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Short communication

Equine coronavirus induces apoptosis in cultured cells

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Abstract

Equine coronavirus (ECoV) was first isolated from a diarrheic foal and was found genetically similar to group II coronaviruses. However, its pathological characteristics were not adequately investigated. In our preliminary *in vitro* investigation, ECoV-induced cell death was observed in bovine kidney-derived MDBK cells. Based on this finding, we investigated whether the ECoV-induced CPE was apoptosis. Following ECoV infection, MDBK cells showed morphological changes such as cell rounding and detachment from the culture surface. Moreover, syncytium formation was observed as the other type of cytopathic effect in ECoV infection. Morphologic and biochemical features of apoptosis, such as nuclear fragmentation and DNA ladder formation, were also detected in ECoV-infected cells. Moreover, as is commonly observed in coronavirus infection in other animals, the activities of effecter caspases – caspase-3/7 – and initiator caspases – caspase-8 and caspase-9 – that are representative factors in the death receptor-mediated apoptotic pathway and mitochondrial apoptotic pathway, respectively, were increased in ECoV-infected MDBK cells. Therefore, it was suggested that ECoV can induce apoptosis in MDBK cells via a caspase-dependent pathway. Apoptotic death of infected cells is detrimental because it causes cell and tissue destruction and inflammatory responses. Although the pathological characteristics of ECoV are largely unknown, apoptosis may be the pathological basis of lesions of the digestive system in ECoV infection.

Keywords: Equine coronavirus; Apoptosis

1. Introduction

Apoptosis is a physiologically important process that eliminates damaged cells and is defined by typical changes in cellular morphology and biochemical features, including DNA fragmentation, cytoplasm vacuolization, chromatin condensation and margination, and cellular breakdown into apoptotic bodies (Kerr et al., 1972). Apoptosis can be triggered by diverse intracellular and extracellular signals, including virus infections. Many viruses have the ability to induce apoptosis, which is one of the cytolytic properties of viral infections, and show the phenotype of cytopathic effect (CPE) *in vitro*; this is particularly

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observed in RNA viruses, including *Coronaviridae* (Collins, 2001; Eleouet et al., 1998; Liu et al., 2001, 2006; Liu and Zhang, 2007; Ruggieri et al., 2007).

The *Coronaviridae* family is a group of enveloped viruses with a large, positive-stranded RNA genome and comprises two genera—*Coronavirus* and *Torovirus*. Coronaviruses are classified into three groups (I–III) based on serological cross-reactivity and phylogenetic analysis (Gonzalez et al., 2003). Equine coronavirus (ECoV) was first identified in the feces of a diarrheic foal. When the amino acid and genome sequences of ECoV were compared with those of other avian and mammalian coronaviruses, ECoV was found to be relatively similar to group II coronaviruses such as murine hepatitis virus (MHV) and human coronavirus (HCoV) OC43 and particularly to bovine coronaviruses (BCoVs) (Guy et al., 2000; Zhang et al., 2007). Although there have been reports on suspected cases of spontaneous ECoV infection showing severe diarrhea, investigations on the pathogenicity of ECoV infection have not been conducted until now.

In *in vitro* cultures, human rectal adenocarcinoma (HRT-18) cells were used for propagation of ECoV, but this cell line could not induce a distinctive CPE (Guy et al., 2000). In our preliminary investigation, we found that MDBK cells (a bovine kidney-derived cell line) infected with ECoV showed more distinctive morphological changes. In this study, to elucidate the mechanisms of cell injury by ECoV, we investigated whether the ECoV-induced CPE was apoptosis by using cytological change, DNA fragmentation, and caspase activity as the indices.

2. Materials and methods

2.1. Virus and cell lines

ECoV NC99 was kindly provided by the Equine Research Institute of the Japan Racing Association. MDBK cells were cultivated in Eagle's minimum essential medium (EMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% fetal bovine serum and 0.3% tryptose phosphate broth. Subconfluent cells infected with ECV at 10^5 $TCID_{50}$ were harvested and collected at 0, 3, 6, 9, 12, 15, 18, 21, and 24 h after treatment (HAT). Mock-infected cells were used as controls.

2.2. Cytological analysis

ECoV-infected and mock-infected MDBK cells were collected at the predetermined time points after infection, fixed in methanol for 5 min, stained with Giemsa's solution for 20 min, and examined microscopically.

2.3. DNA fragmentation analysis by gel electrophoresis

The Apoptotic DNA Ladder Detection Kit (CHEMICON International Inc., CA, USA) was used for DNA extraction from cells. Briefly, ECoV-infected MDBK cells collected at the predetermined time points after infection were lysed by the addition of 20 μl of TE lysis buffer and 5 μl of Enzyme A (RNase A) and incubated at 37 °C for 10 min. Then, 5 μl of Enzyme B (proteinase K) was added, and the lysate was further incubated at 37 °C for 30 min. DNA was precipitated using ethanol. After washing with 70% ethanol, the pellet was resuspended in DNA suspension buffer. For detecting the DNA ladder, the extracted DNA samples were run on a 1% agarose gel in Tris–acetic acid–EDTA buffer. After electrophoresis, the gel was stained with ethidium bromide (Nippon Gene Co. Ltd., Toyama, Japan), visualized with a UV light transilluminator, and photographed.

2.4. Caspase assay

Caspase-3/7 activity was measured by Apo-ONE Homogeneous Caspase-3/7 Assay (Promega Corporation, WI, USA), according to the manufacturer's instructions. In brief, subconfluent MDBK cells seeded in 96-well plates were infected and harvested at the various predetermined times post-infection. ECoV-infected and mock-infected cells were treated with equal volumes of the homogeneous caspase-3/7 reagent and incubated for 12 h. Then, their fluorescence was measured using a fluorescence microplate reader. Individual caspase-3/7 activities were measured as the difference in fluorescence between ECoV-infected cells and mock-infected cells at each time point.

Caspase-8 and caspase-9 activities were measured using a Caspase-Glo Assay kit (Promega Corporation, WI, USA), according to the manufacturer's instructions. In brief, subconfluent MDBK cells seeded in

96-well plates were infected and harvested at the various predetermined times post-infection. ECoV-infected and mock-infected cells were treated with equal volumes of the Caspase-Glo 8 or Caspase-Glo 9 reagent and incubated for 30 min. Then, their luminescence was measured using a luminometer. Individual caspase-8 and caspase-9 activities were measured as the difference in fluorescence between ECoV-infected cells and mock-infected cells at each time point.

3. Results

3.1. Histological and DNA fragmentation analysis

The ECoV-infected MDBK cells gradually developed the CPE, including cell rounding and detachment, at and after 15 HAT. When stained with Giemsa's solution, syncytium formation was also observed as a variant of the CPE (Fig. 1A). In addition to syncytium formation, chromatin fragmentation was observed in many ECoV-infected cells at and after 18 HAT (Fig. 1B and C). DNA fragmentation started at 15 HAT, and the ''ladder'' pattern was apparent at 18 and 21 HAT (Fig. 2).

3.2. Caspase assay

We measured caspase-3/7 activity based on the cleavage of the selective fluorescent substrate. The results showed that caspase-3/7 activity was significantly increased in ECoV-infected cells at and after 15 HAT, and the maximum increase was observed at 21 HAT (2.2-fold increase in activity when compared with that of mock-infected cells) (Fig. 3).

Further, the activities of caspase-8 and caspase-9, which are representative initiator caspases in the death receptor-mediated and mitochondrial apoptotic pathways, respectively, were also measured based on the

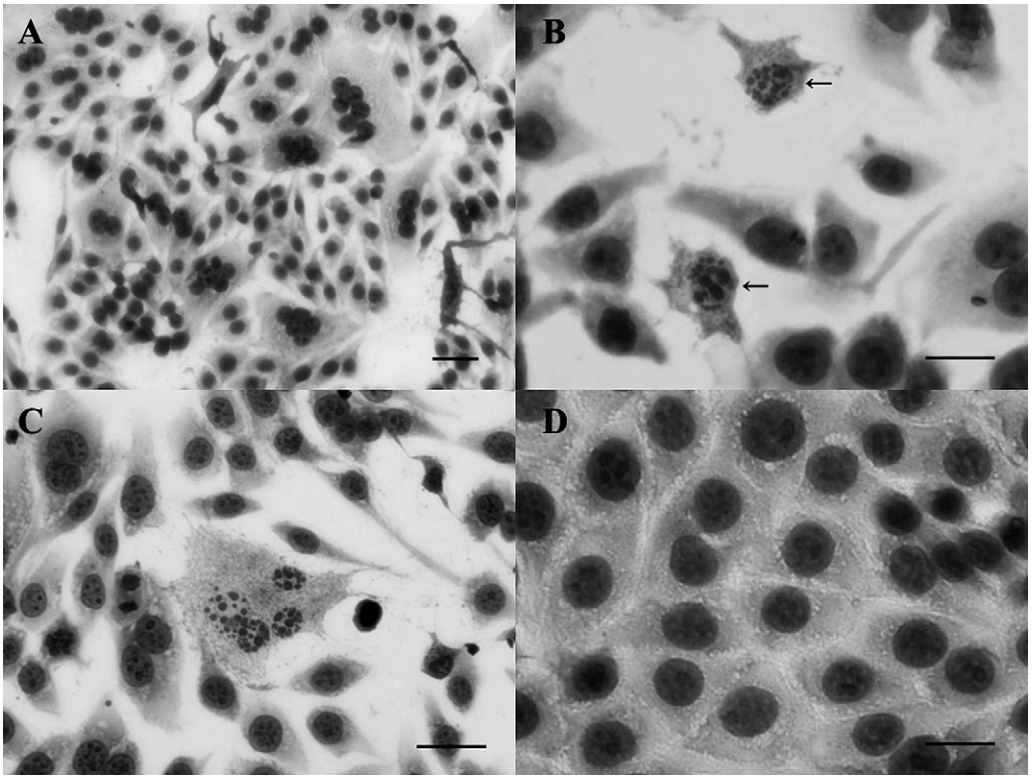

Fig. 1. Histological findings of ECV-infected MDBK cells at 15 HAT. Syncytium formation was observed as a variant of CPE (A), and chromatin fragmentation was observed in mononuclear (B, arrow) and syncytium (C). Mock-infected cells did not shown any change (D). Bar = 20 μm.

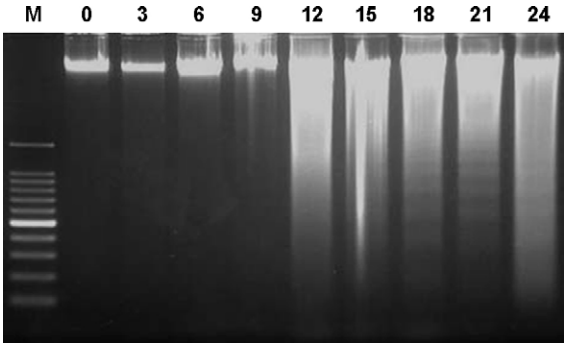

Fig. 2. Agarose gel electrophoresis of DNA from ECV-infected cells. DNA fragmentation was observed at and after 12 HAI, but was prominent at 18 and 21 HAT. M: 100 bp DNA marker (Takara Bio Inc., Shiga, Japan), 0–24: 0, 3, 6, 9, 12, 15, 18, 21, and 24 HAT, respectively.

cleavage of the luminogenic substrates. Caspase-8 and caspase-9 activities were increased in ECoV-infected cells at and after 12 HAT, and the maximal increases were observed at 24 HAT (4.2-fold and 5.0-fold increases in activity, respectively, when compared with those of mock-infected cells).

4. Discussion

Apoptosis represents an important antivirus defense mechanism of the host cell, and viruses have evolved strategies to counteract and regulate apoptosis in order to maximize the production of virus progeny and promote the spread of virus progeny to neighboring cells. ECoV was isolated from the feces of a diarrheic foal, and investigations revealed that it was genetically similar to BCoV (Guy et al., 2000). However, no other study has examined ECoV after this. Therefore, as the first step to clarify the biological characteristics of ECoV, we investigated whether ECoV could induce apoptosis.

We characterized the apoptotic cell death of ECoV-infected MDBK cells. At and after 15 HAT, there was a gradual increase in the number of infected cells that showed the morphological changes of cell rounding and detachment from the culture surface. In addition, chromatin fragmentation was observed at 18 and 21 HAT. Moreover, DNA ladder formation was observed at and after 15 HAT and was most prominent at 21 HAT.

ECV-infected cells also showed syncytium formation prior to apoptosis induction. Syncytium formation has been previously reported in some coronaviruses (Kusanagi et al., 1992; Lavi et al., 1996; Li and Cavanagh, 1992). In a study on group II coronaviruses, MHV-3-infected macrophages from BALB/c mice showed syncytium formation and apoptosis, but the cell populations that showed the 2 phenomena were different (Belyavsky et al., 1998). In contrast, infectious bronchitis virus (IBV)-infected Vero cells showed syncytium formation followed by caspase-dependent apoptosis (Liu et al., 2001). These findings indicate that since IBV belongs to group III coronaviruses, ECoV and IBV are considerably separated on the phylogenetic tree based on genome sequences but are similar with respect to biological behavior (Zhang et al., 2007). IBV is known as a prototype virus of the *Coronaviridae* family. Therefore, it is conceivable that ECoV has genetically evolved into a group II coronavirus but has conserved the functions of the *Coronaviridae* family.

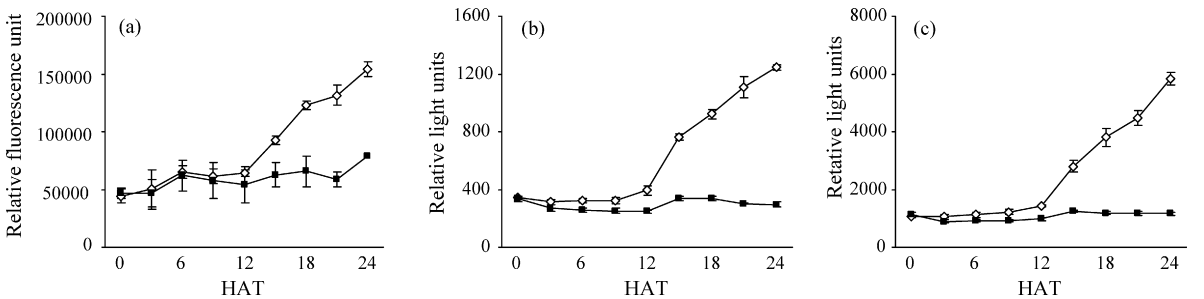

Fig. 3. Kinetics of activity of caspase-3/7 (a), caspase-8 (b) and caspase-9 in ECV-infected (□) and mock-infected (■) cells. Data represent the mean ± S.D.

Caspases are cysteine proteases that play fundamental roles in the apoptotic responses of cells to different stimuli. In particular, caspase-3, caspase-6, and caspase-7 are classified as effecter caspases that are activated by the respective initiator caspases in the death receptor-mediated and mitochondrial apoptotic pathways and can be considered as key enzymes involved in apoptosis since they are indispensable for chromatin condensation and DNA fragmentation. In the death receptor-mediated apoptotic pathway, caspase-8 is the initiator caspase that is activated by the interaction between external apoptotic factors and cell surface molecules such as Fas or Fas ligand via adaptor proteins such as Fas-associated death domain (FADD) (Cohen, 1997). On the other hand, in the mitochondrial apoptotic pathway, caspase-9 is the initiator caspase whose activity is regulated by the expression balance between Bcl-2 and Bax via cytochrome *c*/Apaf-1 complex formation (Cohen, 1997; Strasser et al., 2000). In ECoV-infected MDBK cells, caspase-3/7 activity was increased at and after 15 HAT, and caspase-8 and caspase-9 activities were increased at and after 12 HAT. These results indicate that ECoV can induce caspase-dependent apoptosis in MDBK cells via both the death receptor-mediated and mitochondrial apoptotic pathways. However, the activation of the initiator caspase-8 can also be related to the mitochondrial apoptotic pathway via cleavage of Bid (a member of the proapoptotic Bcl-2 family) and translocation of the truncated Bid to mitochondria (Wang et al., 1996). In some coronaviruses, both initiator caspases are activated in the course of apoptosis; the Fas-signaling pathway in which both initiator caspases are activated via cleavage and translocation of Bid was suggested as the mechanism of apoptosis, particularly in MHV infection (Eleouet et al., 2000; Liu et al., 2006; Liu and Zhang, 2007). Therefore, it was speculated that ECoV also induces apoptosis via a caspase-dependent pathway that may be activated by an interaction between virus protein and cell surface signaling factors with participation of both the death receptor-mediated and mitochondrial apoptotic pathways. However, further investigations that will focus on the kinetics of signal transduction factors are necessary to clarify the detailed mechanisms of ECoV-induced apoptosis.

In conclusion, this report showed that ECoV-induced apoptosis via caspase-dependent pathway. This finding indicated that MDBK cells can be use for propagation and establishment of serological diagnosis system for ECoV. However, because the findings from bovine-derived cell line may not faithfully reflect on the in vivo situation, further investigation using equine-derived cell line will be needed to clarify the pathobiological characters of ECoV.

References

Belyavsky, M., Belyavskaya, E., Levy, G.A., Leibowitz, J.L., 1998. Coronavirus MHV-3-induced apoptosis in macrophages. Virology 250, 41–49.

Cohen, G.M., 1997. Caspases: the executioners of apoptosis. Biochem. J. 326 (Pt 1), 1–16.

Collins, A.R., 2001. Induction of apoptosis in MRC-5, diploid human fetal lung cells after infection with human coronavirus OC43. Adv. Exp. Med. Biol. 494, 677–682.

Eleouet, J.F., Chilmonczyk, S., Besnardeau, L., Laude, H., 1998. Transmissible gastroenteritis coronavirus induces programmed cell death in infected cells through a caspase-dependent pathway. J. Virol. 72, 4918–4924.

Eleouet, J.F., Slee, E.A., Saurini, F., Castagne, N., Poncet, D., Garrido, C., Solary, E., Martin, S.J., 2000. The viral nucleocapsid protein of transmissible gastroenteritis coronavirus (TGEV) is cleaved by caspase-6 and -7 during TGEV-induced apoptosis. J. Virol. 74, 3975–3983.

Gonzalez, J.M., Gomez-Puertas, P., Cavanagh, D., Gorbalenya, A.E., Enjuanes, L., 2003. A comparative sequence analysis to revise the current taxonomy of the family Coronaviridae. Arch. Virol. 148, 2207–2235.

Guy, J.S., Breslin, J.J., Breuhaus, B., Vivrette, S., Smith, L.G., 2000. Characterization of a coronavirus isolated from a diarrheic foal. J. Clin. Microbiol. 38, 4523–4526.

Kerr, J.F., Wyllie, A.H., Currie, A.R., 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 26, 239–257.

Kusanagi, K., Kuwahara, H., Katoh, T., Nunoya, T., Ishikawa, Y., Samejima, T., Tajima, M., 1992. Isolation and serial propagation of porcine epidemic diarrhea virus in cell cultures and partial characterization of the isolate. J. Vet. Med. Sci. 54, 313–318.

Lavi, E., Wang, Q., Weiss, S.R., Gonatas, N.K., 1996. Syncytia formation induced by coronavirus infection is associated with fragmentation and rearrangement of the Golgi apparatus. Virology 221, 325–334.

Li, D., Cavanagh, D., 1992. Coronavirus IBV-induced membrane fusion occurs at near-neutral pH. Arch. Virol. 122, 307–316.

Liu, Y., Zhang, X., 2007. Murine coronavirus-induced oligodendrocyte apoptosis is mediated through the activation of the Fas signaling pathway. Virology 360, 364–375.

Liu, C., Xu, H.Y., Liu, D.X., 2001. Induction of caspase-dependent apoptosis in cultured cells by the avian coronavirus infectious bronchitis virus. J. Virol. 75, 6402–6409.

Liu, Y., Pu, Y., Zhang, X., 2006. Role of the mitochondrial signaling pathway in murine coronavirus-induced oligodendrocyte apoptosis. J. Virol. 80, 395–403.

Ruggieri, A., Di Trani, L., Gatto, I., Franco, M., Vignolo, E., Bedini, B., Elia, G., Buonavoglia, C., 2007. Canine coronavirus induces apoptosis in cultured cells. Vet. Microbiol. 121, 64–72.

Strasser, A., O'Connor, L., Dixit, V.M., 2000. Apoptosis signaling. Annu. Rev. Biochem. 69, 217–245.

Wang, K., Yin, X.M., Chao, D.T., Milliman, C.L., Korsmeyer, S.J., 1996. BID: a novel BH3 domain-only death agonist. Genes Dev. 10, 2859–2869.

Zhang, J., Guy, J.S., Snijder, E.J., Denniston, D.A., Timoney, P.J., Balasuriya, U.B., 2007. Genomic characterization of equine coronavirus. Virology 369, 92–104.