Differential effect of cholesterol on type I and II feline coronavirus infection

Tomomi Takano 1 · Yui Satomi 1 · Yuu Oyama 1 · Tomoyoshi Doki 1 · Tsutomu Hohdatsu 1

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Abstract Feline infectious peritonitis (FIP) is a fatal disease of domestic and wild felidae that is caused by feline coronavirus (FCoV). FCoV has been classified into types I and II. Since type I FCoV infection is dominant in the field, it is necessary to develop antiviral agents and vaccines against type I FCoV infection. However, few studies have been conducted on type I FCoV. Here, we compare the effects of cholesterol on types I and II FCoV infections. When cells were treated methyl-β-cyclodextrin (MβCD) and inoculated with type I FCoV, the infection rate decreased significantly, and the addition of exogenous cholesterol to MβCD-treated cells resulted in the recovery of the infectivity of type I FCoV. Furthermore, exogenous cholesterol increased the infectivity of type I FCoV. In contrast, the addition of MβCD and exogenous cholesterol had little effect on the efficiency of type II FCoV infection. These results strongly suggest that the dependence of infection by types I and II FCoV on cholesterol differs.

Introduction

Coronaviruses are single-positive-strand RNA viruses with a genome size of approximately 30 kbp, belonging to the family Coronaviridae, subfamily Coronavirinae. The subfamily Coronavirinae has been further classified into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus [6]. In cats, feline coronavirus (FCoV) of the genus Alphacoronavirus has been identified.

The FCoV virion is mainly composed of nucleocapsid (N), envelope (E), membrane (M), and peplomer spike (S) proteins [6]. There are two genotypes/serotypes of FCoV: FCoV has been classified into types I and II based on the amino acid sequence of its S protein [10, 19]. Type II FCoV was previously suggested to be produced by recombination between type I FCoV and type II canine coronavirus (CCoV) [7, 31]. Separate from these genotypes/serotypes, FCoV has been classified into two biotypes: weakly pathogenic feline enteric coronavirus (FECV; low-virulent FCoV) and strongly pathogenic feline infectious peritonitis virus (FIPV; virulent FCoV) [20]. The etiology of the pathogenesis of FECV/FIPV has not yet been elucidated in detail. Two theories currently exist: an “internal mutation theory” and a “circulating virulent/avirulent theory” [1, 3, 21]. FIPV causes a fatal disease called FIP in wild and domestic cats [20]. Although antiviral agents and vaccines against FIPV infection have been investigated, no method has yet been established for practical use.

Since type I FCoV infection is dominant in the field [9, 16, 18, 26], antiviral agents and vaccines need to be developed against type I FCoV infection. However, type II FCoV is mainly used in studies on FIP because the propagation of type I FCoV in feline cell lines and monocytes/macrophages is weaker than that of type II FCoV. Although recombinant type I FCoV has recently been prepared by reverse genetics [30], studies on type I FCoV have not progressed. Differences have been identified in the following events between types I and II FCoV: replicative ability in cell lines and monocytes/macrophages [29], the amino acid sequence and immunogenicity of the S protein [10, 19], and the virus receptor used to enter cells.
Cholesterol is an essential component in animal cells [12]. It has been shown to play a role in viral infection of cells. For example, host cell cholesterol levels have been shown to influence the replication of human hepatitis C virus (HCV), human immunodeficiency virus, and dengue virus [14, 17, 36], but not that of vesicular stomatitis virus (VSV) [13, 22]. Furthermore, cholesterol contained in the envelope of influenza virus has been shown to affect infection of cells [27]. Cholesterol contained in host cells was previously reported to be involved in host cell infection by mouse hepatitis virus (MHV) and severe acute respiratory syndrome coronavirus (SARS-CoV) [5, 33], which, like FCoV both belong to the family Coronaviridae. Transmissible gastroenteritis virus (TGEV) and type II CCoV infections involve cellular and viral cholesterol [23, 25]. The S protein of type II FCoV is homologous to those of TGEV and type II CCoV, and the infection of cells by type II FCoV was previously shown to be only slightly influenced by cholesterol [35]. On the other hand, the relationship between type I FCoV and cholesterol remains to be investigated.

In the present study, we determined whether cellular and viral cholesterol levels influenced the infectivity of FCoV types I and II in cell culture.

Materials and methods

Cell cultures and viruses

Felis catus whole fetus (fcwf)-4 cells (kindly supplied by Dr. M. C. Horzinek of State University of Utrecht) were grown in Eagle’s minimum essential medium containing 50 % L-15 medium, 5 % fetal calf serum (FCS), and 100 U of penicillin and 100 μg of streptomycin per ml. The type I FCoV KU-2 strain (FCoV-I KU-2) was isolated in our laboratory, and the type I FCoV Black strain (FCoV-I Black) and the type I FCoV UCD-1 strain (FCoV-I UCD-1) were kindly supplied by Dr. J. K. Yamamoto of the University of Florida. The type II FCoV 79-1146 strain (FCoV-II 79-1146) was kindly provided by Dr. M. C. Horzinek. VSV (New Jersey strain) was obtained from the Laboratory of Veterinary Microbiology at Kitasato University. These viruses were grown in fcwf-4 cells at 37 °C.

Compounds

Methyl-β-cyclodextrin (MβCD) and water-soluble cholesterol were obtained from Sigma Aldrich (USA). These compounds were diluted to the desired concentrations in maintenance medium.

Detection of cellular cholesterol

The cellular cholesterol content of fcwf-4 cells was evaluated using a Cholesterol Cell-Based Detection Assay Kit (Cayman Chemical, USA) according to the manufacturer’s instructions. Briefly, fcwf-4 cells were grown on an 8-well Lab-Tek Chamber Slide (Thermo Fisher Scientific, USA). Confluent fcwf-4 cell monolayers were cultured in medium containing 16 mM MβCD at 37 °C for 30 min. After washing with PBS, cells were cultured in medium containing 0.5 mg of water-soluble cholesterol per ml at 37 °C for 30 min. After fixing and staining, filipin-III-binding cells were analyzed using a Leica FW4000 fluorescence microscope (Germany).

Cytotoxic effects of compounds

Cell viability was measured using a WST-8 assay, as described previously [28]. Briefly, fcwf-4 cells were seeded in 96-well plates. MβCD or water-soluble cholesterol was added in triplicate to the wells. After 48 hours, the culture supernatants were removed, WST-8 solution (WST-8 Cell Proliferation Assay Kit; Kishida Chemical Co., Ltd., Japan) was added, and the cells were returned to the incubator for 1 hour. The absorbance of formazan produced was measured at 450 nm using a 96-well spectrophotometric plate reader as described by the manufacturer. Cytotoxicity was calculated using the following formula: Cytotoxicity (％) = [(OD of untreated cells - compound-treated cells)/(OD of compound-untreated cells)] × 100.

Effects of cellular cholesterol depletion on FCoV infection in fcwf-4 cells

The method for the depletion of cholesterol from cells was modified from that described by Ren et al. [25]. Briefly, confluent fcwf-4 cell monolayers were cultured in medium containing MβCD ranging from 0 to 16 mM in 24-well multi-plates at 37 °C for 30 min. After washing with phosphate-buffered saline (PBS), the virus (multiplicity of infection [MOI], 0.01) was adsorbed into the cells at 37 °C for 1 hour. After washing, cells were cultured in maintenance medium with 1.5 % carboxymethyl cellulose (CMC-MEM). Cell monolayers were incubated at 37 °C for 48 hours, fixed, and stained with 1 % crystal violet solution containing 10 % buffered formalin, and the plaques were then counted. The percentage of plaque decrease or increase was determined using the following formula: Percentage of the plaque number (%) = [(plaque number of compound-treated cells)/ (plaque number of untreated cells)] × 100.
**Effects of cellular cholesterol replenishment on FCoV infection in fcwf-4 cells**

The method of replenishment of cholesterol-depleted cells was modified from that described by Ren et al. [25]. Briefly, confluent fcwf-4 cell monolayers were cultured in medium containing 16 mM MβCD in 24-well multi-plates at 37°C for 30 min. After washing with PBS, cells were cultured in medium containing water-soluble cholesterol ranging from 0 to 2.5 mg/ml at 37°C for 30 min. Cells were washed and the virus (MOI, 0.01) was adsorbed onto the cells at 37°C for 1 hour. After washing, cells were cultured in CMC-MEM or medium without carboxymethyl cellulose (MEM). In the case of cultured in CMC-MEM, the cell monolayers were incubated at 37°C for 48 hours, fixed, and stained with 1% crystal violet solution containing 10% buffered formalin, and the resulting plaques were then counted. The percentage of plaques (%) was determined by the method described above. When MEM was used, the culture supernatants were collected 48 hours postinfection, and virus titers were determined by the TCID50 assay.

**Immunofluorescence assay**

Fcwf-4 cells were grown on an 8-well Lab-Tek chamber slide (Thermo Fisher Scientific, USA). Confluent fcwf-4 cell monolayers were cultured in medium containing 16 mM MβCD at 37°C for 30 min. After washing with PBS, cells were cultured in medium containing 0.5 mg of water-soluble cholesterol per ml at 37°C for 30 min. Cells were washed, and the virus (MOI, 0.01) was adsorbed into the cells at 37°C for 1 hour. After washing, cells were cultured in MEM. The cell monolayers were incubated at 37°C. After 12 hours, N protein levels were determined by an immunofluorescence assay (IFA) as described previously [11].

**Effects on infectivity of depletion and replenishment of cholesterol from the virus**

The method for depletion and replenishment of cholesterol from the virus was modified from that described by Pratelli and Colao [23]. Briefly, the virus (MOI, 0.1) was incubated with medium containing 16 mM MβCD at 37°C for 1 hour. In order to specifically determine whether cholesterol depletion by MβCD affected the number of plaques caused by FCoV, MβCD-treated FCoV was adsorbed onto the cells at 37°C for 1 hour. MβCD-treated viral suspensions were diluted 1:9 in medium before infection to avoid the carry-over effects of MβCD on fcwf-4 cells. After washing, cells were cultured in CMC-MEM or MEM. In order to analyze infection efficiency after viral cholesterol replenishment, the virus (MOI, 0.1) was incubated with medium containing 16 mM MβCD at 37°C for 30 min. MβCD-treated viral suspensions were then incubated with medium containing water-soluble cholesterol ranging from 0 to 2.5 mg/ml at 37°C for 1 hour. Cholesterol- and MβCD-treated FCoV were adsorbed onto cells at 37°C for 1 hour in order to specifically determine whether cholesterol replenishment affected the number of plaques caused by FCoV. In an attempt to avoid the carry-over effects of cholesterol and MβCD on fcwf-4 cells, cholesterol- and MβCD-treated viral suspensions were diluted 1:9 in medium before infection. Cells were washed, and the virus was adsorbed onto the cells at 37°C for 1 hour. After washing, cells were cultured in CMC-MEM at 37°C for 48 hours. The cells were fixed and stained with 1% crystal violet solution containing 10% buffered formalin, and the resulting plaques were counted. The number of plaques, expressed as a percentage of the control, was measured by the method described above.

**Statistical analysis**

Data from only two groups were analyzed by Student’s t-test (Welch’s t-test or Bartlett’s test), and multiple groups were analyzed by one-way ANOVA followed by Tukey’s test.

**Results**

**The cytotoxic effects of MβCD and exogenous cholesterol**

The cytotoxic effects of MβCD and exogenous cholesterol in fcwf-4 cells are shown in Tables 1 and 2. Exogenous cholesterol slightly exhibited cytotoxicity at 5 mg/ml.

**Effects of MβCD and exogenous cholesterol on cellular membranes**

We investigated changes in the cellular cholesterol content of fcwf-4 cells treated with MβCD and water-soluble cholesterol (exogenous cholesterol) using filipin III as a fluorescent stain for cholesterol. Cellular cholesterol levels specifically decreased in fcwf-4 cells treated with 16 mM MβCD (Fig. 1A and B) and slightly decreased in cells treated with 4 mM MβCD (data not shown). The cellular cholesterol level in fcwf-4 cells treated with 16 mM MβCD followed by the addition of 0.5 mg/ml of exogenous cholesterol returned to a level similar to that in untreated-fcwf-4 cells (Fig. 1A and C).
Efficiency of depletion and replenishment of cellular cholesterol on FCoV

In order to confirm the effects of the cellular depletion of cholesterol on FCoV infection, fcwf-4 cells were pretreated with MβCD ranging from 0 mM to 16 mM, followed by virus inoculation. The number of plaques caused by FCoV-I KU-2 was reduced by MβCD treatment in a dose-dependent manner (Fig. 2). The number of plaques caused by FCoV-II 79-1146 was reduced in cells treated with 16 mM MβCD but did not show a significant difference between treatment and control (0 mM MβCD). In contrast, the number of plaques caused by VSV was not affected by pretreatment with MβCD. Cholesterol-depleted fcwf-4 cells were replenished by addition of exogenous cholesterol before virus inoculation in order to determine whether cellular cholesterol is important for FCoV-I infection. The number of plaques caused by FCoV-I KU-2 was increased by treatment with exogenous cholesterol in a dose-dependent manner (Fig. 3). In contrast, the number of plaques caused by FCoV-II 79-1146 increased slightly, but VSV was not affected by replenishment with exogenous cholesterol.

The influence of cellular cholesterol on the replication of other type I FCoVs was investigated. The number of plaques caused by FCoV-I Black, and FCoV-I UCD-1 was reduced by treatment with 16 mM MβCD (Fig. 4A). However, exogenous cholesterol restored the number of plaques caused by these viruses in a dose-dependent manner. When 0.5 mg of exogenous cholesterol per ml was added, the number of plaques caused by FCoV-I Black and FCoV-I UCD-1 was significantly higher than without treatment with MβCD and exogenous cholesterol. In contrast, cellular cholesterol

### Table 1 Cytotoxic effects of MβCD in fcwf-4 cells

| MβCD (µM) | 0    | 2    | 4    | 8    | 16   | 32   |
|-----------|------|------|------|------|------|------|
| Cell viability (%) | 98.7 ± 1.6<br>1) | 100.7 ± 3.0<br>1) | 97.7 ± 1.8<br>1) | 98.6 ± 2.1<br>1) | 98.4 ± 3.3<br>1) | 96.3 ± 2.6<br>1) |

1) Cell viability (% mean ± SE); n = 3

### Table 2 Cytotoxic effects of exogenous cholesterol (water-soluble cholesterol) in fcwf-4 cells

| Exogenous cholesterol (mg/ml) | 0   | 0.1 | 0.5 | 1.0 | 2.5 | 5    |
|-------------------------------|-----|-----|-----|-----|-----|------|
| Cell viability (%) | 99.1 ± 1.9<br>1) | 98.5 ± 1.8<br>1) | 98.9 ± 1.7<br>1) | 96.9 ± 2.3<br>1) | 91.9 ± 2.5<br>1) | 80.2 ± 7.3<br>1) |

1) Cell viability (% mean ± SE); n = 3

**Fig. 1** Effects of MβCD and exogenous cholesterol on cellular membrane content. Fcwf-4 cells were incubated with 16 mM MβCD at 37 °C for 30 min. After washing, cells were incubated with 0.5 mg of cholesterol per ml. The cellular cholesterol content of fcwf-4 cells was evaluated using a filipin-cholesterol stain. (A) Untreated cells, (B) cholesterol-depleted cells (MβCD-treated cells that were then incubated with vehicle), and (C) cholesterol-replenished cells (MβCD-treated cells that were then incubated with exogenous cholesterol).
depletion and replenishment only slightly affected the number of plaques caused by FCoV-II 79-1146. In order to determine the amount of FCoV released into the supernatant of cholesterol-depleted and -replenished fcwf-4 cells, we measured virus titers in culture supernatants using the TCID$_{50}$ assay. The titers of FCoV-I KU-2, FCoV-I Black, and FCoV-I UCD-1 in the supernatant of MβCD-treated cells were significantly decreased by approximately 100-fold (Fig. 4B), and exogenous cholesterol restored the titers of these viruses in a dose-dependent manner. In
contrast to type I FCoVs, the cellular depletion and replenishment of cholesterol slightly affected the titer of FCoV-II 79-1146 in the supernatant of fcwf-4 cells. We investigated the expression of viral proteins in order to further investigate the effects of cholesterol on FCoV infectivity. The N protein levels of FCoV-I KU-2 specifically decreased in fcwf-4 cells treated with 16 mM MβCD (Fig. 5). The N protein levels of fcwf-4 cells treated with 16 mM MβCD, followed by the addition of 0.5 mg of exogenous cholesterol per ml, returned to levels similar to those in untreated-fcwf-4 cells. In contrast to type I FCoV KU-2, the depletion and replenishment of cellular cholesterol did not affect the N protein levels of FCoV-II 79-1146 in fcwf-4 cells.

**Facilitation of type I FCoV infection by the addition of cholesterol to fcwf-4 cells**

As shown in Figure 4, number of plaques caused by type-I FCoV increased in exogenous-cholesterol-treated fcwf-4 cells. In order to confirm the influence of cholesterol on type-I FCoV in cells untreated with MβCD, untreated cells were incubated with exogenous cholesterol and then inoculated with the virus. Incubation with 0.1 and 0.5 mg of exogenous cholesterol per ml significantly increased the number of plaques caused by FCoV-I KU-2, FCoV-I Black, and FCoV-I UCD-1 (Fig. 6A). However, addition of 2.5 mg of exogenous cholesterol per ml did not increase the number of plaques caused by type-I FCoV. Virus titers in culture supernatants showed similar changes; however, no significant differences were noted (Fig. 6B).

**Efficiency of depletion and replenishment of cholesterol in virions**

In order to confirm the effects of cholesterol depletion on the envelope of FCoV, the virus was treated with 16 mM MβCD prior to inoculation. Treatment with MβCD reduced the number of plaques caused by FCoV-I KU-2 (Fig. 7), and exogenous cholesterol restored the number of plaques caused by FCoV-I KU-2 in a dose-dependent manner; however, no significant differences were noted (Fig. 7). In contrast to FCoV-I KU-2, viral cholesterol depletion and replenishment did not affect the number of plaques caused by FCoV-II 79-1146.

**Discussion**

Between 70 and 98 % of FCoV-infected cats in the field have been reported to be infected with type I FCoV [9, 16, 18, 26]. This finding demonstrates that type I FCoV needs to be examined in more detail in studies on the prevention and treatment of feline FCoV infection. However, no study using type I FCoV has been conducted to date because the propagation of type I FCoV in feline cell lines is low. Type II FCoV uses feline aminopeptidase N (fAPN) as a receptor [8, 34], and infection of cells with this virus has been studied in detail. In contrast, the virus receptor for type I FCoV has not been identified, and the mechanism underlying cell infection has not yet been elucidated. Therefore, a basic analysis of type I FCoV is needed for the development of effective antiviral drugs against type I FCoV, which is prevalent in the field. In the present study, we discovered that the dependence of the infection on cholesterol differs between types I and II FCoV. We recently reported that the rate of inhibition of viral replication by a viroporin inhibitor differed between types I and II FCoV [28]. Based on these findings, it is desirable to use type I FCoV, in addition to type II FCoV, for the development of effective antiviral drugs.

We depleted cellular cholesterol in fcwf-4 cells using MβCD. Cell membrane cholesterol is extracted by binding to the hydrophobic cores of MβCD [15]. Nystatin is
frequently used, as well as MβCD, to analyze cholesterol-dependent viral cell infection; however, it sequesters, rather than removes cell membrane cholesterol [32]. Moreover, strong cytotoxicity was noted in fcwf-4 cells treated with nystatin in a preliminary experiment. Therefore, we selected MβCD.

The involvement of cholesterol in the entry of an enveloped virus has been reported previously [24]. Based on the type of cholesterol dependence they display, viruses can be divided into four groups: i) envelope cholesterol (e.g., influenza virus) [27], ii) cell membrane cholesterol (e.g., Ebola virus) [2], iii) both envelope and cell membrane cholesterol (e.g., human immunodeficiency virus) [36], and iv) no cholesterol involvement in cell infection (e.g., VSV) [13, 22]. The coronaviruses MHV and SARS-CoV of the genus Betacoronavirus belong to the second group [5, 33], while TGEV and type II canine coronavirus (CCoV) of the genus Alphacoronavirus belong to the third group [23, 25]. Based on our results, type I FCoV belongs to the second or third group, and type II FCoV belongs to the second or fourth group, suggesting that the cholesterol dependence of infection differs between FCoV types I and II.

MβCD slightly inhibited type II FCoV replication. A previous report showed that cholesterol had a slight effect on infection by FCoV-II 79-1146. It has been suggested that type II FCoV does not require much cholesterol for infection.

The virus receptor for type II FCoV is fAPN [8, 34], whereas that for type I FCoV has not yet been identified. Tekes et al. [29] suggested that fAPN functions as a virus receptor for type I FCoV, although it is very inefficient. Based on this hypothesis, type I FCoV might enter cells using fAPN and a cofactor (coreceptor). MHV fuses with the cell membrane, utilizing cholesterol after binding to carcinoembryonic antigen-related cell adhesion molecule (CEACAM), and enters cells using cholesterol in the absence of CEACAM [33]. Cholesterol may also function as an essential cofactor in type I FCoV infection.
Furthermore, the envelope cholesterol of type I FCoV has been suggested to be involved in infection, as shown in Figure 7. Further investigations are needed in order to determine whether the envelope cholesterol of type I FCoV plays an important role in cell infection.

Although it currently remains unclear whether the results of the present study provide direct evidence regarding the cell entry mechanism of field strains of type I FCoV, they may be useful for determining the mechanism responsible. Previous studies on the FCoV replication mechanism were mainly performed using type II FCoV. Therefore, our study may be more useful than previous studies using type II FCoV for elucidating the cell entry mechanism of field strains of type I FCoV.

In this study, the dependence of the infection on cholesterol markedly differed between types I and II FCoV. Since type I FCoV is dominant in the field, studies using type I FCoV are desirable. Our results may be useful for obtaining a deeper understanding of type I FCoV.

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