Pathological and Molecular Biological Studies on Canine Distemper

CHAPTER IV

Pathogenesis and Phylogenetic Analyses of Canine Distemper Virus Strain 007Lm, a New Isolate in Dogs

Summary

The pathogenesis of a new isolate of canine distemper virus (CDV), strain 007Lm, was investigated from lymph node tissue by using Vero cells that express canine signalling lymphocyte activation molecules with a tag (Vero-DST) in dogs. Two CDV sero-negative Beagle dogs were inoculated intranasally and intraconjunctively with a virus suspension. Both infected dogs showed clinical signs of severe bloody diarrhea, conjunctivitis, ocular discharge, nasal discharge and coughing, lymphopenia, fever and weight loss. Titers of CDV-IgM and CDV-IgG in the blood were measured. CDV was detected by using reverse transcriptase-PCR and was recovered in swabs from one dog from 9 days and from the other dogs from 10 days after inoculation. Molecular and phylogenetic analyses of H and P genes showed that nucleotide and amino acid sequences of these genes of strain 007Lm after isolation in Vero-DST cells are identical to those of the original virus from fresh tissue and that strain 007Lm joins to the Asia 2 group cluster of CDV strains that is distinct from other clusters. These results indicate that, 1) CDV strain 007Lm isolated in Vero-DST cells is virulent, 2) nucleotide and amino acid sequences of H and P genes of strain 007Lm do not change after isolation in Vero-DST cells compared with the original virus from fresh tissue and 3) strain 007Lm isolated from a vaccinated dog belongs to a cluster far from the vaccine strains in the phylogenetic trees of H and P genes.

Key words: canine distemper; dog; pathogenesis; canine signaling lymphocyte activation molecules; sequence; Vero-DST cells; virulence.

Introduction

Canine distemper virus (CDV), a *Morbillivirus* of the family Paramyxoviridae, causes a highly infectious, systemic, often fatal disease in dogs (Krakowka et al., 1985). The initial infection is in epithelial cells and lymphoid tissue in the nasopharynx. The virus replicates primarily in lymphatic tissues of the respiratory tract. Temporary fever and the onset of lymphopenia appear from 3 to 6 days after infection (Krakowka et al., 1980; Sakaguchi et al., 1986; Iwatsuki et al., 1995). Commonly, acute infection is associated with respiratory or gastrointestinal tract disease or both and includes early or late infection of the central nervous system (Alldinger et al., 1993).

Recently, Vero cells expressing canine signalling lymphocyte activation molecules (SLAM) with a tag (Vero.Dog SLAM tag; Vero-DST cells) were developed to efficiently isolate CDV from clinical samples (Seki et al., 2003). Previously we showed that CDV strains could grow in Vero-DST and behave differently against Vero cells without SLAM (Lan et al, 2005a). Vero-DST cells were used not only for isolation of CDV from clinical samples but also for titration and researching growth profiles of new isolated CDV. We isolated recently some new CDVs from clinical cases that were propagated well by using Vero-DST cells. One of these CDVs, strain 007Lm, grew very well in Vero-DST cells and had a distinct high titer of 3.16×10^7 $TCID_{50}$ /25ul with a clear cytopathogenic effect at one day post inoculation (dpi) (Lan et al, 2005b). Isolating and keeping virulent highly virulent viruses are needed to produce vaccines and to investigate pathogenesis in animal experiments. The virulence of CDV has been investigated by inoculating strain A75/17 into susceptible animals (CDV-sero negative dogs) that then have clinical signs of distemper (Hamburger et al., 1991). We previously found CDV strains isolated in Vero-DST cells and B95a cells have two amino acid differences in the hemagglutinin sequence (Seki et al., 2003). However, we did not examine if the genes of isolated

viruses in Vero-DST cells are identical or almost identical to those of the original virus in fresh tissues. Therefore, in this study, we investigated the ability to cause canine distemper disease in dogs by strain 007Lm after isolation in Vero-DST cells and we made a molecular analysis.

Materials and methods

Experimental animals: Two 2-month old, sero-negative to CDV, female Beagle dogs, which we numbered 1 and 2, were purchased from Nosan Corporation (Yokohama, Japan) and were raised in isolated cages.

Virus infection: Under anesthesia with propofol (Schering-Plough, Osaka, Japan), a viral suspension (1 ml) containing $1.2x10^8$ $TCID_{50}$ of CDV strain 007Lm directly isolated from lymph node tissue of a dog showing pathological changes consistent with canine distemper at necropsy and then passaged at least 5 times in Vero-DST cells was dropped into the right conjunctiva and nostril of the two dogs by using a syringe without a needle.

Clinical signs and rectal temperatures were daily recorded until the dogs were euthanized at 28 and 21 dpi for dogs 1 and 2, respectively, with Nembutal (Dainippon Pharmaceutical Co., Ltd, Japan).

Sample collection: Serum to check the IgG and IgM, blood for a complete blood count and nasal, tonsilar, conjunctival, rectal and vaginal swabs to detect CDV by using reverse transcriptase (RT)-PCR and virus reisolation were collected at 0 (before inoculation of virus), 5, 7, 9, 10, 12, 14, 19, 21, 23 and 28 dpi. Samples for isolation were stored at -70^0C until used.

Titration of IgM and IgG against CDV: Sera IgM and IgG against CDV were measured by using enzyme-linked immunosorbent assay (ELISA). Samples (100 μ l), each serially diluted 2-fold, were added to each well of 96-well ELISA plates coated with

CDV antigen, and were incubated for 40 min at 37℃. After washing three times with 1% Tween 20- phosphate-buffered saline (PBS), anti-IgM and anti-IgG goat anti-sera labeled with secondary peroxidase were added, respectively, and were incubated for 1 hr. After washing with Tween 20-PBS , 100 μl color reagent, 3,3',5,5'-tetramethylbenzidine, dehydrochloride, dehydrate (Dojindo Laboratories, Japan), was added, the mixture was incubated for 10 min and the absorbance was measured at 450 nm.

Virus recovery: An each suspension of the nasal, tonsilar, conjunctival and vaginal swab (40 μ l) with 1000 units/ml of penicillin and 1000 μ g/ml of streptomycin was inoculated into one well of a 24-well plate seeded with Vero-DST cells. The cytopathogenic effect was observed by phase contrast microscope and the presence of CDV was confirmed by immunohistochemistry with a specific CDV–Nucleoprotein monoclonal antibody.

Results

Clinical features: The clinical signs of the dogs were mainly conjunctivitis with oculonasal discharge, depression, anorexia, coughing and moderate to severe diarrhea with blood. Dog 1 started to show mild to severe diarrhea with blood at 8 to 12 dpi and subsided with soft feces at 13 to 23 dpi. Ocular discharge and mild to severe depression without coughing occurred at 6 to 23 dpi. Dog 2 began to show soft feces, mild diarrhea to severe diarrhea with blood at 4 to 8 dpi and subsided with soft feces at 14 to 21 dpi. No neurological signs appeared in the two dogs.

Dogs 1 and 2 showed one and two peaks of rectal temperature at more than 40°C, respectively (Fig.11). The body weights of both dogs decreased. The feces of both dogs during severe diarrhea were checked for parasite eggs and bacteria, such as *Anaerophytes*, *E.coli*, *Salmonella*, *Clostridium perfringens*, *Cryptospodium*, *coccidium* and rotavirus,

but none were detected.

Lymphopenia started from 5 or 7 dpi in both dogs and continued to 14 dpi. Then, the number of lymphocytes increased very fast to the normal range (Fig. 12).

Serology: Anti-CDV IgM and anti-CDV IgG were not detected in the serum before infection with CDV at day 0 in the dogs. At 7 dpi, anti-CDV IgM and anti-CDV IgG started to increase (Fig. 13a and b). Anti-CDV IgM was detected at higher level than anti-CDV IgG and they both decreased from 21 dpi in dog 1. Anti-CDV IgM increased fast from 7 dpi of dog 2. The titer of anti -CDV IgM was higher than that of anti-CDV IgG in dog 2.

CDV detection by RT-PCR and virus recovery: CDV in the swabs from both dogs was detected by using RT-PCR (Table 8). It was not detected until 9 and 7 dpi in dogs 1 and 2, respectively. CDV was still detected in conjunctival, nasal and throat swabs of dog 1 at 28 dpi. The virus was also reisolated from swabs of both dogs in Vero-DST cells (Table 8). CDV was still recovered from the rectal swab of dog 1 at 14 dpi.

Sequencing and phylogenic analysis: The nucleotide sequence and phylogenetic analysis of the P and H genes of the isolated 007Lm strain and the original 007LmT virus in homogenated tissue were done. The nucleotide sequence analysis of a 390 bp fragment of the P gene showed a 100% identity of the nucleotide sequence between CDV strain 007Lm and the original strain 007LmT (Fig. 7). The homologies between strain 007Lm and CDV strains Hamamatsu, Jujo, Yanaka and Onderstepoort were 95.15%, 96.92%, 96.41% and 95.87%, respectively. A phylogenetic tree was constructed based on the nucleotide sequence of the 390 bp fragment of P gene (Fig.8).

The H gene of strains 007Lm and original 007LmT was sequenced and the predicted amino acid sequence was analysed. The H gene consisted of a fragment of 1824 bp and a single reading frame encoding 607 amino acids (Fig. 9). A 100% identity of the

nucleotide amino acid sequence was predicted between strain 007 and 007LmT. The predicted amino acid homologies between strain 007Lm and strains 5VD, 98-002, KDK1, Yanaka, Onderstepoort were 100%, 99.67%, 93.57%, 93.44%, and 90.41%, respectively. In the H protein of strain 007Lm, 12 cystein residues were at positions identical to those of the other compared strains (Fig. 9). One major hydrophobic region (amino acid 35-56) and eight potential glycosylation sites for asparagines N-linked glycosylation were at amino acid positions 19-21,149-151, 309-311, 391-393, 422-424, 456-458, 587-589 and 603-605. Fig. 10 shows the phylogenetic tree of the predicted amino acid sequences of strain 007Lm and other CDV strains. Strain 007Lm joined a tight cluster of CDV strains in the Asia 2 group that separated from known CDV clades (Hashimoto et al., 2001).

Discussion

Dogs infected with CDV strain 007Lm showed marked clinical signs of canine distemper, such as conjunctivitis, ocular discharge, nasal discharge, depression, coughing, diarrhea, lymphopenia, high body temperature and body weight loss (Appel, 1969; Appel and Summers, 1995). All dogs began to show lymphopenia after 5 or 7 dpi. Lymphoid depletion begins in the lymph nodes and thymus without necrosis at 6 dpi (Krakowka et al., 1980). Lymphopenia is the most important clinical sign reflecting immunosuppression (Krakowka et al., 1975) and may be affected by apoptosis (Kumagai et al., 2004). However, lymph node follicles of dogs naturally infected with CDV have pathological findings ranging from necrosis to lymphoid depletion (Iwatsuki et al.,1995). In this study, the degree of lymphopenia in dogs was severe from 5 to 14 dpi (Fig. 12) and increased after 19 dpi. Titers of IgM and IgG to CDV, body temperature and clinical signs with severe bloody diarrhea showed an acute form of canine distemper. High serum IgM titers are shown in acute clinical distemper cases, but high IgG titers indicate either

past or present infection by CDV (Greene and Appel, 1998).

The clinical signs of central nervous system may develop after systemic disease as acute encephalomyelitis (Zurbriggen et al., 1987; Baumgartner et al., 1989). In this study, both dogs showed no neurological signs during the 28-day observation. The occurrence of nervous signs might have been possible if the observation period had been longer.

Recently, we experienced several CDV cases in our laboratory from several areas in Japan. They showed mainly severe diarrhea with oculonasal discharge. In this study, all dogs predominantly showed severe hemorrhagic diarrhea with oculonasal discharge. Strain 007Lm may be a recent prevalent representative strain of CDV as judged by clinical signs in Japan.

However, until 7 dpi, no viral RNA was detected and no virus was recovered from swabs of both dogs suggesting that infection occurred in lymphoid tissue because of high temperature and lymphopenia. Before euthanasia, only viral RNA was detected from swabs, but no virus was recovered probably because of accumulation of RNA or few viruses in the dogs. RT-PCR is sometimes more sensitive than other methods, so it could detect very small amounts of RNA.

Biochemical analysis of blood was done to investigate the function of the liver and kidney. BUN, Cre, ALP, and GTP were still in normal range at the peak of diarrhea, implying that CDV does not affect the liver and kidney directly.

Two amino acid differences are at positions 530 and 548 in the hemagglutinin sequence between the CDV strains isolated in Vero-DST cells and in B95a cells (Seki et al., 2003). In this study, 100% identities of nucleotide and amino acid sequences of H and P genes between strain 007Lm isolated in Vero-DST cells and the original virus from infected fresh tissue implies that CDV might be genetically stable after isolation within a few passages and that Vero-DST cells are suitable as a cell line to isolate CDV from

fresh tissues. Strain 007Lm was isolated from the lymph node of a diseased dog that had been vaccinated against CDV (Lan et al, 2005b). The possibility that this dog was infected with recovered virus from vaccine was doubtful. In this study, the phylogenetic trees of P and H genes (Fig. 8, 10) showed that strain 007Lm joined to the clade of the Asia 2 virus group that was far from the vaccine virus group. The identites of the nucleotide sequence of the P gene and the amino acid sequence of the H gene between strain 007Lm and strain Onderstepoort were 95.87% and 90.41%, respectively. The hydrophobic region, including 20 amino acids, of strain 007Lm is one amino acid longer than for strains Hamamatsu, Yanaka and Ueno (Iwatsuki et al., 1997), KDK1 and strain Onderstepoort. Strain 007Lm has eight N-linked glycosylation sites, which are the same as in the strains in the Asia 2 group, but Japanese isolates in the Asia 1 group have nine sites.

We clarified by investigating the virulence and by molecular analysis the strain 007Lm isolated from a diseased dog in Japan, which was a recent representative of CDV in Japan. The results of this study indicated that CDV strain 007Lm, isolated from the lymph node of a dog by autopsy, in Vero-DST cells can cause disease in dogs, that the P and H genes of the 007Lm strain isolated in Vero-DST cells do not change compared with the original virus from fresh tissue and that strain 007Lm is far from the vaccine strains in phylogenetic trees of the P and H genes.