Pathological and Molecular Biological Studies on Canine Distemper

CHAPTER I

The Growth Profiles of The Laboratory Strain Onderstepoort, Strain MD77 and Strain KDK1 of Canine Distemper Virus on Vero Cells Expressing Canine Signaling Lymphocyte Activation Molecule

Summary

To know growth profiles of canine distemper virus (CDV) on Vero cells stably expressing canine signaling lymphocyte activation molecule (Vero-DogSLAMtag; Vero-DST cells), the propagation of three strains of CDV was tested in Vero-DST cells in comparison with parental Vero cells. Strain MD77 could grow well in both cell lines, but demonstrated no syncytium formation or indistinguishable rounding cytopathic effects (CPE) in Vero cells. Strains Onderstepoort and KDK-1 also grew well in Vero-DST cells with apparent syncytium CPE, while they grew less or no efficiently, respectively, in Vero cells. All three CDV strains demonstrated the peak titers, in Vero-DST cells before reaching to an extensive CPE and drastic decrease of titers at/after full CPE. Immunohistochemistry revealed that viral antigens of all CDV strains were found exclusively in the syncytia in Vero-DST cells, while in Vero cells, viral antigen was identified in their single cells for strain MD77 but none for other strains. Thus, every strain of CDV could grow well in Vero-DST cells and behaved differently against Vero cells. These results would be of practical value for workers of CDV because 1) In Vero DST cells, by observation of distinct syncytium CPE, the highest titer or the best growth of virus could be identified; 2) In Vero cells, various CDV strains could be readily classified after propagation in Vero-DST cells.

Key words: CDV, CPE, growth properties, Vero cells, Vero-DST cells

Introduction

Canine distemper virus (CDV) is a member of the Morbillivirus genus in the family Paramyxoviridae. So far both isolation and titration of CDV without adaptation have been difficult because there is no appropriate cell line to propagate the virus with CPE. Vero cells derived from African green monkey kidney, were often used to isolate CDV strains, but failed to propagate wild type CDV strains in many cases (Appel et al., 1992; Metzler et al., 1984; Seiki et al., 2003; Shin et al., 1997). Infection of CDV to the mitogen-stimulated lymphocytes from SPF dogs (Appel et al., 1992), to the peritoneal macrophages from SPF ferrets (Poste, 1971; Whetstone et al., 1981), or to the B95a cells derived from marmoset B lymphoblastoid cell resulted in successful propagation of CDV (Kai et al,. 1993). But these methods are not convenient for titration of CDV. The use of canine epithelial kidney (MDCK) cell line also resulted in successful isolation of clinical CDV sample (Karber, 1931). However, in this case, it took so long before development of CPE, being impractical for titration of the virus.

Recently, signaling lymphocyte activation molecule (SLAM; CD150) of various animals (human, canine and bovine) was identified to be the proper receptor of *Morbilliviruses* including measles, canine distemper and rinderpest viruses (Tatsuo et al., 2001). Seki et al. reported that Vero cells stably expressing canine SLAM were highly sensitive to detect CDV in clinical samples and could successfully recover the representative strains of CDV without selection and/or adaptation (Seiki et al., 2003). However, they did not describe about the quantitative assay and growth profiles of CDV including antigen distribution, highest titer and suitable harvesting time, relevance of virus growth in syncytial cells and comparison between cell associate virus and released virus. In this report, we tried to study the growth profiles of 3 CDV strains in Vero-DST cells which were maintained in the laboratory in cell lines and showed different growth

characteristics in normal Vero cells.

Materials and methods

Cell lines: Vero cells stably expressing dog SLAM (Vero-DST cells) was generated from Vero cells with pCXN2 (Tatsuo et al., 2001) and pCAGDogSLAMtag which was constructed to express plasmid that encoded the membrane-bound form of canine SLAM fusing to H tag at N terminus. H tag does not interfere the binding of CDV H protein (Tatsuo et al., 2001). Vero-DST cells and their original Vero cells were used in this study. Vero-DST and Vero cell lines were kindly provided by Dr. Yanagi (Kyushu University, Fukuoka, Japan). Both cell lines were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 unit/ml of penicillin and 100 μg/ml of streptomycin and 0.4 mg of geneticin (G418) per ml and grown in a CO2-incubator at 37 ℃.

Viruses: Three CDV strains were used in this study. The virulent strain of MD77 (Hirayama et al., 1986) provided by Dr. Tokiyoshi (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was isolated from a dog in 1977 in Japan, and passaged twice in primary dog kidney cells and three times in Vero cells. The recent field isolated strain of KDK-1 (Mochizuki et al., 1999) provided by Dr. Mochizuki (Kyoritsu Pharmaceutical Company LTD, Tokyo, Japan) was isolated from a diseased dog in B95a cells in Japan in 1991 and passaged three times in Vero cells. The attenuated strain of Onderstepoort (Bolt et al., 1997; Bussel & Karzon, 1965; Haig, 1948) provided by Dr. Summers (Cornell University, USA) was isolated from a North American ranched fox diseased under outbreak of canine distemper in 1930s, and passaged serially in ferrets 57 times, in chicken embryos 208 times, chicken embryo cell culture 62-66 times, ferret kidney cells 13-14 times and in Vero cells more than 100 times. These viruses were

passaged once in Vero-DST cells for the stock preparation and stored at –70 ℃ until used.

Virus titration: Vero-DST cells were prepared on 96 well culture plate ($2.0x10^4$ cells/well). Supernatants were removed and the virus suspensions (diluted serial 10-fold) were overlaid on the cells (25 ul/well; three wells for each dilution). After 1 hr incubation, maintenance medium of DMEM with 10% tryptose phosphate broth (TPB) was added to each well (100ul/well). CPE formation was observed daily up to day 5 of infection. Tissue culture infective dose ($TCID_{50}$) was determined according to the method of Behrens-Karber.

Virus growth: Vero-DST and Vero cells were seeded on 48-well multiplates (1.0×10^5 cells/well) and cultivated as described above. Strains MD77, Onderstepoort and KDK-1 were infected to the cells at a multiplicity of infection (MOI) of 0.01. After 1 hr adsorption of virus, the virus suspensions were removed and the cultures were washed once with 0.5 ml of phosphate buffered saline (PBS). Then maintenance medium was added to the cultures (0.5 ml). Cell-free and cell-associated viruses were recovered from culture supernatant and residual cells at 6, 12, 24, 36, 48, 72, 96, 120, 144 and 168 hr post inoculation (hpi) until detachment of cells from the surface of culture plate. Cell-associated virus was liberated from the infected cells by freezing and thawing. The harvested virus was stored at –70 ℃ until titration. CPE was recorded at each harvested time.

Immunohistochemistry: Sub-confluent cultures of Vero-DST and Vero cells were infected with each CDV strain at a MOI of 0.01. After appearance of CPE, medium was replaced with 2 ml of PBS solution containing 1% (w/v) methylcellulose 4000 (Nakarai chemical Co. Ltd, Kyoto), then incubated at 37°C for 30 min. Cells were fixed by adding 2 ml of absolute methanol for over 4 hr at 4°C and washed thoroughly with distilled water

and then absolute methanol. The cultures were dried at room temperature (RT) and rinsed once with PBS containing 1% Tween 20 before using for immunohistochemistry (Kai et al., 1992). The fixed cells were immersed in absolute methanol with 3% H_2O_2 for 10 min at RT and then incubated with 3% bovine serum albumin (BSA) for 30 min at 37°C. After that, the cells were incubated with monoclonal antibody against the nucleo-protein (NP) of CDV (purchased from Monotope of Virostat, USA) diluted to 1:20 at 37°C for 1 hr followed by reaction with anti-mouse and anti-rabbit antibody Envision polymer, HRP (DAKO, USA). After each incubation step, the cells were washed with PBS for 5 min three times. The reactions were visualized with 3,3'-diaminobenzidine tetra-hydrochloride. The immuno-labelled cells were observed directly or after counterstained with Mayer's hematoxyline under a phase contrast microscope.

Results

Infection of three CDV strains to Vero-DST and Vero cells: The CDV strains of MD77, Onderstepoort, KDK-1 prepared through Vero-DST cells were tested on both Vero-DST and normal Vero cells. As shown in Fig. 1, all of three CDV strains induced apparent cytopathic effects (CPE) of syncytium formation in Vero-DST cells (Fig.1a, b, and c). On the other hand, the CPE in Vero cells was induced by only strain Onderstepoort (Fig.1e) but not by MD77 or KDK-1 (Fig.1d and f). To know if virus could infect to a cell and grow without CPE induction, the cells were further tested immuno-histochemically with a monoclonal antibody to NP protein of CDV. As shown in Fig. 1, in infected Vero-DST cells, the antigen of NP protein (visualized as brown color) was found exclusively in syncytia but not in single cells (Fig. 1a, b, and c). On the contrary, in Vero cells, the viral antigen was found in single cells infected with strain MD77, probably being indistinguishable CPE of rounding cells (Fig. 1d). The finding

was also found in syncytia induced by strain Onderstepoort at less intensity as compared to that occurring in Vero-DST cells (Fig. 1e), while no such change was induced by strain KDK-1 (Fig. 1f).

Since the appearance of CPE in Vero-DST cells infected with each strain of CDV differed from others by incubation time, detailed observations were carried out (Table 1). Vero-DST cells with strain MD77 developed firstly CPE at 24 hpi, reaching a 40% of maximum appearance of CPE, and 100% by 96 hpi. After a 100% CPE, the infected cells began to detach from the surface of culture plate. Vero cells with strain MD77 developed no syncytia during the observation period, although the viral antigen was found in the cells at 48 hpi (Fig. 1d). Vero-DST cells with strain Onderstepoort developed CPE almost in the same manner as or slightly faster than those with strain MD77. In contrast, strain Onderstepoort produced CPE in Vero cells slower than in Vero-DST cells in day 1-2 of infection. Strain KDK-1 developed CPE in Vero-DST cells slower than the other two strains, but a 100% CPE was attained by 96 hpi as done by strain MD77. Strain KDK-1 failed to develop CPE in Vero cells during the observation period.

Growth profiles of CDV: The results obtained above may indicate the following notions: 1) Vero-DST cells were appropriate for propagation of three types of CDV strain, although the optimum conditions to recover virus was not determined. 2) Vero cells infected with strain MD77 might sustain virus propagation in single cells, and spread out virus without forming syncytium. 3) The intensity of viral antigen visualized in brown color might reflect the replication level of strain Onderstepoort in Vero-DST and Vero cells (Fig. 1b and e). To make it clear, the growth profiles of three CDV strains were studied in Vero-DST and Vero cells.

In the growth profile studies the titers of cell-free and cell-associated virus were determined separately (Fig. 2 a, b, and c) and expressed as $TCID_{50}$/25 ul. As shown in Fig. 2a, strain MD77 grew well in both Vero-DST and Vero cells. In Vero-DST cells, cell-associated virus appeared at 12 hpi and reach the highest state, $3.2x10^6$ $TCID_{50}$ at 48 hpi, while cell-free virus appeared slightly slowly reaching a peak titer, $3.2x10^3$ $TCID_{50}$ at 72 hpi. In Vero cells, both cell-associated and cell-free virus titers increased slowly than those in Vero-DST cells but showed a steep rise later on until reaching a peak of $1.4x10^3$ and $3.2x10^3$ $TCID_{50}$ at 144 hpi, respectively, with much higher titers (4.4 as fold for cell-associated and 20 fold for cell-free virus calculated from Fig. 1) than those recovered from infected Vero-DST cells. The slow increase of cell-free virus titer might support the notion 2) mentioned above.

As to strain Onderstepoort (Fig. 2 b), the growth patterns of cell-associated and cell-free virus in Vero-DST or in Vero cells resembled well each other. The virus titers of both cell-associated and cell-free virus slowly increased in Vero cells than those in Vero-DST cells. In contrast to MD77, the virus recovery from Vero-DST cells was better than that in Vero cells.

Strain KDK-1 could grow only in Vero-DST cells with almost a comparable peak titer to that in strain MD77 (Fig. 2 c). Curiously, strain KDK-1 could hardly grow in Vero cells although it had been passed three times in Vero cells as done for strain MD77 (see Materials and Methods).

Discussion

In this report, we could demonstrate that Vero-DST cells are possibly useful not only for virus propagation but also virus assay of various strains of CDV such as MD77, Onderstepoort, and KDK-1. This indicates that the dog SLAM is the most important

molecule to establish the binding between the H protein of CDV and a cellular receptor. However, strains MD77 and Onderstepoort could grow in Vero cells that had not any dog SLAM molecule, while KDK-1 strain could not grow. One possible explanation for this phenomenon is that Vero cells have a different type of CDV receptor from SLAM and CDV strains which provide a ligand domain in their envelope H protein(s) applicable to this receptor can infect to Vero cells but those without the ligand domain can not infect. In fact, it is reported that strains MD77 and Onderstepoort could bind to another receptor of CD46, although the property of their ligand domain may be slightly different each other. Strain MD77 could infect to Vero cells more efficiently than strain Onderstepoort, but grew slowly probably due to lack of CPE formation by virion in the course of cell expansion (Fig.2 a). In contrast, strain Onderstepoort, because of extensive passages in Vero cells (see Materials and Methods), changed its ligand domain to a suitable form for CD46 of Vero cells, enabling a firm binding to the receptor and and effective CPE formation (Fig.1 e). However, it seems that the binding affinity of strain Onderstepoort to dog SLAM is more dominant than to CD46, since the recoveries of cell-free and cell-associated virus from infected Vero-DST cells were more superior than those from infected Vero cells. Strain KDK-1 seems not to have the domain against CD46. The H protein gene of strain KDK-1 differed from that of other CDV strains as evidenced by digestion pattern analysis with the RT-PCR amplified DNA and restriction enzymes (Metzler et al., 1984).

CPE characteristics of all morbilliviruses are syncytium formation (Tatsuo et al., 2001). The viral envelope proteins, H and F proteins, have the role in attachment and invasion to the susceptible cells following syncytium formation (Iwatsuki et al., 1997). It was known that cell-to-cell spread of virus in tissue culture was related to the capability of the virus to induce membrane fusion (Poste, 1971). H protein determines the extent

and efficiency of cell-cell fusion, resulting in decisive influence on the CPE. It seems that each CDV strain has different efficacy in fusion to host cells, ex. Vero cells, presenting a possible rationale why CPE is induced by some CDV strains but not others (von Messling et al., 2001).

Strain KDK-1 could not induce any CPE in Vero cells in our case, although it was passaged through Vero cells for three times along with apparent CPE (personally communicated by Dr Mochizuki, *Kyoritsu Co. LTD*). How can we account for this disparity? Since Vero cells have been distributed widely in the world since early 1960s, they might vary and develop differently as "sub lines". In any way, CDV strains of MD77, Onderstepoort and KDK-1 showed quite different phenotypes behaviors to our Vero cells from each other. This fact may indicate that our Vero cells are very useful for classification of various CDV strains after propagation in Vero-DST cells. It is also useful and convenient to know the relationship between CPE and virus titer of virus. Since then, the good harvested time of virus for stock and further purposes could be identified easily.