



# Apoptosis induced by Ibaraki virus does not affect virus replication and cell death in hamster lung HmLu-1 cells

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*J. Vet. Med. Sci.*

81(2): 197–203, 2019

doi: 10.1292/jvms.18-0366

Received: 28 June 2018

Accepted: 2 December 2018

Published online in J-STAGE:  
12 December 2018

**ABSTRACT.** Ibaraki virus (IBAV) is an arbovirus that is transmitted by biting midges and causes Ibaraki disease in cattle. IBAV induces apoptosis in several mammalian cell lines, and apoptosis in turn facilitates IBAV replication. In addition, virus-induced apoptosis may contribute to mammalian-specific pathogenicity considering that some arboviruses induce apoptosis in mammalian cells but not in insect cells. In this study, we found that when hamster lung cells (HmLu-1) are used as a virus host, IBAV causes severe cytopathic effects with little induction of apoptosis. Furthermore, pharmacological inhibition of apoptosis did not affect IBAV-induced cytotoxicity. These results indicate the existence of an apoptosis-independent pathway in which IBAV replicates and exerts cytotoxicity in mammalian cells.

**KEY WORDS:** apoptosis, HmLu-1, Ibaraki virus, IBAV

Orbiviruses (genus *Orbivirus* in the *Reoviridae* family) are viruses that can exhibit severe pathogenicity to livestock animals and include the epizootic hemorrhagic disease virus (EHDV), bluetongue virus (BTV), and African horse sickness virus (AHSV) [3]. Orbiviruses are arboviruses and thus infect both mammalian and insect cells. Different host cell responses depending on the host cell species have been reported. BTV, for example, induces apoptosis and severe cytopathic effects (CPE) in mammalian cells but not in insect cells [8]. Similarly, viral replication without CPE in EHDV-infected insect cells is also reported [16].

In this study, we investigated a strain of EHDV called Ibaraki virus (IBAV). IBAV is transmitted by biting midges (*Culicoides* species) and causes Ibaraki disease, which is characterized by hemorrhagic lesions in the upper gastrointestinal tract and swallowing difficulty in cattle [4, 10]. IBAV exploits the endocytosis pathway to enter the host cell [14], as is shown for BTV [7]. Additionally, previous studies have reported that infection with IBAV, and the related EHDV, induces apoptosis in multiple mammalian cell lines (ovine kidney cells, calf pulmonary aortal endothelial cells, Vero cells, and bovine carotid artery endothelial cells), which is also the case with BTV infections [2, 12, 13]. Moreover, pharmacological inhibition of apoptosis suppressed IBAV replication and cell death, suggesting that apoptotic signaling induced by IBAV accelerates IBAV replication and contributes to IBAV-induced cell death [12].

Here, we examined IBAV-induced apoptosis using hamster lung cells (HmLu-1), which are routinely used for studying IBAV, since HmLu-1 cells are known to exhibit CPE when infected with this virus. Our aim was to determine whether IBAV induces apoptosis in HmLu-1 cells as previously reported in other cell lines, and if this is the case, to determine whether apoptosis contributes to IBAV replication and IBAV-induced cell death.

## MATERIALS AND METHODS

### *Cells and viruses*

HmLu-1 cells and IBAV (epizootic hemorrhagic disease virus serotype 2, strain Ibaraki) were obtained from the National Institute of Animal Health, Japan. HmLu-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM; Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (0.29 mg/ml). IBAV was propagated in HmLu-1 cells. Virus titer was determined by plaque assays with HmLu-1 cells and results are reported as plaque forming units (pfu).

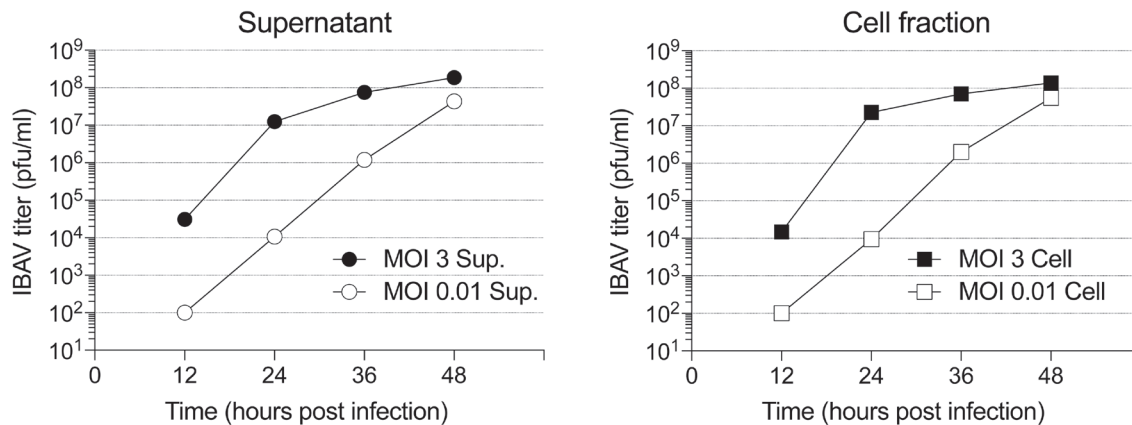
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**Fig. 1.** Time-dependent replication of IBAV in HmLu-1 cells. HmLu-1 cells were plated in 6-well plates and infected with IBAV at a multiplicity of infection (MOI) of 0.01 or 3. The virus titers in the culture supernatants and cell fractions were determined by the plaque assay. For the plaque assay, HmLu-1 cells were prepared in 6-well plates and incubated with appropriate dilutions of virus samples for 2 hr, followed by overlaying with DMEM containing 5% FBS and 0.75% agar and incubation for 4 days. After incubation, the cells were fixed and stained with staining solution. Plaques were counted and the virus titer in each sample was calculated.

### Antibodies

The following antibodies and reagents were used in this study, rabbit anti-cleaved caspase-3 (#9661) was purchased from Cell Signaling Technology (Danvers, MA, U.S.A.), mouse anti- $\alpha$ -tubulin (T5168) from Sigma-Aldrich (St. Louis, MO, U.S.A.), and goat anti-rabbit IgG (H+L)-HRP, goat anti-mouse IgG (H+L)-HRP, and goat anti-mouse IgG-DyLight 488 from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Goat anti-rabbit IgG-Alexa 488, goat anti-rabbit IgG-Alexa 568, goat anti-mouse IgG-Alexa 647 was purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.), and mouse anti-IBAV antiserum was generated in our laboratory by intraperitoneal injection of ddY mice with 50–100  $\mu$ g purified IBAB particles/mouse along with Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (second and third injections). IBAB purification was performed as detailed in a previous study [11].

### Quantification of IBAB replication in HmLu-1 cells

HmLu-1 cells were plated in 6-well plates and infected with IBAB at a multiplicity of infection (MOI) of 0.01 or 3. Culture supernatants (Figs. 1 and 4) and cell fractions (Fig. 1) were harvested at 12, 24, 36 and 48 hr post-infection (hpi) after virus infection. In order to obtain the cell fractions, HmLu-1 cells were washed with phosphate buffered saline (PBS), the supernatant was removed, the cells were scraped from the bottom of the well, and collected by suspending in 1 ml PBS. The collected cell fractions were sonicated for 2 min, centrifuged at 3,000 rpm (800  $\times$  g) for 10 min, and the supernatant was used for measuring the titer of cell-associated virus. The virus titers in the supernatant and the cell fraction were determined by plaque assays. Briefly, HmLu-1 cells were prepared in 6-well plates and incubated with the appropriate dilutions of virus samples in a CO<sub>2</sub> incubator at 37°C for 2 hr. After incubation, the media was removed and DMEM containing 5% FBS and 0.75% agar was overlaid. Plates were then incubated for 4 days, after which the cells were fixed and stained with staining solution (0.1% crystal violet in 10% buffered formalin and 20% methanol). Plaques were counted and the virus titer in each sample was calculated.

### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

HmLu-1 cells were prepared in 6-well plates and infected with IBAB at an MOI of 0.01 or 3. At 12, 24, 36 and 48 hpi, cells were washed with phosphate buffered saline (PBS), lysed with cold lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM orthovanadate, 1% Triton X-100, and a protease inhibitor cocktail [cOmplete; Sigma-Aldrich]), sonicated for 2 min, and then supernatants were mixed with sample buffer and boiled for 5 min. Samples were subjected to SDS-PAGE with 10 or 15% acrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Merck-Millipore, Burlington, MA, U.S.A.). Membranes were treated with blocking buffer (Tris-buffered saline and 0.1% Tween 20 containing 2% skim milk). Proteins of interest were detected with specific primary and secondary antibodies by using EzWestLumi plus (Atto, Tokyo, Japan) as the substrate. Images were taken with the LAS 3000 luminescent imager (Fuji Film, Tokyo, Japan) and proteins were quantified using ImageJ software version 1.52b (National Institutes of Health, Bethesda, MD, U.S.A.).

### Immunofluorescence

HmLu-1 cells were plated on glass coverslips prepared in 6-well plates and infected with IBAB at an MOI of 0.1. Cells were washed with PBS, fixed with 4% paraformaldehyde at 24 or 48 hpi, and then permeabilized with PBS containing 0.1% IGEPAL CA-630 (Sigma-Aldrich) for 5 min on ice. After blocking with DMEM containing 10% FBS, cells were incubated with primary

antibodies (mouse anti-IBAV and rabbit anti-cleaved caspase-3) for 1 hr at room temperature, washed with PBS, and then incubated with secondary antibodies (anti-mouse-IgG-DyLight 488 and anti-rabbit-IgG-Alexa 568) for 1 hr at room temperature. Coverslips were then mounted onto glass slides with ProLong Gold antifade mountant with DAPI (Thermo Fisher Scientific). Fluorescent signals were observed using a fluorescence microscope (IX53; Olympus, Tokyo, Japan) equipped with UPlan FL N 60x/1.25 NA objective lens (Olympus).

#### *Flow cytometry and TUNEL staining*

HmLu-1 cells were plated in 6-well plates at a density of  $5 \times 10^4$  cells/well. After 18 hr, cells were infected with IBAV at an MOI of 0.1 and incubated in a CO<sub>2</sub> incubator for 24 or 48 hr. Positive control cells were prepared by treating cells with 1  $\mu$ M staurosporine (Wako Pure Chemical) for 4 hr. Cells were washed with PBS, treated with 500  $\mu$ l/well trypsin-EDTA (Wako Pure Chemical), and resuspended in DMEM containing 10% FBS. Cells were fixed with 4% paraformaldehyde, and then permeabilized with PBS containing 0.1% Triton X-100 (Wako Pure Chemical) for 5 min at room temperature. After blocking with 0.2% gelatin in PBS for at least 30 min at room temperature, cells were either subjected to immunofluorescence or TdT-mediated dUTP nick end labeling (TUNEL) staining. For immunofluorescence, cells were incubated with primary antibodies (mouse anti-IBAV and rabbit anti-cleaved caspase-3) for 1 hr at room temperature, washed with PBS, and then incubated with secondary antibodies (anti-mouse-IgG-Alexa 488 and anti-rabbit IgG-Alexa 647) for 1 hr at room temperature. For TUNEL staining, cells were incubated with the TUNEL staining solution of MEBSTAIN Apoptosis TUNEL Kit Direct (MBL, Nagoya, Japan) at 37°C for 1 hr, according to the manufacturer's manual. Flow cytometric analysis was performed with BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, U.S.A.). At least 20,000 cells were counted for each sample, and the obtained data were analyzed with FlowJo software (ver. 10.5.3, FlowJo, Ashland, OR, U.S.A.).

#### *Quantification of cytotoxicity of Z-VAD-FMK*

Cytotoxicity of Z-VAD-FMK (3188-v; Peptide Institute Inc., Osaka, Japan) in HmLu-1 cells was examined by the MTT assay using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) following manufacturer's instructions. In brief, HmLu-1 cells plated in 96-well plates were treated with DMEM containing 10% FBS and Z-VAD-FMK. After incubating for 48 hr, the Cell Counting Kit reagent was added to each well and incubated at 37°C. After 1 hr, the absorbance at 450 nm was measured with an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, U.S.A.).

#### *Effect of Z-VAD-FMK on IBAV-induced apoptosis*

HmLu-1 cells plated in 6-well plates were infected with IBAV at an MOI of 0.01 for 2 hr. After washing with PBS, cells were further incubated with DMEM containing 10% FBS and Z-VAD-FMK. At 48 hr after virus infection, cells were lysed with cold lysis buffer and subjected to western blot analysis as described in "SDS-PAGE and western blot analysis".

#### *Quantification of IBAV replication in Z-VAD-FMK-treated HmLu-1 cells*

HmLu-1 cells plated in 6-well plates were infected with IBAV at an MOI of 0.01 for 2 hr at 37°C. After washing with PBS, cells were incubated with DMEM containing 10% FBS and Z-VAD-FMK. After incubating for 48 hr in a CO<sub>2</sub> incubator, the culture supernatants were collected and subjected to a plaque assay.

#### *Effect of Z-VAD-FMK on IBAV-induced cytotoxicity*

HmLu-1 cells plated in 24-well plates were infected with IBAV at an MOI of 0.1, 1, or 10 for 2 hr at 37°C. After washing with PBS, cells were incubated with DMEM containing 10% FBS and Z-VAD-FMK. After incubating for 24 or 48 hr, cells were washed with PBS and then DMEM containing 10% FBS and the Cell Counting Kit reagent was added to each well. After incubating for 1 hr in a CO<sub>2</sub> incubator, 100  $\mu$ l/sample of the culture medium was transferred to a 96-well plate and the absorbance at 450 nm was measured with the iMark microplate reader.

#### *Statistical analysis*

To analyze the statistical significance of each group against the mock treated group, the statistical software R was used to run one-way ANOVA and *t*-tests. *P* values of <0.05 were considered statistically significant.

## RESULTS

To determine the experimental conditions for investigating the effect of apoptosis on IBAV replication, we first tested time-dependent replication of IBAV in HmLu-1 cells (Fig. 1). HmLu-1 cells were infected with IBAV at an MOI of 0.01 or 3. Culture supernatants and cell fractions (containing cell-associated virus) were collected at 12, 24, 36, and 48 hpi, and virus titers in supernatants or cell fractions were determined by plaque assays. Virus titers of supernatants were similar to those of cell fractions (Fig. 1, circles vs. squares). When IBAV was infected at MOI=0.01, the virus titer continued to increase up to 48 hpi. On the other hand, when infected at MOI=3, the virus titer increased quickly and the replication rate slowed down after 24 hpi (Fig. 1).

We next examined the cleavage of caspase-3, a hallmark event of apoptosis [5], in IBAV-infected HmLu-1 cells. We infected HmLu-1 cells with IBAV at MOI=0.01 or 3 and harvested cell lysates at 12, 24, 36, and 48 hpi. When infected at MOI=0.01 (Fig. 2A), the detection of both IBAV capsid protein VP5 and cleaved caspase-3 began at 48 hpi. On the other hand, when cells

were infected at MOI=3 (Fig. 2B), the IBAV capsid protein VP5 was detected at as early as 24 hpi, consistent with the finding that virus titers rapidly increased at 24 hpi at MOI=3 (Fig. 1). Interestingly, even with active virus replication at 24 hpi, cleaved caspase-3 was not detected at this time point. The detection of cleaved caspase-3 started later at 36 hpi and the amount of cleaved caspase-3 continued to increase up to at least 48 hpi (Fig. 1).

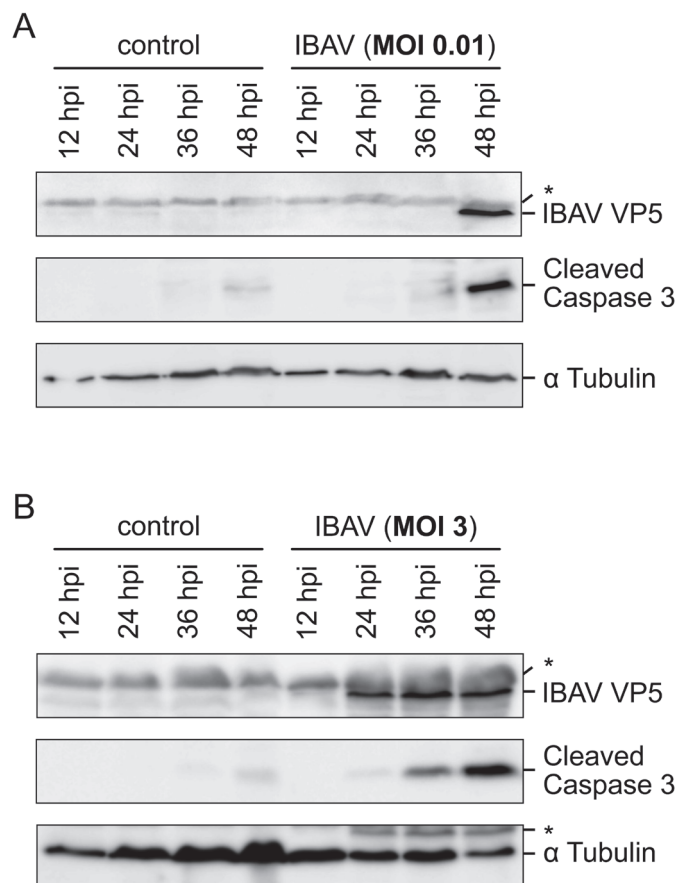
To examine the timing and penetrance of caspase-3 cleavage in relationship with IBAV replication, we performed immunofluorescence and counted individual cells (Fig. 3). We infected HmLu-1 cells with IBAV at MOI=0.1, fixed cells at 24 hpi and 48 hpi, and stained cells with anti-IBAV and anti-cleaved caspase-3 antibodies. Here, we employed MOI=0.1 as it was a suitable MOI for observing the increase in IBAV-infected cells during the tested time window (24–48 hpi). At 24 hpi, a small population of cells were positive for IBAV and no cells with cleaved caspase-3 were found. Although many cells became IBAV-positive at 48 hpi, only a small fraction was clearly positive for cleaved caspase-3 (Fig. 3A). In order to quantify immunofluorescence, we employed flow cytometric analysis. Consistent with microscopic observation (Fig. 3A), induction of IBAV viral protein expression (Fig. 3B, left columns) and cleaved caspase-3 (Fig. 3B, middle columns) were not discernible at 24 hpi of IBAV infection, but were detected in a small population of cells at 48 hpi. Furthermore, TUNEL staining, which is widely used as a readout for apoptosis, detected a small population of apoptotic cells at 48 hpi (Fig. 3B right columns). The apoptosis-inducing reagent staurosporine was used as a positive control. Treatment of cells with 1  $\mu$ M staurosporine for 4 hr induced more apoptotic cells with higher intensities of cleaved caspase-3 and TUNEL staining than IBAV-infected cells at 48 hpi.

A previous study showed that pharmacological inhibition of caspases with the pan-caspase inhibitor Q-VD-OPH decreased IBAV replication in ovine kidney cells [12], suggesting that IBAV benefits from apoptosis signaling. We thus attempted to examine the effect of pharmacological inhibition of apoptosis with the pan-caspase inhibitor Z-VAD-FMK. It has been reported that Z-VAD-FMK inhibits multiple caspases—similar to Q-VD-OPH [1]—and effectively inhibits virus-induced apoptosis in the case of BTV [9]. We first confirmed that no cytotoxicity was observed with 50 and 100  $\mu$ M Z-VAD-FMK for at least 48 hr (Fig. 4A). We also confirmed that 100  $\mu$ M Z-VAD-FMK effectively inhibited the IBAV-induced cleavage of caspase-3 at 48 hpi (Fig. 4B). We then examined if the inhibition of apoptotic signaling alters replication efficiency of IBAV. In the same experimental conditions as Fig. 4B (MOI=0.01 and 100  $\mu$ M Z-VAD-FMK at 48 hpi), however, Z-VAD-FMK did not affect IBAV replication (Fig. 4C).

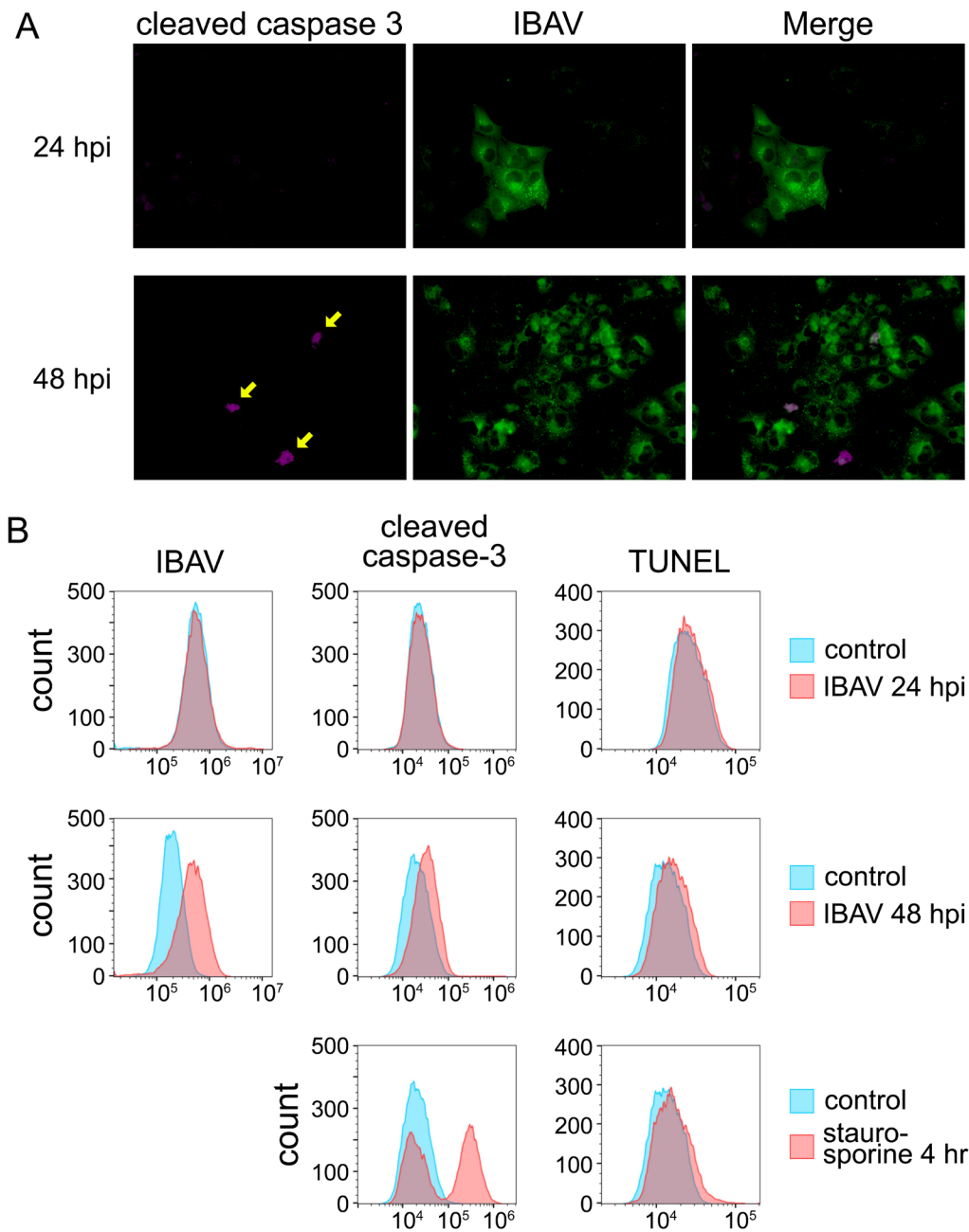
Finally, we examined whether IBAV-induced apoptosis contributes to the cytotoxicity of IBAV infection. To this end, HmLu-1 cells were infected with IBAV at an MOI of 0.1, 1, or 10 and then total cell metabolic activity (as a readout of cell viability) was measured with an MTT assay. As shown in Fig. 5, apoptosis inhibition by 100  $\mu$ M Z-VAD-FMK did not affect total cell metabolic activity.

## DISCUSSION

Previous studies have shown that IBAV [12], EHDV [13] and the related BTV [2] induce apoptosis in mammalian host cells. In the case of BTV, apoptosis induction does not require active viral replication as ultraviolet (UV)-irradiated BTV particles can induce apoptosis [8]. Moreover, extracellularly applied viral outer capsid proteins VP2 and VP5 were sufficient to induce apoptosis, while inhibitors of endosomal acidification, which prevent virus uncoating, completely suppressed BTV-induced apoptosis, suggesting that virus uncoating triggers apoptosis induction [8]. Likewise, mammalian and avian reoviruses induce apoptosis without requiring virus replication [6, 15]. On the other hand, UV-irradiation of IBAV and EHDV significantly decreased their apoptosis-inducing ability [12, 13]. Therefore, EHDV, including IBAV, may utilize a mechanism of apoptosis induction that is different from BTV and reoviruses. It has been shown that pharmacological inhibition of caspases decreased IBAV replication [12].

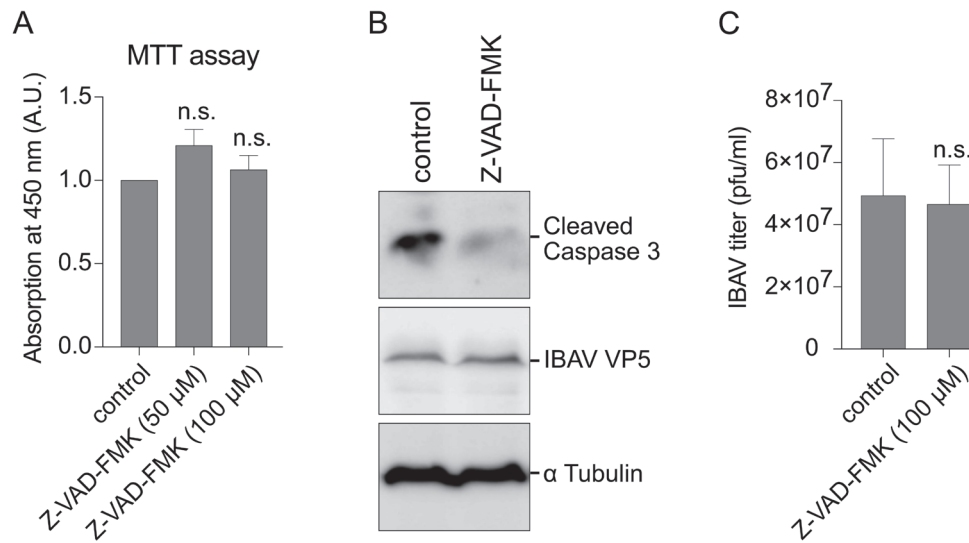


**Fig. 2.** Induction of apoptosis by IBAV infection. HmLu-1 cells were either untreated (control) or infected with IBAV at an MOI of (A) 0.01 or (B) 3. Cell lysates were harvested at the indicated time points after infection and subjected to western blot analysis with anti-IBAV, anti-cleaved caspase-3, and anti- $\alpha$ -tubulin antibodies. Asterisks indicate non-specific signals. hpi, hr post-infection.

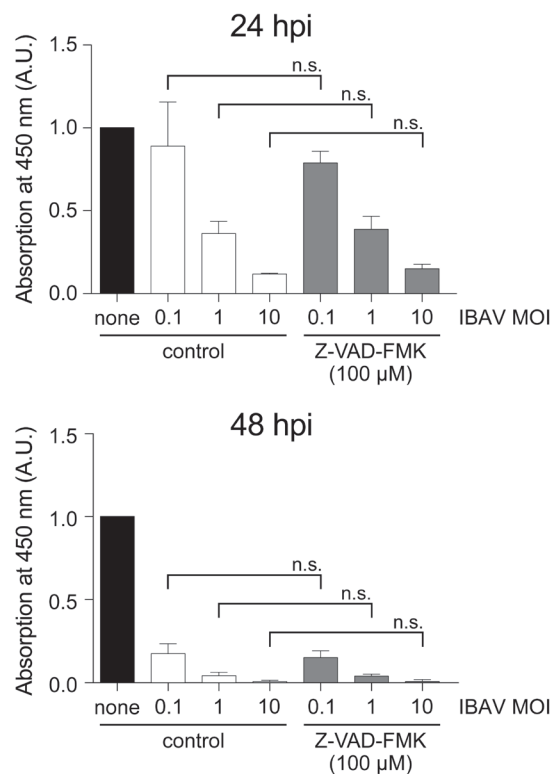


**Fig. 3.** Single cell analysis of IBAV-positive and apoptotic cells. (A) HmLu-1 cells were infected with IBAV at an MOI of 0.1, fixed at 24 or 48 hr post-infection, and stained with anti-IBAV and anti-cleaved caspase-3 antibodies. Arrows indicate cleaved caspase-3-positive cells. The results shown are representative of two independent experiments. Scale bar=10  $\mu$ m. (B) Immunofluorescence with anti-IBAV (left columns) and anti-cleaved caspase-3 (middle columns) as well as TUNEL staining (right columns) were quantified by flow cytometric analysis. The results shown are representative of three independent experiments. hpi, hr post-infection.

Thus, it was suggested that IBAV induces apoptosis signaling and then exploits the induced apoptosis to facilitate viral replication. This study, however, demonstrated that (i) apoptosis signaling becomes activated during late stages of IBAV replication (Fig. 2), (ii) apoptosis signaling is activated in only a small subset IBAV-infected cells, thus most IBAV-infected cells remain negative for apoptosis signaling after infection (Fig. 3), and (iii) IBAV replication efficiency was independent of apoptotic caspase activation (Fig. 4). Our findings also suggested that apoptosis does not contribute to IBAV-induced cytotoxicity in HmLu-1 cells (Fig. 5). The discrepancy between the study by Shai *et al.* [12] and our study may result from the different cell lines used. Nevertheless, our study indicates that, depending on the host cell, IBAV may choose an apoptosis-independent pathway of replication. The effect of apoptosis on virus replication and host cell death has not been assessed in the study on EHDV, conducted by Sharma *et al.* [13]. It remains possible that the phenomenon observed in this study (i.e. apoptosis-independent IBAV replication and cytotoxicity) occurs also in other situations, including the experimental condition employed by Sharma *et al.*



**Fig. 4.** Effect of Z-VAD-FMK on IBAV replication in HmLu-1 cells. (A) Cytotoxicity of Z-VAD-FMK was examined by the MTT assay. HmLu-1 cells were incubated with DMSO (control) or Z-VAD-FMK for 48 hr and then cell metabolic activity was measured with an MTT reagent. Values represent the means of three independent experiments. Error bars indicate standard deviations. n.s., not statistically significant. (B) HmLu-1 cells were infected with IBAV at an MOI of 0.01 for 2 hr and then the medium was replaced with growth medium (DMEM supplemented with 10% FBS) containing 0.4% DMSO (control) or 100  $\mu$ M Z-VAD-FMK. After incubating for 48 hr, cell lysates were harvested and subjected to western blot analysis with anti-cleaved caspase-3, anti-IBAV, and anti- $\alpha$ -tubulin antibodies. (C) HmLu-1 cells were infected with IBAV at an MOI of 0.01 for 2 hr and then the medium was replaced with growth medium containing 0.4% DMSO (control) or 100  $\mu$ M Z-VAD-FMK. After incubating for 48 hr, the culture supernatants were collected and subjected to plaque assays. Values represent the means of three independent experiments. Error bars indicate standard deviations. n.s., not statistically significant.



**Fig. 5.** Effect of Z-VAD-FMK on cell viability of IBAV-infected HmLu-1 cells. HmLu-1 cells were infected with IBAV at an MOI of 0.1, 1, or 10 for 2 hr and then the medium was replaced with growth medium containing 0.4% DMSO (control) or 100  $\mu$ M Z-VAD-FMK. After incubating for 24 or 48 hr, cell metabolic activity was measured with an MTT reagent. Values represent the means of three independent experiments. Error bars indicate standard deviations. n.s., not statistically significant. hpi, hr post-infection.

IBAV infection in cattle is mediated by biting midges. Similar to other arboviruses, orbiviruses do not exhibit serious pathogenicity in their arthropod vectors. Consistent with this host-specific pathogenicity, BTV causes apoptosis in cultured mammalian cells but not in cultured insect cells [8]. Our previous study also showed that IBAV replication occurs with a robust CPE in HmLu-1 cells but without CPE in mosquito (*Aedes albopictus*) C6/36 cells [16]. In the case of HmLu-1, IBAV-induced CPE appears independent of apoptosis (Fig. 5). Therefore, the mammalian cell-specific pathogenicity of IBAV and insect cell-specific cytotoxicity-free replication may not be explained solely by the presence or absence of apoptosis induction. Our previous studies indicated that IBAV protein NS3 is specifically glycosylated in mammalian cells but not in insect cells and that glycosylation increases toxicity of NS3 expressed in mammalian cells [16, 17]. In addition to the mammalian-specific glycosylation of NS3, further investigation is required to clarify the mechanism of IBAV replication without inducing cytotoxicity in insect cells, as insect cells may possess a mechanism that avoids both apoptosis-dependent and -independent CPE.

**ACKNOWLEDGMENTS.** This study was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant Number 15K07745; to H.I.), Funds for the Development of Human Resources in Science and Technology under Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan through the Home for Innovative Researchers and Academic Knowledge Users (HIRAKU) consortium (to S.S.), and Yamaguchi University Foundation, Japan (to S.S.). The authors thank the members of the Laboratory of Veterinary Hygiene at Yamaguchi University for the insightful discussions.

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