The role of feline aminopeptidase N as a receptor for infectious bronchitis virus

Brief Review

B. Miguel*, G. T. Pharr, and C. Wang

Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, U.S.A.

Received March 26, 2002; accepted July 11, 2002
Published online September 18, 2002 © Springer-Verlag 2002

Summary. Feline aminopeptidase N (fAPN) has been shown to serve as a receptor for feline, canine, porcine and human coronaviruses. Our objective was to determine if fAPN can serve as a receptor for infectious bronchitis virus (IBV). Feline kidney cells that express fAPN and hamster kidney fibroblasts that do not express fAPN were inoculated with IBV and monitored for replication by indirect fluorescent assay and confocal microscopy and in chicken embryonated eggs. The results showed that the feline cells were permissive to IBV but the hamster cells were not. The hamster cells became permissive to IBV after transfection with a fAPN cDNA suggesting that the feline APN molecule plays a role in IBV entry.

Introduction

Members of the family Coronaviridae infect a wide range of hosts and have been classified into three groups. One major group (group I) was recently shown to use aminopeptidase N (APN) as its cell surface receptor [7, 9, 10, 11, 16, 21–23, 28, 43]. Aminopeptidase N, also called CD13 in humans [31], is a zinc metalloprotease. It is a 150-kDa glycoprotein with zinc located in its globular region that is believed to be the active enzymatic site playing a major role in the cleaving of peptides [1, 2, 12, 15, 33]. APN is expressed on the plasma membranes of granulocytes [2, 36], lymphocytes and monocytes [26, 30, 31, 35]. It is also expressed on

*Present address: Department of Microbiology and Immunology, Cornell University Duck Research Laboratory, P.O. Box 217, Eastport, NY 11941-0217, U.S.A.
non-hematopoetic tissue including fibroblasts [35], synaptic membranes in the central nervous system [6], epithelial cells from the renal proximal tubules and the intestinal brush border, and endothelial and epithelial cells of the respiratory tract [8, 12, 34, 35]. Most recently APN has been isolated from chicken embryo yolk [32] and chicken intestine [14]. The feline aminopeptidase N has been shown to serve as a receptor for feline, canine, porcine and human coronaviruses in group I [38, 39]. The coronaviruses from group I cause disease only in their usual target host species. However, it has been reported that some strains of canine coronavirus (CCV) and human coronavirus 229E can infect other non-target species without causing disease [4, 5]. Cats inoculated with human coronavirus 229E or CCV seroconverted and in many cases shed the virus with no signs of clinical disease [4, 5].

Infectious bronchitis virus (IBV) is a member of the group III of Coronaviridae. This virus causes severe respiratory disease in chickens, and its receptor has not been identified. The objectives of this study were to: (1) determine if feline cells were permissive to Arkansas 99 (Ark 99) serotype of IBV, (2) evaluate if the IBV can replicate in feline cells, and (3) determine the contribution of APN to permissiveness of feline cells.

Materials and methods

Tissue culture

A cell line derived from a normal kidney cortex of a 12-week-old female feline (Felis catus) (CRFK-CCL-94) was purchased from American Type Culture Collection (Manassas, VA) and is henceforth designated as FEK. The cells were cultured in Dulbecco’s modified eagle medium (DMEM) (Sigma Chemical Co., St. Louis, MO) consisting of 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 250 µg/ml streptomycin (Sigma). The cells were incubated in 5% CO₂, 90% humidity at 37 °C until they were confluent. For the IFA assay, 4 × 10⁴ cells/ml were seeded into 6-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) containing 18 × 18 mm coverslips. The cells were used once attachment had occurred (24 h). A cell line of normal hamster kidney fibroblasts (BHK-21) was used as control cells for the indirect immunofluorescence assay (IFA). The medium and culture conditions for BHK cells were identical to those used for FEK cells.

Virus

A field strain of Ark 99 of IBV provided by Dr. J. Gelb Jr. (Dept. of Animal and Food Sciences, University of Delaware, Newark, DE) was propagated by passage in specific pathogen free (SPF) 10-day-old embryonated chicken eggs (Hy-Vac, Adel, Iowa). The allantoic fluid was harvested 24 h after infection, clarified by centrifugation at low speed (1,075 × g for 15 min), and the pellet was discarded. The virus was collected from the supernatant and concentrated by overnight high-speed centrifugation (10,000 × g) at 4 °C. The supernatant was discarded and the pellet was resuspended in 1 ml of TNE buffer (10 mM Tris-HCl pH 7.4, 0.1 M NaCl, 1 mM EDTA) [16]. The virus suspension was aliquoted and stored at −70 °C until used. For the calculation of 50% embryo infectious dose (EID₅₀) an aliquot was titrated in SPF embryonated chicken eggs [40].
The feline aminopeptidase cDNA (pBK-fAPN) subcloned into the plasmid pBK-CMV vector (Stratagene) was kindly provided by Dr. Andreas Kolb, Hannah Research Institute, Ayr, Scotland, UK. BHK-21 cells were transfected with pBK-fAPN or pBK-CMV (control plasmid) using LipofectAMINE reagent (Stratagene). Transfected cells were seeded in 25-cm² flasks for 24 h, at which time the medium was changed. The cells incubated for an additional 48 h before evaluation by flow cytometry.

Indirect fluorescent assay (IFA) and confocal microscopy

To evaluate the permissiveness of cells to IBV, monolayers of transfected (pBK-fAPN and pBK-CMV) BHK-21, non-transfected BHK-21 and FEK on coverslips were infected with a $10^{3.2}$ EID$_{50}$ of Ark/IBV virus. The cells were washed, fixed in ethanol, rinsed again with PBS (pH 7.4) and incubated with a monoclonal antibody to the S1 glycoprotein of Ark serotype IBV monoclonal (Dr. S. A. Naqi, Dept. of Microbiology and Immunology, Cornell University, Ithaca, NY) at a 1:50 dilution for 45 min at $37^\circ$C. The coverslips were washed in PBS three times for 5 min each and then incubated with a goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Southern Biotechnologies Associates Inc., Birmingham, AL) at a dilution of 1:50 for 45 min at $37^\circ$C. The coverslips were washed three times for 5 min in PBS, then mounted on microscope slides with mounting medium (Dako Corporation, Carpinteria, CA). Slides were viewed using epifluorescent microscopy (Olympus America, Inc., Lake Success, N.Y.), and confocal microscopy (Leica, Heidelberg, Germany).

Titration of the virus from FEK

FEK grown on 6-well tissue culture plates were infected with a $10^{3.2}$ EID$_{50}$ of Ark/IBV virus. The supernatant of these infected cell cultures was collected at 1, 2, 3 and 10 d post infection. The media in this last culture was collected twice, pooled and frozen at $-80^\circ$C until titrated. Virus titer was calculated using the method of Reed and Munch described in [40].

Flow cytometry analysis

Expression of fAPN on the surface of transfected BHK-21 cells was determined using a monoclonal antibody to human CD13 clone WM-47 (Accurate Antibodies, Westbury, NY) that cross-reacts with the feline APN (personal communication with Dr. K. V. Holmes). BHK-21 cells were washed two times with phosphate buffered saline (PBS), incubated with anti-human CD13 or an isotype matched control, washed, and then incubated with FITC conjugated goat anti-mouse IgG. An additional control included cells with the secondary antibody only. All incubations were done for 5 min on ice. After the final wash, all cells were suspended in 500 µl of PBS and analyzed by flow cytometry (Calibur Flow Cytometry System, San Jose, CA).

Results

Infection of FEK cells

IFA and confocal analyses of FEK cells inoculated with Ark/IBV demonstrated that the FEK cells were susceptible to the virus. Fluorescence was detected on the surface of inoculated cells and the accumulation of viral antigen in the cytoplasm, the site of replication for IBV, was detected (Figs. 1A and 1E). Viral antigen was detected as soon as 1 h post infection (PI). The BHK-21 cells, which were shown
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to be non-permissive to IBV infection by IFA, were used as a negative control cell line (Figs. 1B and IF).

*Replication of IBV in FEK cells*

To determine if IBV could replicate in the FEK cells, cell cultures were infected with Ark/IBV and the supernatants from infected cells were collected at 1, 2, 3 and 10 d PI. The cell supernatants were titrated in chicken embryos and the EID$_{50}$ was calculated. The results indicated that Ark/IBV does replicate in the FEK cells in vitro. The EID$_{50}$/ml values were $10^{3.8}$, $10^{5.8}$, $10^{7.2}$ and $10^{9.2}$ for the 1, 2, 3, and 10 d incubation, respectively. The pathological lesions, including dwarfed and hemorrhagic embryos with curled toes typically present in IBV infected embryos [40] were produced in SPF embryonated chicken eggs infected with virus passaged in FEK cells (Fig. 2).

**Fig. 2.** Chicken embryo lesions. First row: chicken embryos were inoculated with saline; Second to fifth rows: Chicken embryos were inoculated with dilutions of $10^{-2}$ to $10^{-5}$ IBV propagated in FEK CCL-94

**Fig. 1.** Indirect fluorescent assay (A to D) and confocal (E to H) images of FEK CCL-94, transfected BHK-21 and non-transfected BHK cells infected with ARK/IBV. A FEK cells, B BHK cells, C BHK cells transfected with pBK-fAPN, D BHK transfected with pBK-CMV, E FEK cells, F BHK cells, G BHK cells transfected with pBK-fAPN, and H BHK transfected with pBK-CMV
Fig. 3. Overlay of histograms generated by flow cytometry analysis of different cell types which were labeled with anti-CD13 monoclonal antibody. A Dotted line: BHK-21 transfected with pBK-CVM, B Dashed line: BHK-21 cells, C Solid line: FEK94 cells, and D Dark solid line: BHK-21 cells transfected with pBK-fAPN

Transfection of non-permissive BHK-21

Previous reports have shown fAPN to be the molecule that allows entry of the coronavirus from other species into the feline cells [38, 39]. To test the hypothesis that the feline APN had a role in permissiveness of FEK cells to Ark/IBV, BHK-21 cells were transfected with pBK-CMV plasmid or pBK-fAPN. Surface expression of fAPN was assayed by flow cytometry. The BHK-21 cells transfected with pBK-fAPN were shown to express the fAPN receptor on the cell surface (Fig. 3), and were permissive to Ark/IBV (Figs. 1C and 1G). Viral antigen labeled with fluorescein was detected in the cell cytoplasm, and further examination of the cells with confocal microscopy also showed that the virus was intracytoplasmic and not membrane bound (Fig. 1G). However, fluorescence was not detected on the surface or in cytoplasm of BHK cells transfected with pBK-CMV (Figs. 1D and 1H).

Discussion

This study showed that FEK cells in culture were permissive to Ark/IBV and the virus was capable of replicating within the cells. Previous studies have shown that cats may become infected with coronavirus from other animal species and seroconvert without developing clinical signs of disease [4, 5]. Thus, it is possible that cats may become infected with Ark/IBV and serve as carriers of the virus for chickens if cats shed the virus. This RNA virus lacks proof reading by RNA
polymerases, and therefore may undergo recombination [3, 13, 17] in cats coinfected with different coronaviruses. The resulting recombinant viruses may have properties different from either parent, infect different hosts and have different tissue tropism, antigenicity and virulence, possibly resulting in the emergence of a new disease [38].

Because feline cells can harbor coronaviruses from several species, this may explain why recombination of coronaviruses occurs somewhat frequently. Cats may serve as a vehicle for the recombination event and may contribute to new IBV emerging serotypes. There have been more than 20 serotypes of IBV identified and new IBV variants continue to emerge. Recombination has been experimentally proven to occur in IBV [19, 24, 25, 27, 29, 41] particularly in the hypervariable region of S1 [42]. This region of the S1 glycoprotein of IBV has also been postulated to be one of the major elicitors of the immune response [18, 20, 37]. Consequently, current vaccines generally do not cross protect against the different serotypes, resulting in a serious problem in the control and prevention of this disease.

BHK-21 cells are not naturally permissive to this virus but became permissive when transfected with the fAPN cDNA. This result indicates that the fAPN plays a role in IBV entry. Previous work demonstrated that fAPN serves as the receptor for Group I coronaviruses infecting feline cells [38, 39]. This is the first report indicating that IBV of the Group III coronaviruses also uses this receptor. While the fAPN appears to play a role as a receptor molecule for the Ark/IBV in feline cells, further investigation is needed to determine if the chicken APN plays a role in the entry of the Ark/IBV in its natural host.

**Acknowledgments**

We thank Dr. Andreas Kolb, Cell Physiology Group, Hannah Research Institute Ayr, KA6 5HL, UK for supplying us with the feline APN plasmid. This study was supported by the College of Veterinary Medicine and the Mississippi Agriculture and Forestry Experiment Station. Contribution No. J-9932 from the Mississippi Agriculture and Forestry Experiment Station.

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Author’s address: Chinling Wang, DVM, MS, PhD. P.O. Box 6100, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS 39762, U.S.A.; e-mail: wang@cvm.msstate.edu