Pathological and Molecular Biological Studies on Canine Distemper

CHAPTER VII

Stability of Canine Distemper Virus (CDV) after Twenty Passages in Vero-DST Cells Expressing the Receptor Protein for CDV

Summary

Isolates 007Lm, S124C and Ac96I and a Vero cell-adapted Onderstepoort strain of canine distemper viruses (CDV) were examined for stability after passages in Vero cells expressing the canine signaling lymphocyte activation molecule (dogSLAM, the intrinsic receptor to CDV). These viruses passage once in Vero cells expressing dogSLAM (Vero-DST) cells (original) and after 20 passages (20p) were compared by using sequence analyses and growth characteristics. All four strains of 20p grew well and were slightly better than their originals. The 20p viruses developed a cytopathic effect slightly lower than the original strains. A few changes in amino acids in the H gene were between the 20p and the original viruses, but the sites of changes were not specific. Fragments of P, M and L genes of all strains showed no nucleotide changes after the passages. These results showed that: 1) passages of CDVs in Vero-DST cells induced amino acid changes only in the H gene, not in the P, M and L genes, unlike in a previous study with Vero cells; 2) passages did not markedly affect the growth characteristics of every viral strain. These results indicate that Vero cells expressing canine SLAM allow the isolation and passaging of CDV without major changes in viral genes.

Key words: adaptation, CDV, growth, passage, sequence; Vero-DST cells.

Introduction:

Canine distemper virus (CDV) (genus *Morbillivirus,* family Paramyxoviridae) causes systemic disease in domestic and wild dogs, foxes and wolves. CDV infection has also

caused deaths of large cats, including tigers in lions in zoos in the United States. CDV outbreaks have occurred among small carnivores, like minks, ferrets, raccoons and seals (Summers and Appel, 1994; de Swart et al., 1995; Harder and Osterhaus, 1997; Barrett, 1999). CDV may be related to some human diseases, like Paget's disease and multiple sclerosis (Mee and Sharpe, 1993; Fraser, 1997; Hodge and Wolfson, 1997). The CDV genome has 15,690 nucleotides and contains six non-overlapping genes encoding nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), attachment (H) and large (L) proteins (Barrett 1985,1999; Bellini et al., 1986; Curran et al.,1991; Sidhu et al.,1993; McIlhatton et al., 1997). The P gene encodes the C and V non-structural proteins (Lamb and Kolakofsky, 1996; Barrett, 1999). Adaptation of CDVs to Vero cells causes loss of pathogenicity (Hamburger et al., 1991) and changes in growth characteristics or cytopathic effect (CPE) (Plattet et al., 2005). Vero cell adaptation of wild-type CDV requires no amino acid changes in the H protein (Nielsen et al., 2003). A comparison of nucleotides of Vero-adapted A75/17-V strain passaged in Vero cells for 17 times with nucleotides of wild-type strain A75/17 showed only seven nucleotide changes: at nucleotide positions 2275, 2381 and 2399 in the P/V/C gene, at nucleotide positions 3610, 4422 and 4434 in the M gene and at nucleotide position 14940 in the L gene (Plattet et al., 2004). Wild-type and Vero-adapted strains showed no nucleotide differences in the H gene.

Vero cells expressing canine signaling lymphocyte activation molecules (dogSLAM) have been developed to isolate CDVs efficiently from clinical samples (Seki et al., 2003). Previously, we showed that CDV strains grow in Vero cells expressing dogSLAM (Vero-DST) with an apparent CPE of cell-fusion and that they behave differently in normal Vero cells (Lan et al, 2005a). Vero-DST cells were used not only for isolation of CDV from clinical samples, but also for titration and research of biological properties of

newly isolated CDVs. Field CDV strains isolated in Vero-DST cells do not change nucleotide sequences of P and H genes and keep virulence in dogs (Lan, et al., 2005 c) compared with samples of diseased animals. However, the effects of passages in Vero-DST cells on growth characteristics and genetic changes of CDVs isolated through Vero-DST cells were not tested. Thus, in this study, we investigated the growth characters in Vero-DST cells and genetic changes in H, P, M, and L genes of new isolates and a Vero cell-adapted Onderstepoort strain of canine distemper viruses after one and twenty passages in Vero-DST cells.

Materials and methods

Viruses: The attenuated strain of Onderstepoort (Bussel and Karzon, 1965; Haig, 1948; Bolt et al., 1997) was provided by Dr. Summers (Cornell University, USA). This strain was isolated from a North American ranched fox diseased in an outbreak of canine distemper in the 1930s and has been passaged serially in ferrets 57 times, in chicken embryos 208 times, chicken embryo cell culture 62-66 times, ferret kidney cells 13-14 times, in Vero cells more than 100 times and in Vero-DST cells once. CDV strains 007Lm, Ac96I and S124C were used in this study as wild-type strains, which were isolated from the lymph node, large intestine and cerebellum of autopsied dogs. All strains passaged once in Vero-DST are referred to as original viruses. Viruses at a multiplicity of infection of 0.01 were used for every passage. After observing the CPE, viruses were harvested, were stored at -80^0C and were used for next passage. Viruses serially passaged at 20 times the passage of the original strains in Vero-DST are called 20p strains in this study

Results

Growth kinetics: Fig. 19 compares the growth kinetics of the original (ori) and 20p strains. Both Onderstepoort-ori and -20p strains (Ond-ori and Ond-20p) showed that titers of cell-associated viruses were higher than for released viruses (Fig.19a). Maximum titers of cell-associated viruses of strain Ond-20p and Ond-ori were $3.16x10^7$ $TCID_{50}$/25µ at 48 h and $3,16x10^5$ $TCID_{50}$/25µ at 36 h, respectively; maximum titers of released viruses of strain Ond-20p and Ond-ori were $1.4x10^4$ $TCID_{50}$/25µ at 48 h and $6.76x10^2$ $TCID_{50}$/25µ at 24h to 48 h , respectively. Strain Ond-20p grew better than Ond-ori in Vero-DST cells. At 24 h, Ond-ori showed 50% extensive CPE, but only 30% extensive CPE with Ond-20p virus. Vero-DST cells infected with strain Ond-ori developed CPE slightly faster than for cells infected with strain Ond-20p. Figs. 1b and c show the growth kinetics of strains 007Lm-ori and 007Lm-20p and S124C-ori and S124C-20p in Vero-DST cells. Titers of both 20p strains were slightly higher than for the original strain. For Ac96I, the maximum virus titers of cell-associated viruses of 20p and ori of this strain were $1.4x10^6$ $TCID_{50}$/25µ and $3.16x10^4$ $TCID_{50}$/25µ, respectively at 36 h; and for released viruses were $3.16x10^2$ $TCID_{50}$/25µ and $1.4x10^2$ $TCID_{50}$/25µ, respectively, at 36 h. All four 20p strains grew well and were slightly better than the original strains. All strains kept the similar growth curves. CPE characteristics of the four 20p and ori strains were also examined in Vero cells without dogSLAM (normal Vero cells). None of them showed syncytia in Vero cells except strain Ond-20p and Ond-ori. After 20 passages in Vero-DST cells, the CPE characteristics in normal Vero cells of all strains did not change.

Sequence analysis: P, M, H and L genes were sequenced for ori and 20p strains of Onderstepoort, 007Lm, Ac96I and S124C. Table 12 and Fig.20 summarized the nucleotide and deduced amino acid differences, respectively, of the H gene of the four ori

and 20p strains. The H genes of all strains consisted of 1821 nucleotides in one open reading frame encoding 607 amino acids, except for only 604 amino acids of strains Ond-ori and Ond-20p, and 12 cystein (C) residues that determined the secondary structure of protein at an identical position (Fig. 20).

The deduced amino acid sequences of both strains Ond-ori and Ond-20p had 4 N-linked glycosylation sites and a hydrophobic region of 19 amino acids at an identical position. These strains had two nucleotide changes, but only one amino acid difference at amino acid position 500: isoleucine (I) with isoelectric point (p*I*) of 6.02 in strain Ond-ori and arginine (R) with p*I of* 10.76 in Ond-20p. Amino acid sequences of strain 007Lm-ori and 007Lm-20p contained a hydrophobic region of 20 amino acids and 8 N-linked glycosylation sites at an identical position. The H gene at amino acid position 548 of strain 007Lm-ori contained amino acid threonine (T) with p*I* of 6.16 and of strain 007Lm-20p contained methionine (M) with p*I* of 5.74, thus showing only one amino acid difference. Only one sense mutation at nucleotide position 8721 was found in the H gene of strain 007Lm-20p. The H genes of strains S124C-ori and S124C-20p showed 2 sense amino acid differences at each of amino acid positions 534 and 540, respectively, and had 9 N-linked glycosylation sites and a hydrophobic region of 19 amino acids at an identical positions. The H genes of strains Ac96I-ori and Ac96I-20p also had a hydrophobic region of 19 amino acids, 9 N-linked glycosylation sites and 2 amino acid differences at each of amino acid positions 212 and 225, respectively, different from strain S124C. Strains S124C-ori and S124C-20p had three nucleotide changes, but only two amino acid differences: from M to I at amino acid position 534 and from G (*pI 5*.97) to D (p*I* 2.77) at amino acid position 540 in H genes (Fig.20).

Fragments of P, M, and L of the ori and 20p strains of 007Lm, S124C and Ac96I and Onderstepoort were compared. No nucleotide differences were found in P, M and L genes between ori and 20p strains..

Discussion

Nucleotide and amino acid changes between the original virus and cell line-passaged or adapted viruses depend on different virus strains and different cell lines used for passage or adaptation. CDV adaptation to Vero cells do not necessarily require amino acid changes in the H protein (Nielsen et al., 2003). Comparison of nucleotide and amino acid sequences between 9301B (fresh isolate in B95a cells) and 9301V (Vero cell-adapted form) of measles virus, also of the genus *Morbillivirus* in the family *Paramyxoviridae*, show eight nucleotide differences in P/V/C, H and L proteins: three differences in the H gene at nucleotide positions 7311, 8538 and 8906, two differences in the P/V/C gene at positions 1969 and 2160, and three differences in the L gene at positions 12976, 12977 and 14632 (Takeda, et al., 1998). After adaptation to Vero cells, the measles virus does not induce syncytia and shows no amino acid differences in H and F proteins (Kouomou, et al., 2002). The measles virus passaged five times in Hep-2 or Vero cells shows no nucleotide or amino acid differences from their progenitor wild-type passages. Changes in the H protein of Vero cell-adapted measles viruses might occur if viruses are passaged more than five times in Vero cells (Nielsen et al., 2000).

Comparison of the biological characteristics between CDVs adapted to human neural cells (glioblastoma, oligodedroglioma and neuroblastoma cells) and the unadapted original virus suggests a difference in the p*I* of the viral envelope proteins H and F between the original and adapted viruses because of viral genomic changes during adaptation (Morikawa et al., 1988). Vero-adapted CDV strain A75/17-V was obtained

after 17 serial passages in Vero cells from highly virulent A75/17-CDV. The 15,690 nucleotide genome of A75/17-V differs from that of the wild-type strain by only seven nucleotides in the P/V/C gene at positions 2275, 2381 and 2399, in the M gene at positions 3610, 4422 and 4434 and in the L gene at position 14940 (Plattet et al., 2004). Therefore, in this study, we investigated changes in those positions in the genome of CDV after 20 passages in Vero-DST cells. Those positions in the P, M and L genes of the four Vero-DST cell-passaged CDV strains and their original strains showed no differences. In the H genes of these strains, one or two amino acid differences were between passaged strains and their original strains, but the rule of amino acid changes could not be shown clearly. Plattet et al., (2004) passaged and compared the sequence of only one CDV strain A75/17 with the Vero-adapted strain. In previous studies, we showed that different strains of CDV have different molecular and biological characteristics (Lan et al., 2005e). Hence, in this study, we used four CDV strains, including the laboratory strain Onderstepoort and three new isolates; they showed almost the same results. Vero-DST cells have the receptor for CDV (dog SLAM) that is different from Vero cells (Seki et al., 2003). A SLAM molecule acts as a host cell factor favoring cell - cell fusion and does not affect virus growth. In SLAM, all homotypic and heterotypic combinations of F and H proteins are fusogenic. SLAM has a crucial role in modulating the level of viral cell-cell fusion. In the presence of SLAM, viruses induce the ability of cell-cell fusion (Plattet et al., 2005). Because of Vero cells without SLAM, the ability of virus cell-cell fusion is less than for Vero DST cells. The H protein binds to the CDV receptor of the host cell membrane and allows the virus genome to enter the cytoplasm. H and F proteins are necessary to induce cell fusion. The H protein is the major determinant of tropism and cytopathogenicity (von Messling et al., 2001) and has the highest antigenic variation (Blixenkrone- Moller et al., 1992). In our

results, only one or two random amino acid changes were between the H gene of Vero-DST cell-passaged viruses and that of their original viruses. However, to give the complete picture of nucleotide changes between Vero-DST cell-passaged viruses and their original viruses, the sequence analysis of complete genomes would be carried out in the further research. Viruses after passage in Vero-DST cells grew slightly better than their original virus, but kept the same growth patterns. For example, the titer of the cell-associated virus of the Onderstepoort strain was higher than for the released virus. All Vero-DST cell-passaged viruses and their original viruses showed CPE clearly and early because Vero-DST cells express canine SLAM that is a suitable receptor for CDV. We also checked the CPE of the original and 20p of the four strains in normal Vero cells and determined the growth of viruses by using immunocytochemistry. Both strains Ond-ori and Ond-20p showed syncytia and good growth as an Onderstepoort type (Lan et al, 2005a). The other strains showed no syncytia even after 20 passages in Vero-DST cells.

In this study, CDV strains passaged in Vero-DST cells and their original viruses showed moderate amino acid nucleotide changes in the H gene, no changes in P, M and L genes, and typical growth kinetics of each strain.