixed with 50 μl of 0.5% human type O erythrocytes (provided by the Hyogo Red Cross Blood Center, Hyogo, Japan) and was further incubated for 3 hr at 4°C. The HA titer was defined as the reciprocal of the greatest dilution of virus suspension with which complete HA was observed.

Immunocytochemical staining

JCI cells, which had been cultured for 36 days in the absence or presence of compounds, were washed twice with PBS and then fixed in cold acetone for 10 min at −20 °C. The cells were treated with H_2O_2-methanol to inactivate endogenous peroxidase. A mouse monoclonal antibody (CLA375) against JCV and BKV (13) was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY, USA) and used at a dilution of 1:40 in PBS containing 1.0% BSA according to the manufacturer's instructions. Antibody CLA375 is recommended to detect JCV-infected cells using immunofluorescence and immunocytochemistry. Fixed JCI cells were reacted overnight at 4°C with the above-mentioned antibody, labeled with Histofine SAB-PO (MULTI) kit (Nichirei, Tokyo, Japan), and stained with DAB. The immunostained cells were counterstained with hematoxylin, mounted in Entellan neu (Merck-Rahway, NJ, USA), and examined under a microscope (Olympus, Tokyo, Japan). The percentages of JCV-positive cells were

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calculated with reference to the total cell number (≈ 1000 cells) in each of three fields.

Plasmid

The standard plasmid for quantification of JCV genome DNA copies using real-time PCR was constructed as described below. Cellular and viral DNA was extracted from IMR-32 cells, which had been transfected with Mad-1/CR-JCI DNA containing the viral genome of the JCV Mad-1 strain (9). The above-mentioned DNA (1 μg) was mixed with 25 μl reaction mixture containing PCR buffer, 200 μM of each dNTP, 0.4 μM of each primer, and 2.5 U Ex *Taq* polymerase according to the manufacturer's recommendations (Takara Bio, Kyoto, Japan). A pair of primers termed JC-VP3 (5′-CATAggtaccATGGCCCC-AACAAAAAGAAAAG-3′; nt 1459–1490) and JC-VP4 (5′-ATTAatcgatAGGCTTTTGATTACAGCATT-3′; nt 2553–2524) was designed to amplify the full-length sequence of the JCV *VP1* gene. Lowercase letters in primer sequences represent *Kpn* I (ggtacc) and *Cla* I (atcgat) restriction sites. After enzyme activation at 95°C for 45 s, the amplification reaction was carried out for 30 cycles. The cycle profile was 94°C for 45 s, 53°C for 45 s, and 72°C for 4 min. Both activation and amplification were carried out in a Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). The amplified fragments were cleaved with a combination of *Kpn* I and *Cla* I (Takara Bio) and ligated to pBluescript SK(+) (Stratagene, La Jolla, CA, USA), which had been digested with *Kpn* I and *Cla* I and dephosphorylated with CIAP (Gibco BRL, Rockville, MD, USA). The ligation products were used to transform competent cells, *Escherichia coli* DH5α (TOYOBO, Osaka, Japan), and recombinant plasmids were prepared using a Qiagen Plasmid Mini kit (Qiagen, Hilden, Germany.). The identity of the inserted fragment was confirmed by DNA sequencing using a BigDye Terminator v1.1 Cycle Sequencing Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Real-time PCR analysis

JCI cells were cultured in the absence or presence of ketanserin or ritanserin at a final concentration of 10 μM for 21 days, and then cellular and viral DNA was extracted from four cell cultures using a QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). Each DNA sample was recovered by ethanol precipitation and dissolved in 50 μl distilled water. For real-time PCR analysis, PCR primers and a TaqMan probe were designed using Primer Express Software (Applied Biosystems). The nucleotide sequences of the primers and the TaqMan probe were as follows: primer JC-VP-F, 5′-TTGAAAGTGACTCCCCAAATAGG-3′ (nt 1671–1693); primer JC-VP-R, 5′-CCACAGGTTA-GATCCTCATTTAGATTG-3′ (nt 1761–1735); TaqMan probe JC-VP1, 5′-FAM-ACATGCTTCCTTGTTACAGT-GTGGCCAGA-TAMRA-3′ (nt 1695–1723). Aliquots of each DNA extract (1 μl) were mixed with 24 μl reaction mixture containing 2×TaqMan universal PCR master mix (Applied Biosystems), JC-VP-F (300 nM), JC-VP-R (300 nM) and JC-VP1 (200 nM). For the absolute quantification of JCV genome copies, the above-mentioned plasmid containing a DNA fragment of the *JCV-VP1* gene was serially diluted from 10^6 to 10^{10} copies per reaction and was used as a standard DNA template. Real-time PCR amplification was carried out using an ABI PRISM 7900HI (Applied Biosystems). The amplification conditions were as follows: 2 min at 60°C, 10 min at 95°C, 40 cycles of 95°C for 15 s and 60°C for 60 s. As an endogenous reference, the copy number of the housekeeping gene (*β-actin* gene) in each DNA extract was determined by real-time PCR using TaqMan *β*-actin Control Reagents (Applied Biosystems). The copy number of JCV DNA in each sample was normalized to *β-actin* gene copies, and the percentages of the amounts of JCV DNA were calculated with reference to the values for drug-untreated cells.

RESULTS

Detection of $5HT_{2A}R$ expression in JCI cells

We first examined whether JCI cells constitutively express $5HT_{2A}R$. Total RNAs were extracted from JCI cells, and first-strand cDNAs were generated by a reverse transcription reaction. The expression of the $5HT_{2A}R$ gene in JCI cells was examined using RT-PCR analysis. Two reactions were carried out in the absence of RT product as a negative control. The $5HT_{2A}R$-specific product (141 bp) was amplified from total RNA extracted from JCI cells (Fig. 1). These results indicate that $5HT_{2A}R$ is constitutively expressed in JCI cells.

Inhibitory effect of $5HT_{2A}R$ antagonists on viral infection to IMR-32 cells

We examined whether $5HT_{2A}R$ antagonists inhibit JCV infection to IMR-32 cells. The cells were pretreated in the absence or presence of ketanserin or ritanserin (0.1, 1.0 and 10 μM each) for 24 hr at 37°C, and then treated cells were infected with JCV. Infectivity indicated the copy numbers of JCV DNA in cells using real-time PCR. As shown in Figure 2, the amounts of JCV DNA in ketanserin-treated IMR-32 cells were 51.3 ± 7.8% (0.1 μM), 64.3 ± 10.9% (1.0 μM) and 41.2 ± 22.3% (10 μM) compared to that in the DMSO-treated control, respectively. The amount of JCV DNA in ritanserin-treated cells was similar to that seen in ketanserin-treated cells, 75.1 ± 22.8% (0.1 μM), 55.3 ± 22.4% (1.0 μM) and

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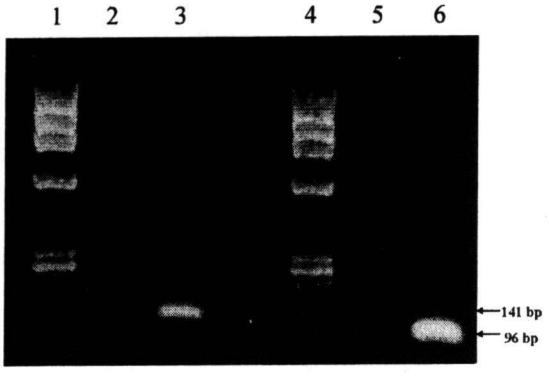

Fig. 1. Detection of $5HT_{2A}R$ expression in JCI cells by RT-PCR. Total RNA was extracted from JCI cells and subjected to RT-PCR analysis using $5HT_{2A}R$-1 primers. GAPDH was used as an internal control. $5HT_{2A}R$-specific products (141 bp) were amplified from JCI cells. The bands of GAPDH DNA were 96 bp. Lane 1, ΦX-174 *Hae* III size marker; Lane 2, $5HT_{2A}R$ of negative control; Lane 3, $5HT_{2A}R$ of JCI cells; Lane 4, ΦX-174 *Hae* III size marker; Lane 5, GAPDH of negative control; Lane 6, GAPDH of JCI cells.

45.2 ± 22.3% (10 μM), compared to that in the DMSO-treated control. These results indicate that $5HT_{2A}R$ antagonists, ketanserin and ritanserin, inhibit JCV infection to IMR-32 cells.

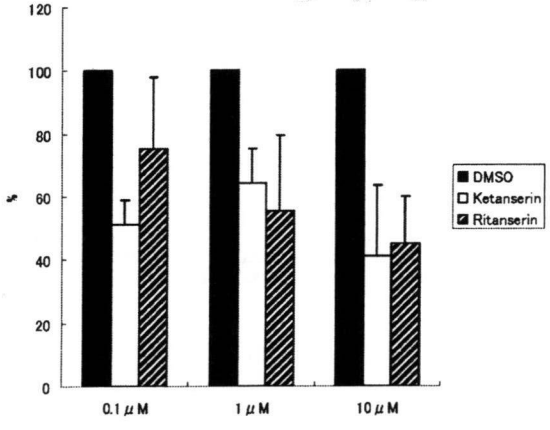

Fig. 2. Real-time PCR analysis of the amount of JCV genomic DNA in IMR-32 cells infected with JCV. IMR-32 cells were pretreated with DMSO (negative control), ketanserin, and ritanserin (0.1, 1.0 and 10 μM each) for 24 hr at 37°C. At 24 hr post-treatment, the cells were infected with JCV derived from JCI cells (512 HAU/well) for 1 hr at 37°C. At 1 day post-infection, cells were subjected to the quantification of viral DNA using real-time PCR analysis. The copy number of JCV DNA was normalized to the number of *β-actin* gene copies. The results are shown as percentages of the amount of JCV DNA with reference to the values for the DMSO-treated control.

Table 1 Inhibition of JCV propagation by $5HT_{2A}R$ antagonists in JCI cells

		HA titers on indicated days after treatment			
Drug	Expt. no.	19	23	28	36
DMSO	1	256	256	512	1024
	2	64	256	512	1024
Ketanserin†	1	32	128	128	128
	2	64	256	256	256
Ritanserin†	1	64	64	64	64
	2	32	64	64	64

†Concentration of 10 μM.

Inhibitory effect of $5HT_{2A}R$ antagonists on JCV propagation in JCI cells

We next examined whether $5HT_{2A}R$ antagonists inhibit the production of virus progenies in JCI cells. JCI cells were harvested on days 19, 23, 28 and 36 after treatment with each compound, and the virus titer was determined using the HA assay, as described earlier (Table 1). In the DMSO-treated control, HA titers of JCI cell extracts gradually increased between 19 and 36 days after incubation and peaked at 1024 HA units on day 36. In the presence of ketanserin, HA titers of cell extracts markedly decreased when compared to that in the DMSO-treated control. A more marked decrease of HA titers of cell extracts was seen when JCI cells were treated with ritanserin. Under the assay conditions, these inhibitors at a concentration of 10 μM did not induce any cytotoxic effects on JCI cells, but these inhibitors could not be examined at a concentration of 100 μM on JCI cells because of toxicity (data not shown). These results indicate that $5HT_{2A}R$ antagonists, ketanserin and ritanserin, inhibit JCV propagation in JCI cells.

Inhibitory effect of $5HT_{2A}R$ antagonists on viral protein production in JCI cells

To confirm that $5HT_{2A}R$ antagonists inhibit JCV production in JCI cells, we examined whether these compounds affect the number of JCV-infected cells (Fig. 3). In the DMSO-treated control, JCV antigens were detected in the nuclei of a small proportion of JCI cells (Fig. 3); occasionally, the nuclei of some giant cells were also stained. On day 36, the percentages of JCV-positive cells in the presence of DMSO, ketanserin and ritanserin (10 μM each) were 2.5%, 1.5% and 0.5%, respectively (Fig. 3). In the presence of ketanserin, the percentage of JCV-positive cells decreased to 57.9 ± 6.1% when compared to DMSO-treated cells. A more significant decrease in the number of JCV-positive cells was seen when JCI cells were treated with ritanserin (19.0 ± 0% compared to that in the

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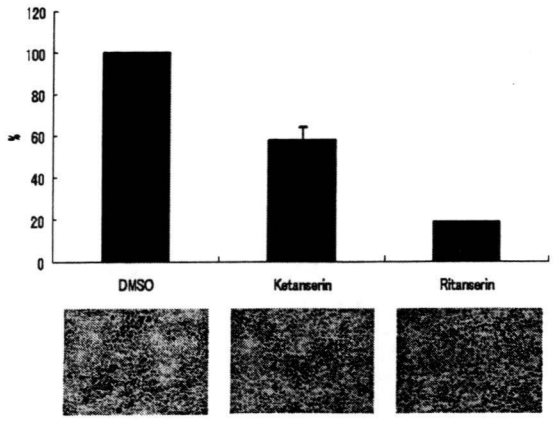

Fig. 3. Immunocytochemical staining of JCV protein in JCI cells in the presence of $5HT_{2A}R$ antagonists. JCI cells were treated with DMSO (negative control), ketanserin, and ritanserin (10 μM each) for 36 days, and JCV proteins were stained with anti-JCV antibody. Representative micrographs of JCV-positive nuclei in JCI cells are shown (brown). Percentages of the numbers of JCV-positive cells were calculated with reference to the values for the DMSO-treated control.

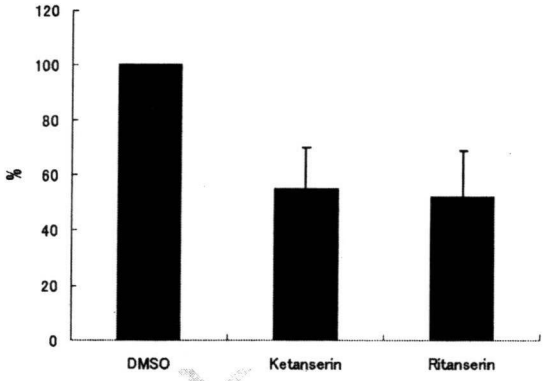

Fig. 4. Real-time PCR analysis of the amount of JCV genomic DNA in JCI cells in the presence of $5HT_{2A}R$ antagonists. JCI cells were treated with DMSO (negative control), ketanserin, and ritanserin (10 μM each) for 21 days and subjected to the quantification of viral DNA using real-time PCR analysis. The copy number of JCV DNA was normalized to the number of *β-actin* gene copies. Results are shown as percentages of the amount of JCV DNA with reference to the values for the DMSO-treated control.

DMSO-treated control). These results indicate that $5HT_{2A}R$ antagonists, ketanserin and ritanserin, inhibit production of JCV protein in JCI cells.

Inhibitory effect of $5HT_{2A}R$ antagonists on synthesis of viral genome DNA in JCI cells

We examined whether $5HT_{2A}R$ antagonists inhibit the amount of JCV genomic DNA in JCI cells. The cells were cultured in the absence or presence of ketanserin or ritanserin (10 μM each) for 21 days, and the copy numbers of JCV DNA were measured using real-time PCR. As shown in Figure 4, the amount of JCV DNA in ketanserin-treated JCI cells was 55.0 ± 15.0% when compared to that in the DMSO-treated control. The amount of JCV DNA in ritanserin-treated cells was similar to that seen in ketanserin-treated cells (51.9 ± 16.7% compared to that in the DMSO-treated control). These results indicate that $5HT_{2A}R$ antagonists, ketanserin and ritanserin, inhibit genome DNA synthesis of JCV in JCI cells.

DISCUSSION

The incidence of PML has increased due to the AIDS pandemic and the widespread use of immunosuppressive drugs (14). Although a common consensus regarding PML therapy has not yet been reached, recent studies suggest that several compounds, such as the cyclin-dependent kinase inhibitor, R-Roscovitine (Calbiochem, Darmstadt, Germany), small interfering RNAs aimed at viral proteins, and $5HT_{2A}R$ antagonists, have an inhibitory effect on JCV infection (6–8, 15–17). Of these compounds, $5HT_{2A}R$ antagonists have generated considerable interest as potential therapeutic agents for PML since they have been used clinically.

JCI cells are derived from human immortalized neuroblastoma cells (IMR-32), a commonly used cell line for pharmacological studies, and are capable of supporting the long-term production of high-titer JCV without virus inoculation (9). Thus, we examined the inhibitory effect of $5HT_{2A}R$ antagonists on JCV propagation to elucidate whether the culture system of JCI cells can be applied to assess the antiviral activity of drug candidates for the treatment of PML.

In our observations, JCV proteins were detected in a small portion of JCI cells, as assessed by immunocytochemical assay, suggesting that JCI cells are a mixed population of JCV-infected and uninfected cells. We also found that a JCV receptor, $5HT_{2A}R$, is constitutively expressed in JCI cells. Based on these data, it is likely that continuous production of JCV in a carrier culture of JCI cells is a sequential multi-step process in which newly produced viruses can infect the remaining uninfected cells. Thus, we think that the culture system of JCI cells may serve as an experimental model that mimics multi-step propagation of JCV in the brain of PML patients.

In the present study, we examined the inhibitory effects of $5HT_{2A}R$ antagonists, ketanserin and ritanserin, both on JCV infection and on propagation using human neuroblastoma cells, IMR-32 and JCI cells. Infection inhibition experiments indicated that $5HT_{2A}R$ antagonists significantly inhibited JCV infection to IMR-32 cells by real-time

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PCR analysis. The inhibitory effect was similar to that seen in previous reports (6–8). Moreover, as assessed from HA assay, immunocytochemical staining, and real-time PCR analysis data, we observed that $5HT_{2A}R$ antagonists, ketanserin and ritanserin, have an inhibitory effect on JCV propagation in JCI cells. The antiviral activity of $5HT_{2A}R$ antagonists against JCV propagation in JCI cells is consistent with earlier results indicating that these compounds inhibit JCV infection of glial cells and oligodendrocyte progenitor cells (6–8). We believe that one of the values of this study is in demonstrating that $5HT_{2A}R$ antagonists inhibit multi-step viral propagation in a mixed population of JCV-infected and -uninfected cells. Considering that $5HT_{2A}R$ antagonists inhibit the entry step of JCV infection but are less effective at reducing viral loads in an already established infection (8), it seems reasonable that these compounds prevent newly produced viruses from binding to the remaining uninfected cells in a carrier culture of JCI cells.

In ritanserin-treated JCI cells, the virus titers of progeny viruses and the percentages of JCV-positive cells were significantly lower than those in ketanserin-treated cells, as judged by HA assay and immunocytochemical staining; however, the reduction of JCV genomic DNA in ritanserin-treated JCI cells was similar to that seen in ketanserin-treated cells. Although the reason for this difference is currently unclear, it is possible that JCV DNA is efficiently replicated after virus entry even when the attachment of JCV to uninfected cells is critically impaired in the presence of ritanserin. Thus, in a culture system of JCI cells, it is thought that HA assay and immunocytochemical analysis are suitable to evaluate drugs targeting the early stage of JCV infection, such as virus binding and entry into cells.

In conclusion, the results obtained in the present study demonstrate that $5HT_{2A}R$ antagonists suppress JCV propagation in human neuroblastoma JCI cells. Our study provides an experimental model for pharmacological evaluation of potential therapeutic compounds for the treatment of PML.

ACKNOWLEDGMENT

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ORIGINAL ARTICLE

Archetype JC virus efficiently propagates in kidney-derived cells stably expressing HIV-1 Tat

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ABSTRACT

Pathogenic JCV with rearranged regulatory regions (PML-type) causes PML, a demyelinating disease, in the brains of immunocompromised patients. On the other hand, archetype JCV persistently infecting the kidney is thought to be converted to PML-type virus during JCV replication in the infected host under immunosuppressed conditions. In addition, Tat protein, encoded by HIV-1, markedly enhances the expression of a reporter gene under control of the JCV late promoter.

In order to examine the influence of Tat on JCV propagation, we used kidney-derived COS-7 cells, which only permit archetype JCV, and established COS-tat cells, which express HIV-1 Tat stably. We found that the extent of archetype JCV propagation in COS-tat cells is significantly greater than in COS-7 cells. On the other hand, COS-7 cells express SV40 T antigen, which is a strong stimulator of archetype JCV replication. The expression of SV40 T antigen was enhanced by HIV-1 Tat slightly according to real-time RT-PCR, this was not closely related to JCV replication in COS-tat cells. The efficiency of JCV propagation depended on the extent of expression of functional Tat. To our knowledge, this is the first report of increased production of archetype JCV in a culture system using cell lines stably expressing HIV-1 Tat. We propose here that COS-tat cells are a useful tool for studying the role of Tat in archetype JCV replication in the development of PML.

Key words archetype JCV, COS-7, HIV-1 Tat, stable expression.

JCV is an etiologic agent of PML, a fatal demyelinating disease of the central nervous system in immunocompromised patients (1). The promoter/enhancer region (designated regulatory region) is located between the DNA replication origin and agnoprotein gene in JCV genomic DNA. It is well known that the regulatory region of JCV isolated from PML-type JCV contains hypervariable sequences (2).

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List of Abbreviations: AIDS, acquired immunodeficiency syndrome; BKV, BK virus; BKVAN, BK virus-associated nephropathy; CPE, cytopathic effect; DAB, 3,3'-diaminobenzidine tetrahydrochloride; FBS, fetal bovine serum; HA, hemagglutination; HIV-1, human immunodeficiency virus-1; JCV, JC virus; LTR, long terminal repeat; luc, luciferase; MEM, minimum essential medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pLTR-luc, HIV-1 LTR-dependent reporter gene; PML, progressive multifocal leukoencephalopathy; PML-type JCV, JCV isolated from the brain of PML patients; pRSV-β gal, RSV promoter-dependent expression vector for β-galactosidase; RLU, relative light units; RT, reverse transcriptase; SV40, simian virus 40.

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On the other hand, archetype JCV infection in children is asymptomatic and results in establishment of persistent infection in the kidneys. Virus progenies of archetype JCV which have persistently infected renal tissues are excreted in urine. It has been reported that the regulatory region of archetype JCV isolated from the urine of healthy individuals has a single enhancer and no rearrangements (3). Archetype JCV has not been described as an etiologic agent in urogenital disease but has been found in the urine of various individuals throughout the world (4–6). Thus, it is postulated that archetype JCV circulates in the human population.

AIDS-related PML has become prevalent, and it is now found in approximately 4% of all AIDS patients (7). The high incidence of PML among individuals with AIDS in comparison to other immunocompromised patients implies that the presence of HIV-1 in the brain of infected individuals is closely associated with the pathogenesis of AIDS-related PML. It has been proposed that, during persistence in PML patients, non-pathogenic archetype JCV is converted to pathogenic PML-type virus (8,9). Although rearrangement of the regulatory region of archetype JCV is thought to be an important event in the pathogenesis of PML, little is known regarding what induces this rearrangement (1).

The host cell range of archetype JCV is strictly restricted in cultured cells. A previous report demonstrated that archetype JCV replicates only poorly in POJ cells, which constitutively express JCV T antigen (10). It has also been reported by others that archetype JCV efficiently replicates in simian kidney-derived COS-7 cells expressing simian virus 40 (SV40) T antigen, indicating that this cell line is a suitable model system for studying the growth characteristics of archetype JCV (11).

HIV-1, the causative agent of AIDS, encodes Tat protein, which is a potent trans-activator and is essential for virus transcription (12). A previous report has shown that Tat protein significantly enhances expression of the late promoter-dependent reporter gene of JCV in glial cells (13). Recently, it has also been shown that Tat protein weakly activates replication of archetype JCV in glial cells (14). Since archetype JCV replicates mainly in renal tissue, a culture system using kidney-derived cells expressing Tat was considered to be a useful tool for studying HIV-induced replication of archetype JCV. For this purpose, we have established and characterized COS-7 cells stably expressing Tat (COS-tat cells). Using COS-tat cells, we have studied whether expression of HIV-1 Tat protein affects propagation of archetype JCV. Our results demonstrate that, compared with COS-7 cells, COS-tat cells support efficient replication of archetype JCV. As far as we know, this is the first report of increased production of archetype JCV in a culture system using cell lines stably expressing HIV-1 Tat. We propose that COS-tat cells are useful tools for assessing the roles of Tat in archetype JCV replication.

MATERIALS AND METHODS

Cells and plasmids

COS-7 cells (ATCC CRL 1651) were incubated at 37°C with 5% CO_2 in MEM containing 10% FBS. The expression vector for HIV-1 Tat was constructed as described below. Wild-type *tat* cDNA encoding 86 amino acids was cloned into pUC19 at *Bam* HI and *Pst* I sites (pUC19-tat86) (15). *Tat* cDNA prepared from pUC19-tat was subcloned into pcDNA 3.1/Zeo (+) (Invitrogen, Carlsbad, CA, USA). The resulting plasmid, pcDNA-tat86, was purified using a plasmid purification kit (Qiagen, Hilden, Germany). pLTR-luc contains the HIV-1 LTR sequence upstream of the luciferase gene (16). To generate pRSV-β gal, the CMV promoter sequence (*Bgl* II-*Hind* III fragment) in pcDNA3.1/His B/lacZ (Invitrogen) was replaced with the RSV promoter (corresponding to the *Bgl* II-*Hind* III fragment) of pRc/RSV (Invitrogen).

Establishment of COS-7 cells stably expressing HIV-1 Tat

COS-7 cells were cultured in a 35-mm dish containing 2 ml MEM-10% FBS until the density of monolayer cells reached 50–80% confluency. The culture medium was then replaced with serum-free medium (OPTI-MEM I; Gibco BRL, Gaithersburg, MD, USA), and cells were transfected with 8 μg pcDNA-tat86 using Lipofectamine reagent (Gibco BRL), according to the manufacturer's instructions. The cells were incubated with transfection complex for 5 hr at 37°C, and then the complex was removed and replaced with complete growth medium.

When the cell monolayer reached confluence, cells were reseeded at 1:15 and cultured at 37°C in an CO_2 incubator for 16 hr. Then, 800 μg/ml Zeocin (Invitrogen) was added to the culture medium, and cells were further cultured for two weeks to select the cells expressing HIV-1 Tat. Finally, Zeocin-resistant colonies were isolated using cloning rings (Iwaki, Tokyo, Japan). Cell clones expressing *tat* mRNA were screened by RT-PCR. Total cellular RNA was extracted from Zeocin-resistant cell clones with TRIzol (Gibco BRL). One microgram of RNA was treated with 3 units of DNase I, Amp Grade (Gibco BRL) for 15 min at room temperature, heated to 65°C for 10 min, and then introduced into the RT reaction, as follows: DNase I-treated RNA was mixed with 30 μl of a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 7.5 mM $MgCl_2$, 10 mM DTT, 625 μM (each) deoxynucleotide triphosphate, 20 U RNase inhibitor (Toyobo, Osaka,

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Japan), 10 U Moloney murine leukemia virus reverse transcriptase RNase H^- (Toyobo), and 40 pmol oligonucleotide dT_{15} (Roche, Indianapolis, IN, USA). The RT mixture was incubated for 1 hr at 37°C, heated to 95°C for 5 min, and then added to the PCR mixture.

ExTaq polymerase, PCR buffer and other reagents were obtained from Takara Shuzo (Shiga, Japan). RT-PCR assay primers were designed as described previously (15). PCR amplification was performed using a Thermal Cycler (Perkin-Elmer, Cetus, CT, USA). The PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Reporter gene assay

Seven clones of COS-7 cells expressing HIV-1 *tat* mRNA, as well as COS-7 cells, were seeded at a density of 2×10^4 cells/well, and cultured until the cell monolayer reached 50–80% confluency. Then, cells were transfected with 100 ng each of pLTR-luc and pRSV-β gal using Lipofectamine reagent (Gibco BRL), as described above. As a positive control, cells were transfected with 100 ng each of pcDNA-tat86, pLTR-luc, and pRSV-β gal. Plasmid pRSV-β was used to monitor transfection efficiency. At 48 hr after transfection, the cells were washed once with PBS and lysed by freeze-thawing in 100 μl reporter lysis buffer (LCβ PCG-51; Toyo Ink, Tokyo, Japan). The supernatants (lysates) from COS-7 and COS-tat cells were clarified at 200 *g* for 5 min at 4°C, and luciferase activity in 20 μl lysate was determined using a luciferase assay system (Promega, Madison, WI, USA). In addition, lysates (20 μl) were assayed for β-galactosidase activity using the Galacto-light Plus system (Applied Biosystems, Foster City, CA, USA). Both luciferase and β-galactosidase activities were measured using a microplate luminometer, TR717 (Applied Biosystems). Luciferase activity was normalized with reference to β-galactosidase activity, and the results are shown as the relative luciferase activity.

Real-time RT-PCR for expression of *tat* and SV40 T antigen

Real-time RT-PCR analyses were performed to examine the extent of expression of *tat* (16) and SV40 T antigen (17), essentially as described previously. Briefly, RNA extraction and RT reaction were performed according to the RT-PCR protocol to screen cell clones expressing *tat* mRNA. In addition, simian β-actin was quantitatively amplified with a probe and primers designed based on the sequence data of *Macaca fuscata*, using Primer Express Software (Applied Biosystems). For real-time RT-PCR analysis of simian β-actin, sense primer, 5′-CCTGAGGCTCTCTTCCAACCTT-3′; antisense primer, 5′-CGTCACACTTCATGATGGAGTTGA-3′; and probe, 5′-VIC-CATGGAGTCCTGTGGCATCCATGAAACTAC-TAMRA-3′ were used.

Real-time PCR analyses were performed in a total volume of 25 μl containing 12.5 μl of 2×TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM of each primer, 200 nM TaqMan probe and 1 μl RT product. Real-time PCR was carried out with ABI PRISM 7900 HI (Applied Biosystems), according to the manufacturer's instructions. The plasmid cloned SV40 for generating a linear standard curve was a gift from Dr. Nakanishi (National Center for Geriatrics and Gerontology, Aichi).

JCV DNA transfection

COS-7 and COS-tat cells were cultured in 35-mm dishes containing 2 ml complete growth medium (MEM-10% FBS) until they reached 50–80% confluency. Archetype JCV DNA (CY) was used for the assay of virus production. The plasmid cloned CY was a gift from Dr. Yogo (University of Tokyo).

For transfection, 1.5 μg viral DNA, excised from a recombinant plasmid, was introduced into the cells by Lipofectamine (Gibco BRL), as described above. After 5 hr incubation, the transfection mixture was removed and replaced with complete growth medium. Fresh complete growth medium was changed 24 hr after transfection. Thereafter, the transfected cells were transferred at a split ratio of 1:3 or 1:4 serially every 3 or 4 days to culture flasks containing MEM supplemented with 10% FBS. The cells were harvested on day 32 and the HA titer was determined as described below.

Immunocytochemical staining

COS-7 and COS-tat cells were transfected with JCV CY DNA and cultured for 32 days. The cells were washed twice with PBS and fixed in cold acetone for 10 min at −20°C, and then treated with H_2O_2-methanol to inactivate endogenous peroxidase. A mouse monoclonal antibody (CLA375) against JCV and BKV (18) was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY, USA) and was used at a dilution of 1:40 in PBS containing 1.0% BSA according to the manufacturer's instructions. Antibody CLA375 is recommended to detect JCV-infected cells using immunofluorescence and immunocytochemistry. Fixed COS-7 and COS-tat cells were reacted overnight at 4°C with the above-mentioned antibody, labeled with a Histofine SAB-PO (MULTI) kit (Nichirei, Tokyo, Japan), and stained with DAB. The immunostained cells were counterstained with hematoxylin, mounted in Entellan neu (Merck, Rahway, NJ, USA), and examined under a microscope (Olympus, Tokyo, Japan).

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HA assay and real-time PCR for JCV propagation

HA assay was carried out, essentially as described previously (19). Briefly, COS-7 and COS-tat cells that had been transfected with JCV CY DNA, were resuspended in 1 ml of 1 mM Tris-HCl buffer (pH 7.5) containing 0.2% BSA, and then frozen and thawed. The cell suspension was incubated with 0.05 mg/ml of neuraminidase (Type V; Sigma Chemical, St. Louis, MO, USA) overnight at 37°C, and then further incubated at 56°C for 30 min. After incubation, the samples were centrifuged at 200 *g* for 10 min at 4°C, and the supernatants collected. HA activity was determined using human type O erythrocytes obtained from the Hyogo Red Cross Blood Center.

Real-time PCR was carried out to quantify JCV DNA copies. Viral DNA was extracted from a virus suspension using a QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). Each DNA sample was recovered by ethanol precipitation and dissolved in 50 μl distilled water. Real-time PCR amplification was carried out essentially as described (20) using ABI PRISM 7900HI (Applied Biosystems).

Statistics

The significance of differences between groups was statistically determined by Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Establishment and characterization of COS-tat cells expressing HIV-1 Tat

We established COS-7 cells stably expressing HIV-1 Tat (designated COS-tat cells) to propagate archetype JC virus. After transfection of pcDNA-tat 86 into COS-7 cells, cells expressing *tat* mRNA were screened for 25 clones of Zeocin-resistant cells by RT-PCR. HIV-1 *tat* mRNA was detected as 270 bp bands in seven cell clones (COS-tat2, 7, 9, 15, 22, 23 and 24) (Fig. 1).

Next, to assess the trans-activating activity of Tat for HIV-1 LTR, seven cell clones were transiently transfected with the HIV-1 reporter gene, pLTR-Luc. The results show raw luciferase activity (Fig. 2). The extent of expression of pLTR-Luc in COS-tat cell clones was 1.3 to 8.1-fold greater than in COS-7 cells, indicating that COS-tat cell clones express functional Tat proteins. In addition, luciferase activity was 34 ± 6 RLU in COS-7 cells untransfected with pLTR-luc.

For quantitative measurement of *tat* mRNA expression in three cell clones, RNA samples extracted from COS-7 and COS-tat cells were analyzed by real-time RT-PCR assays using a TaqMan probe and specific primers. The detectable range of real-time RT-PCR was above 100 copies in this system. In non-transfected COS-7 cells, the amplified product of HIV-1 *tat* mRNA was not detected. In contrast, the expression of *tat* mRNA was detected in three clones of COS-tat cells (Fig. 3). Among the three cell clones tested, the greatest expression of *tat* mRNA was observed in COS-tat7 cells. These results indicate that all three COS-tat cells express *tat* mRNA, although the extent of expression varies. We selected three cell clones stably expressing Tat (COS-tat7, COS-tat15 and COS-tat22) and used them for further analyses.

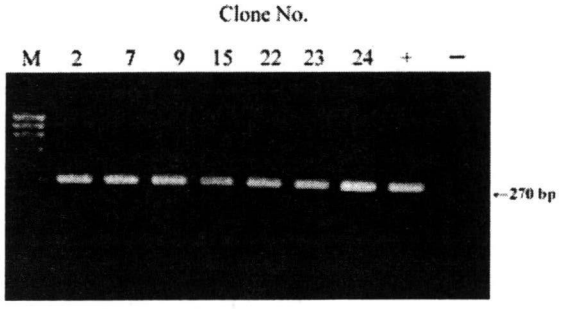

Fig. 1. RT-PCR analyses of *tat* mRNA expression in seven COS-tat cell clones after 11 passages. Lane M, ΦX-174 *Hae* III size marker, Lane −, COS-7 cells as a negative control; lane +, COS-7 cells transiently transfected with pcDNA-tat86. Lane numbers 2, 7, 9, 15, 22, 23 and 24 represent corresponding cell lines. Number on the right represents base pairs of *tat* gene.

HIV-1 Tat enhances protein production of archetype JCV

To examine whether expression of HIV-1 Tat enhances propagation of archetype JCV in cells, CY DNA were introduced into COS-7 and COS-tat cells using Lipofectamine. The transfected cells were maintained at a split ratio of 1:3

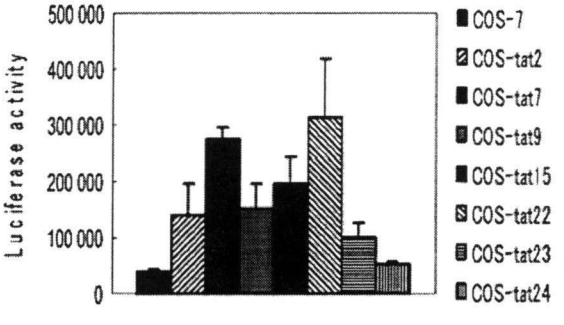

Fig. 2. Luciferase activity. Luciferase activity represented as the average of three samples, or two samples of COS-tat cell lines and COS-7 cells transfected with pLTR-luc. Luciferase activity was normalized to β-galactosidase activity. Luciferase activity was 34 ± 6 RLU in COS-7 cells untransfected with pLTR-luc.

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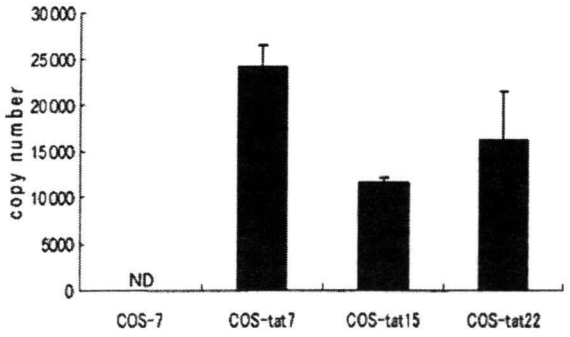

Fig. 3. Expression of HIV-1 *tat*. HIV-1 *tat* mRNA in three samples was determined using real-time RT-PCR. The amounts of HIV-1 *tat* mRNA in each sample were normalized with reference to copy numbers of simian β-actin mRNA. The detectable level is above 100 copies in this system. HIV-1 *tat* mRNA was not detected in untransfected COS-7 cells.

or 1:4 serially every 3 or 4 days in culture flasks containing MEM supplemented with 10% FBS. An obvious CPE, the rounding of cells, was observed in COS-tat clones on day 32. To confirm the enhancement of JCV production by HIV-1 Tat, the production of JCV antigens in COS-7 and COS-tat cells was examined by immunocytochemical staining on day 32 (Fig. 4). In COS-7 cells transfected with JCV CY DNA, JCV antigens were detected in normal-sized nuclei of a few positive cells. In contrast, JCV antigens were detected in enlarged nuclei of several COS-tat cells transfected with JCV CY DNA (Fig. 4). These results indicate that expression of HIV-1 Tat leads to increased production of archetype JCV proteins in kidney-derived cells.

HIV-1 Tat enhances the production of archetype JCV progenies

We next examined the production of progeny viruses of archetype JCV in COS-7 and COS-tat cells. The cells were transfected with archetype JCV DNA and assayed for HA titers, as described in Materials and Methods. Parental COS-7 cells which had been transfected with archetype JCV DNA did not exhibit any detectable HA activity on day 32 (Fig. 5), although they exhibited a detectable HA titer (>64 HA units) on day 42 (data not shown). In contrast, HA activities of extracts were easily detected in COS-tat cells. The HA titers of extracts of COS-tat7, COS-tat15 and COS-tat22 cells were 85 ± 37, 21 ± 9 and 128 ± 0, respectively (Fig. 5). Among the four cell lines tested, COS-tat22 cells showed the highest HA titers, indicating that JCV propagation is closely related to the extent of Tat expression in COS-tat cell clones. These results indicate that expression of HIV-1 Tat leads to increased propagation of archetype JCV in kidney-derived cells.

HIV-1 Tat enhances the genome replication of archetype JCV genome

To assess whether expression of HIV-1 Tat enhances DNA replication of archetype JCV, total DNA was extracted from the cells on day 32, and the amount of JCV DNA copies was determined using real-time PCR, as described in Materials and Methods. In preliminary experiments, we observed that the detectable level of our real-time PCR system was above 100 copies and that JCV DNA was not detected in untransfected COS-7 cells. The results show that JCV DNA was clearly detected (Fig. 6) and in COS-tat

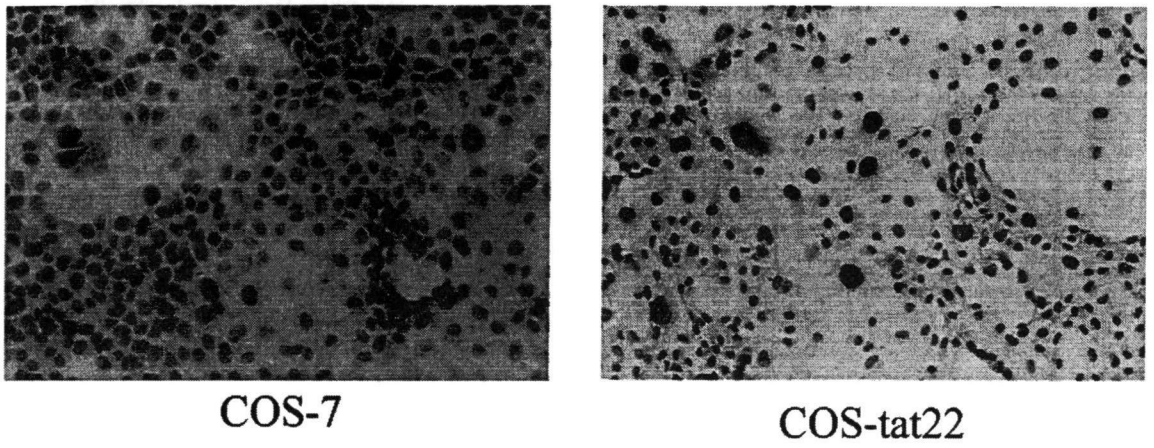

Fig. 4. Immunocytochemical staining of JCV antigen in COS-7 and COS-tat cells on day 32 after transfection with JCV CY DNA. JCV antigens were stained with anti-JCV antibody. Representative micrographs of JCV antigen (brown) in several enlarged nuclei in COS-tat22 cells is shown. In contrast, COS-7 cells, transfected with JCV CY DNA, show JCV antigen in a few cells with normal-sized nuclei (×200).

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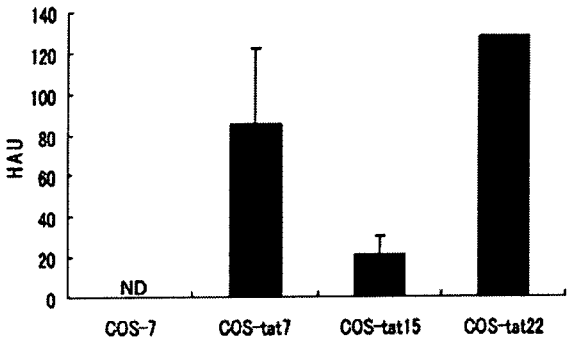

Fig. 5. HA titers. Samples were prepared from three cultures 32 days after transfection with JCV CY DNA. HA titers in the samples were determined by the HA assay using human O erythrocytes. Both untransfected COS-7 cells, and COS-7 cells which had been transfected with CY DNA, did not exhibit any detectable HA (< 2) on day 32.

cell clones was 1.2 to 6.1-fold greater than in parental COS-7 cells. These results indicate that expression of HIV-1 Tat slightly increases the genome replication of archetype JCV genome in kidney-derived cells.

Real-time RT-PCR analyses the expressions of SV40 T antigen

Since SV40 T antigen is a strong stimulator of archetype JCV replication (11), we thought that expression of HIV-1 Tat might lead to enhancement of SV40 T antigen, thereby indirectly enhancing JCV propagation in COS-tat cells. To assess this possibility, the extent of expression of SV40 T antigen in COS-tat cell clones was estimated by real-time RT-PCR, as described in Materials and Methods. The results show that SV40 T antigen mRNA was clearly detected (Fig. 7), and the copy numbers of SV40 T antigen

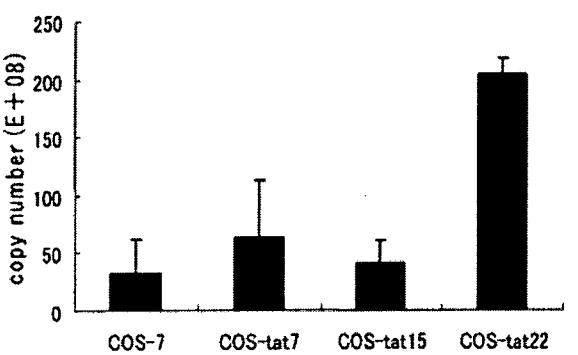

Fig. 6. JCV DNA. JCV DNA in three virus samples derived from transfected cells was quantitatively amplified by real-time PCR. The linear standard curve was generated from a series of dilutions with plasmid VP1. The detectable level is above 100 copies in this system. JCV DNA copies were not detected in untransfected COS-7 cells.

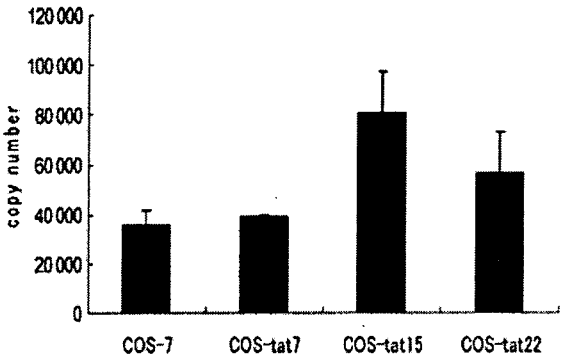

Fig. 7. Expression of SV40 T antigen. Expression of SV40 T antigen from three cultures was quantitatively amplified by real-time RT-PCR. The linear standard curve was generated from a series of dilutions with plasmid pSV40. The amounts of SV40 T antigen mRNA in each sample were normalized with reference to copy numbers of simian β-actin mRNA. The copy number of three COS-tat cell clones was not significantly greater than that of COS-7 (Student's *t*-test).

in three COS-tat cell clones were 1.1 to 2.2-fold greater than in COS-7 cells, but this difference was not significant when evaluated by Student's *t*-test. These results indicate that HIV-1 Tat slightly increases the extent of expression of SV40 T antigen in COS-tat cells.

Discussion

Archetype JCV infects children without causing any symptoms, establishes persistent infection in the kidneys and circulates in the healthy human population without causing illness (21). In contrast, genomic DNA of JCV isolated from the brain and leukocytes of PML patients contain non-archetype regulatory regions with hypervariable sequences (2). These lines of evidence imply that archetype JCV is converted to the PML type during persistence in the kidney. It is also known that conversion of archetype JCV into PML-type JCV increases the infectivity of JCV for extra-renal tissue (8,9).

On the other hand, the high incidence of PML among individuals with AIDS suggests that PML is particularly associated with AIDS. A previous report has shown that HIV-1 Tat protein enhances expression of the JCV late promoter (13); however, the effect of Tat protein on the propagation of archetype JCV in kidney-derived cells is largely unknown. Although propagation of archetype JCV is strictly restricted in cultured cells, COS-7 cells have been shown to support replication of archetype JCV (11). Hence, we have established COS-7 cells that stably express HIV-1 Tat (COS-tat cells) in order to investigate Tat-induced propagation of archetype JCV.

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Using the protocol described in Materials and Methods, we have characterized COS-tat cells using a variety of assay systems. First, HIV-1 *tat* mRNA was detected in seven Zeocin-resistant cell clones by RT-PCR (Fig. 1). Furthermore, the HIV-1 reporter gene assay revealed that Tat proteins stably expressed in COS-tat clones are functional and possess trans-activating activity for HIV-1 LTR (Fig. 2). In addition, high-level expression of *tat* mRNA was observed in COS-tat7, 15 and 22 cells, as judged from the data of real-time RT-PCR analysis (Fig. 3).

It has been previously reported by others that the extent of expression of the HIV-1 reporter gene in T-cell-derived Jurkat cells expressing HIV-1 Tat (Jurkat-tat) is 50-fold greater than in control cell lines (22). In this study, we observed that the extent of expression of the reporter gene in COS-tat cell clones was moderately greater (1.3- to 8.1-fold) than in parental COS-7 cells despite marked expression of HIV-1 *tat* mRNA. Thus, trans-activating activity of Tat in COS-7 cells seems to be less than in Jurkat cells. Although the reason for this difference is not clear, we think that the amount of production of Tat protein may depend on various factors in host cells.

The amount of trans-activating activity of Tat in COS-tat cell clones is, in part, inconsistent with the extent of expression of *tat* mRNA among these cells (Fig. 3); however, a previous report has shown that acetylation of Tat protein regulates its transcriptional activity (23). In other words, Tat activities are regulated not only by the extent of expression of *tat* mRNA, but also by post-translational modifications, such as protein acetylation. Thus, we speculate that the extent of expression of *tat* mRNA may not strictly correlate to Tat activity in COS-tat cell clones.

When COS-tat cell clones were transfected with genomic DNA of archetype JCV, JCV antigen was detected in enlarged nuclei on day 32, as judged by immunocytochemical staining (Fig. 4). We also observed that, as judged from HA assay data, propagation of archetype JCV in COS-tat cells is significantly increased when compared to that in parental COS-7 cells (Fig. 5). These data clearly show that expression of HIV-1 Tat enhances propagation of archetype JCV in kidney-derived cells. Interestingly, the amounts of JCV DNA in COS-tat cell clones were only slightly greater than in parental COS-7 cells (Fig. 6), although the production of progeny viruses was markedly increased by expression of HIV-1 Tat. These observations suggest that HIV-1 Tat protein increases propagation of archetype JCV after synthesis of genomic DNA. This result is consistent with earlier investigations indicating that Tat protein stimulates reporter gene expression under the control of the JCV late promoter (13).

COS-7 cells were established by transforming kidney-derived cells using SV40 and expressing SV40 T antigen (24), which is a strong stimulator of JCV replication (25). According to our observations, the extent of expression of SV40 T antigen in COS-tat cell clones was slightly greater than in parental COS-7 cells (Fig. 7). It therefore seems reasonable that expression of SV40 T antigen is enhanced by HIV-1 Tat protein in COS-tat cell clones; however, the marked enhancement of JCV propagation in COS-tat cell clones cannot be explained by the slight increase in SV40 T antigen expression. Therefore, we think that HIV-1 Tat-induced upregulation of SV40 T antigen expression is not closely related to augmentation of JCV propagation in COS-tat cells.

As far as we know, this is the first report showing increased production of archetype JCV in a culture system using cell lines stably expressing HIV-1 Tat. It is likely that in the kidneys of AIDS patients, replication of archetype JCV is activated by Tat protein secreted from HIV-1-infected cells. We also think that variant strains of JCV are generated during the replication of archetype JCV in the kidney and that these variants may spread to the brain and cause PML. In addition, archetype BKV, which is very closely related to JCV, can also propagate in human renal epithelial cells (26). Reactivation of BKV can occur in association with AIDS, and emerging case reports suggest that it may cause BKVAN (27,28). We believe that COS-tat cells can serve as a useful tool for studying Tat-induced propagation of archetype JCV and BKV for the development of PML and BKVAN.

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