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An RNA-hydrolyzing recombinant antibody exhibits an antiviral activity against classical swine fever virus

Hye-Ryeong Jun [a,1], Chuong D. Pham [a,1], Seong-In Lim [b], Suk-Chan Lee [c], Yong-Sung Kim [d], Sun Park [a], Myung-Hee Kwon [a,*]

[a] Department of Microbiology, Ajou University School of Medicine, San 5, Wonchon-dong, Yeongtong-gu, Suwon 443-721, Republic of Korea
[b] Virology Division, National Veterinary Research Institute, National Veterinary Research and Quarantine Service, Anyang 430-824, Republic of Korea
[c] Department of Genetic Engineering, Sungkyunkwan University, 300 Chunchun-dong Jangan-gu, Suwon 440-746, Republic of Korea
[d] Department of Molecular Science and Technology, Ajou University, San 5, Woncheon-dong, Yeongtong-gu, Suwon 443-749, Republic of Korea

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ABSTRACT

Some proteins with ribonuclease (RNase) activity have been shown to suppress viral replication. A well-characterized recombinant antibody, 3D8 single-chain variable fragment (3D8 scFv), has RNA-hydrolyzing and cell-penetrating activities. Here, we investigated antiviral activity of 3D8 scFv against classical swine fever virus (CSFV). In a cell line expressing 3D8 scFv (C26), intracellular RNA-hydrolysis activity was higher compared to control PK-15 cells and viral replication was strongly suppressed at the viral RNA level, with the evidence of independency of IFN-α/β induction. Exogenous treatment of 3D8 scFv, prior to or post-CSFV infection, was also shown to suppress CSFV replication at the viral RNA level. These observations suggest that antiviral activity of 3D8 scFv may be due to the intrinsic RNase activity of 3D8 scFv, which is capable of targeting viral RNA genomes or transcripts.

1. Introduction

It has been reported that several ribonucleases (RNases) have shown antiviral activity in animal cells and in plants [1–3]. For example, onconase isolated from oocytes of the frog *Rana pipiens*, a cell-penetrating RNase that degrades cellular RNA, inhibited viral replication in chronically HIV-1-infected human cells [1]. Another example is Pac1, an RNase isolated from the yeast *Schizosaccharomyces pombe* [2]. Challenging transgenic potato that expresses Pac1 with potato spindle tuber viroid (PSTVd) resulted in the suppression of PSTVd infection and accumulation without significant undesired effects on the plant [2]. It is known that the anti-viral effect of these RNases depends on their RNA-hydrolyzing activity.

In addition to canonical RNases, some catalytic anti-nucleic acid antibodies are capable of cleaving DNA and RNA strands. Polyclonal antibodies, purified by affinity chromatography on DNA–cellulose, were able to hydrolyze both DNA and RNA [4]; conversely, polyclonal antibodies directed against RNA hydrolyzed both RNA and DNA [5]. Previously, we have introduced the 3D8 single-chain variable (3D8 scFv) antibody as a DNA- and RNA-hydrolyzing recombinant antibody [6,7]. The recent studies showing that canonical RNases can exert anti-viral effect prompted us to investigate the antiviral activity of the 3D8 scFv.

Here, we show the antiviral activity of 3D8 scFv against classical swine fever virus (CSFV), which has (+) stranded RNA as a genome and replicates in the cytoplasm of host cells without causing a cytopathic effect [8], in PK-15 cell line (C26) constitutively expressing 3D8 scFv as well as in the cells treated with 3D8 scFv protein. This the first report describing a RNA-hydrolyzing monoclonal antibody that has antiviral activity against an RNA-genomed virus.

2. Materials and methods

2.1. Cells, virus, and antibody

Porcine kidney cell line PK-15, CSFV strain LOM [9], and mouse monoclonal antibody (3B6) against CSFV E2 were obtained from the National Veterinary Research and Quarantine Service (NVRQS), Korea. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml).

2.2. Establishment of a 3D8 scFv-expressing PK-15 cell line

The 3D8 scFv gene was amplified by PCR from the pIg20-3D8 scFv vector [6] and subsequently cloned into the NotI and BamHI sites of the pQCXIN retroviral vector (Clontech), resulting in pQCXIN-3D8

* Corresponding author. Fax: +82 31 219 5079.
E-mail address: kwonmh@ajou.ac.kr (M.-H. Kwon).
[1] These authors contributed equally to this work.

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scFv fused to myc tag. The primers used for cloning were as follows: 5′-GTT CTA GAG CGG CCG CAT GGA GGT CCA GCT GCA GC-3′ and 5′-TGG ATC CTT ATT AAA GAT CTT CTT CGC TAA TAA GTT TTT GTT CTT TTA TTT CCA GCT TGG TCC C-3′. Bases corresponding to myc tag were underlined. After transfection of the pQCXIN-3D8 scFv vector into PT67 packaging cells (Clontech), G418-resistant PT67 cells were selected by maintaining cells for 2 weeks in DMEM supplemented with G418 (1 mg/ml) and culturing for an additional 3–4 days to allow the accumulation of recombinant retrovirus particles bearing the 3D8 scFv gene in the culture supernatant. PK-15 cells were infected with the supernatant of the PT67 cells in the presence of 4 μg/ml polybrene (Sigma) and selected with G418 as described above. From the isolated G418-resistant colonies, limiting dilutions were performed to clone homogenous cell lines.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Cells were lysed by incubation with lysis buffer (Roche, 150 μl/5×10^5 cells) for 30 min at 25 °C. Cell lysates (100 μl/well) were clarified by centrifugation, then added to wells coated with pEGFP-N2 plasmid DNA (20 μg/ml, Clontech). DNA-bound 3D8 scFv was detected by the incubations with primary rabbit anti-3D8 scFv or mouse anti-myc antibody and alkaline phosphatase-conjugated appropriate secondary antibodies [6]. The purified 3D8 scFv protein lacking myc tag (200 ng/well) was used as a control [6].

2.4. Preparation of 3D8 scFv protein

3D8 scFv protein was purified from bacterial expression [6].

2.5. Immunofluorescence assays

Confocal microscopy and flow cytometry were performed as previously described [7]. 3D8 scFv expressed in C26 cells was detected using rabbit anti-3D8 scFv antibody and secondary TRITC-labeled anti-rabbit IgG antibody. In experiments with treatment of 3D8 scFv protein, PK-15 cells were incubated with 3D8 scFv (5 μM) for 24 h at 37 °C, followed by infection with CSFV at m.o.i (multiplicity of infection) of 10 for 1 h at 37 °C. Cells were washed with phosphate-buffered saline (pH 7.2) twice and further incubated for 24 h. At 24 h post-infection (hpi), cells were washed and fixed, followed by cell staining with anti-CSFV E2 antibody and anti-3D8 antibody, and then secondary FITC-labeled anti-mouse IgG and TRITC-anti-rabbit antibody, respectively.

2.6. FRET (fluorescence resonance energy transfer)-based RNA cleavage assay

FRET-based RNA cleavage assay was performed as previously described [7]. Random ribo-oligonucleotides 21 bases long (500 nM), labeled with 6-carboxyfluorescein (FAM) at the 5′ terminus and a black hole quencher (BHQ) at the 3′ terminus, were synthesized (5′-FAM-CGA TGA GTG CCA TGG ATAT AC-BHQ-3′), annealed to the non-labeled complementary ribo-oligonucleotide, and then delivered into cells in 96-black-well plates using Lipofectamine™ transfection reagent (Invitrogen). Immediately after changing the medium, real-time fluorescence intensity in the cells was read for 2 h in real time using a fluorescence analyzer with 5-min intervals (Molecular Devices).

2.7. Virus yield assay

The virus yield in cells was detected by slight modifications of the immunoperoxidase staining method [10]. Dark-brownish CSFV foci in the infected cells were observed with an inverted light microscope. The virus titer, expressed as 50% tissue culture infection dose per ml ($TCID_{50}$/ml), was determined by an end point method in which infection of six 10-fold serial dilutions was performed in 96-well plates.

2.8. Reverse transcription (RT)-PCR

Total cellular RNA was extracted from cells lysed with TRIzol (Invitrogen). From an RNA aliquot, cDNA was synthesized using the PreMix cDNA synthesis kit (Bioneer) and random hexamer primers (Amersham Pharmacia). CSFV transcripts for the 5′ non-coding region (NCR) and E2 glycoprotein were PCR amplified. Primer sets used for PCRs as follows: 5′-CTA GCC ATG CCC AYA GTA GG-3′ and 5′-CAG CTT CAR YGT TGA TTG T-3′ 5′ for non-coding region (NCR) (150 bp), 5′-AGR CCA GAC TGG TGG CCN TAY GA-3′ and 5′-TTY ACC ACT TCT GTT CTC A-3′ for E2 (190 bp) [11]. IFN-β and β-actin genes were PCR amplified in the cells that were mock-infected, polyinosinic–polycytidylic acid [poly (IC)]-stimulated with transfection (1 μg/ml), or CSFV-infected for 1 h and 6 h. Primer sets used for PCRs as follows: 5′-TGG ATG ACC TGG AGA CAA TC-3′ and 5′-AAA GAG CTT CCC CTG CTT GA-3′ for IFN-β (291 bp), 5′-GTT TGA GAC CTT CAA CAC GC-3′ and 5′-ATG TCC ACG TCG CAC TTC AT-3′ for β-actin (498 bp) [12]. PCR products were analyzed on 1% agarose gel electrophoresis and stained with ethidium bromide.

3. Results

3.1. 3D8 scFv is expressed at a low level in C26 clone

Among 28 PK-15 clones that were selected as positive for the presence of 3D8 scFv mRNA, a representative clone designated as C26 was chosen for the following studies. When we analyze the expression of 3D8 scFv in C26 cells, 3D8 scFv seemed to be expressed at very low levels, that is, flow cytometry analysis showed only a slight shift of the C26 peak from that of control PK-15 cells (Fig. 1A) and confocal microscopy showed weak fluorescence signals in both the cytoplasm and nucleus (Fig. 1B). Intracellular expression of 3D8 scFv in C26 cells was also assayed with ELISA. Total cell lysates were added to ELISA plates coated with a plasmid DNA. The bound 3D8 scFv protein with myc tag was detected using anti-3D8 scFv or anti-myc antibody, respectively. Significant DNA-binding activity was observed in C26 cell lysate by both antibodies, not in PK-15 cell lysate. 3D8 scFv protein lacking myc tag, used as a control, was detected with anti-3D8 scFv antibody but not with anti-myc antibody as expected (Fig. 1C). These results indicate that C26 clone obviously expresses 3D8 scFv protein.

Next we compared the RNA-hydrolyzing activity between control PK-15 and C26 cells by a FRET-based RNA cleavage assay in which the hydrolysis of transfected dsRNA, double-labeled with a fluorophore at the 5′ terminus and its quencher at the 3′ terminus in a single strand, can be read out by the increase in fluorescence intensity of the cells. Cells were transfected with dsRNA and the intensity of fluorescence in the cells was measured by a fluorescence analyzer in real time for 127 min with 5-min intervals. C26 clone exhibited significantly strong fluorescence compared with control PK-15 cells in proportion to the incubation time (Fig. 1D), indicating that C26 cells have the elevated intracellular RNase activity by 3D8 scFv expression. No fluorescence was detected when the double stranded (ds) RNA substrate was incubated with culture medium without cells.

3.2. Intracellular existence of 3D8 scFv confers resistance to CSFV infections

To test whether intracellular expression of 3D8 scFv protein is able to suppress replication of CSFV in cells, the kinetics of viral replication was determined by an immunoperoxidase assay at 2,

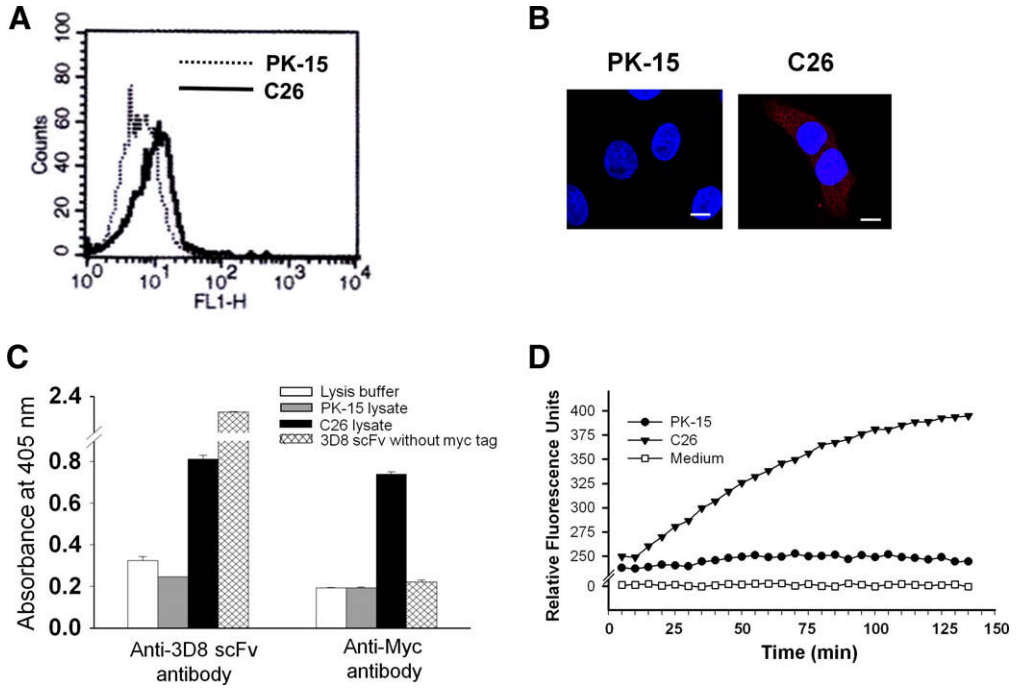

Fig. 1. 3D8 scFv expressed in C26 clone has intracellular RNA-hydrolysis activity. After fixation and permeabilization, cells were stained with anti-3D8 scFv antibody followed by secondary TRITC-anti-rabbit IgG for flow cytometry (A) and confocal microscopy (B). Blue color depicts DAPI-stained nuclei. Bar, 5 μm. (C) ELISA for 3D8 scFv detection in cell lysates. (D) Intracellular RNase activities were analyzed by a FRET-based dsRNA hydrolysis assay in C26 stable cell line and control PK-15 cells. Background fluorescence in the wells without cells was expressed as "Medium". (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

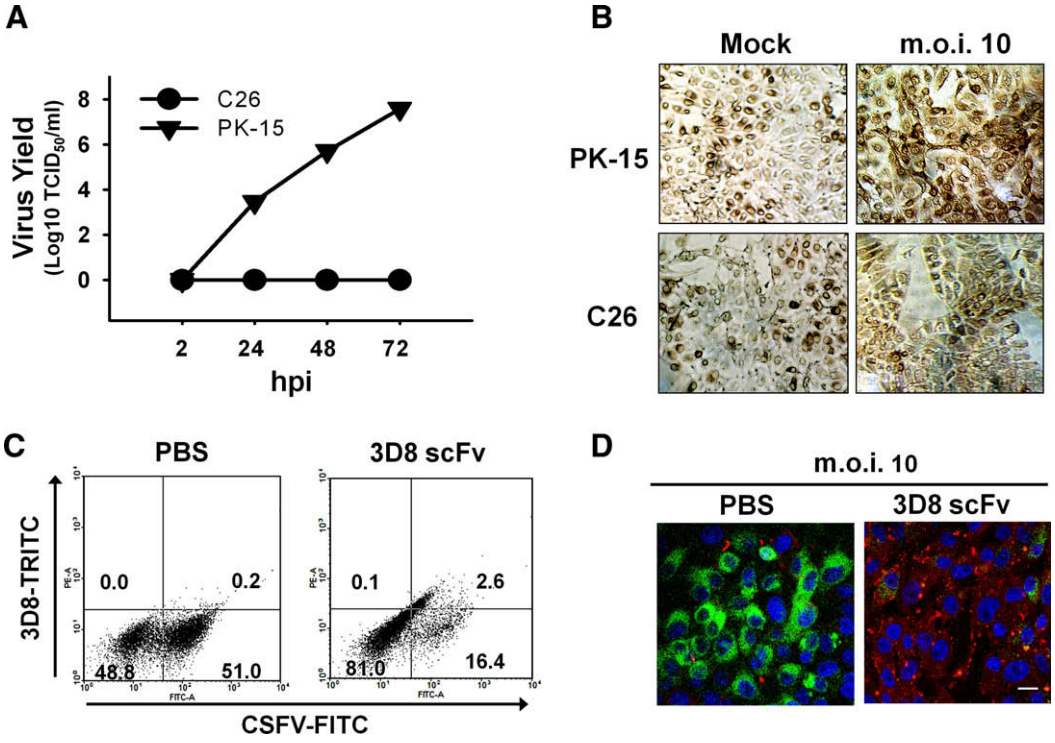

Fig. 2. Intracellular presence of 3D8 scFv confers resistance to CSFV infections. Virus production in cells infected with CSFV was monitored by an immunoperoxidase staining assay (A,B) and immunofluorescence assays (C,D) using an anti-E2 antibody. (A) Viral production in cells infected with 10-fold serial dilution of CSFV was determined at 2, 24, 48, and 72 hpi. (B) Cells were infected with 10 m.o.i. of CSFV, and expression of viral E2 glycoprotein was analyzed at 72 hpi. (C,D) Cells that were infected with CSFV at m.o.i. of 10 after treated with 3D8 scFv (5 μM) for 24 h were stained with anti-3D8 scFv and anti-E2 antibody for flow cytometry (C) and confocal microscopy (D). Numbers in the gated areas indicates the percentage of events (C). Bar, 10 μm. PBS, phosphate-buffered saline.

24, 48, and 72 h hpi using monoclonal anti-E2 antibody (3B6). The virus yield was reduced by a factor of 10^4–10^7 in C26 cells compared with control PK-15 cells at 24–72 hpi, and very surprisingly, no CSFV multiplication in C26 was detected at all through the tested times (Fig. 2A). Under a microscopic observation of the cells at 72 hpi, brownish-stained CSFV foci were observed in control PK-

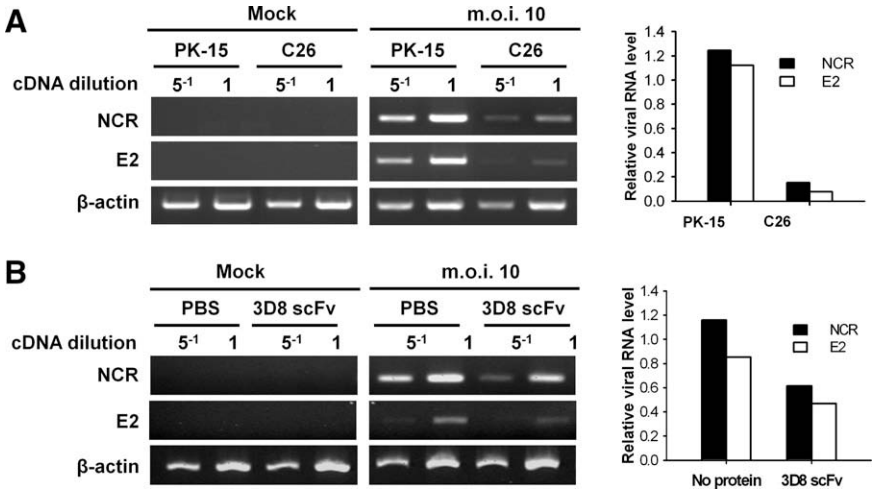

Fig. 3. CSFV replication by 3D8 scFv is down-regulated at the viral RNA levels. (A,B) Viral RNA transcript levels corresponding to the non-coding region (NCR) and E2 glycoprotein were analyzed by RT-PCR from CSFV-infected PK-15 and C26 cells at 72 hpi at m.o.i. of 10 (A) and from PK-15 cells at 24 h after 3D8 scFv treatment (B). cDNA template diluted fivefold against a concentration of "1" is expressed as 5^{-1}. Band intensities of RT-PCR products (A,B) were determined by densitometry and compared with band intensities for β-actin-specific RT-PCR products from the same RNA samples (right panels).

15 cells but not in C26 cells, at infectious dosage of m.o.i. of 10 (Fig. 2B).

Based on the report that the purified 3D8 scFv protein is able to penetrate cell membrane by itself [7], we also tested antiviral activity by exogenous treatment of 3D8 scFv protein to PK-15 cells. PK-15 cells were incubated with 3D8 scFv (5 μM) for 24 h at 37 °C, followed by infection with CSFV at m.o.i of 10 for 1 h at 37 °C. At 24 h post-infection (hpi), E2-positive cells were significantly reduced (19.0%) compared to untreated cells (51.2%) (Fig. 2C). Consistently, much less detection of CSFV E2 in 3D8 scFv-treated PK-15 cells was observed in confocal microscopy (Fig. 2D). These results demonstrate that intracellular existence of 3D8 scFv is capable of conferring antiviral activity on the cells.

4.3. CSFV replication is down-regulated by 3D8 scFv at the viral RNA levels

To examine whether the inhibition of CSFV multiplication is due to the loss of the viral RNA genome or transcripts, we performed reverse transcription-PCR using primers corresponding to the highly conserved 5′ non-coding region (NCR) and E2 gene of CSFV [11]. From C26 cells infected with CSFV with m.o.i. of 10, total cellular RNA was purified at 72 hpi and cDNA was synthesized prior to PCR. Viral RNA levels for NCR and E2 were fivefold lower in C26 cells, than those of control PK-15 cells (Fig. 3A). Reduction of viral RNA levels was observed also in PK-15 cells that were exogenously treated with 3D8 scFv protein (5 μM), for 24 h prior to infection with CSFV (Fig. 3B). In contrast, β-actin mRNA was observed to a similar extent in both control PK-15 and C26 cells, and both 3D8 scFv-treated and -untreated PK-15 cells. These results show that CSFV replication is suppressed by 3D8 scFv at the viral RNA levels.

4.4. Antiviral activity of 3D8 scFv is not associated with induction of type I IFN

Type I interferons, IFN-α and -β, are known as the anti-viral cytokines. CSFV is capable of avoiding the cellular anti-viral defense by suppressing production of IFN-α/β induction [13,14]. To know that reduction of viral RNA level in C26 was caused by induction of IFN-α/β, expression of IFN-β mRNA was measured. Cells were harvested at 1 h and 6 h after stimulation of the cells with poly IC or CSFV and then mRNA levels for IFN-β were analyzed by RT-PCR. Poly IC, a synthetic double stranded RNA analog, was used as a positive control to stimulate the production of IFN-β. Interestingly, the level of IFN-β mRNA induction at 6 h after stimulation was slightly lower in C26 cells compared to PK-15 cells, in both cases of poly IC treatment (1 μg/ml) and CSFV infections at m.o.i of 1 and 10 (Fig. 4A and B). This result suggests that antiviral activity shown in C26 was from the direct down-regulation of viral RNA by 3D8 scFv, but not from the turn-on of IFN-β production that is kept suppressed by the CSFV proteins.

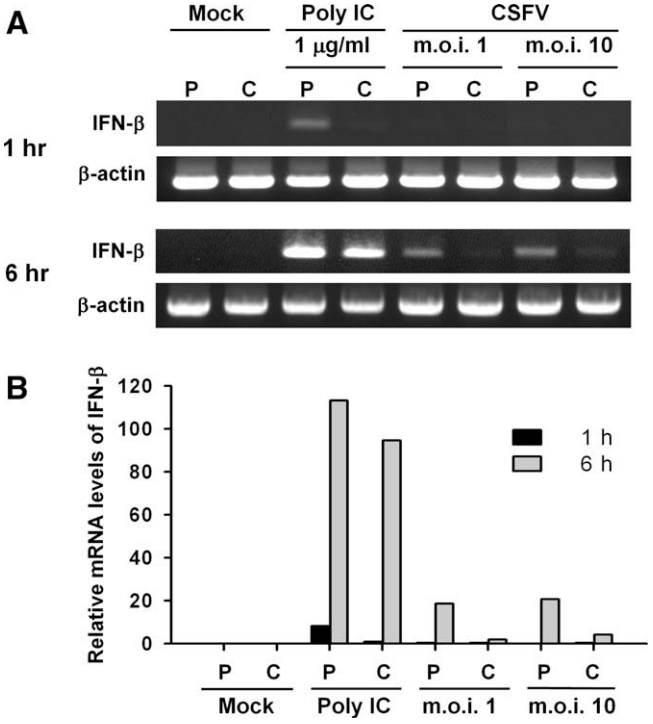

Fig. 4. Antiviral activity of 3D8 scFv is not associated with induction of type I IFN. (A) mRNA levels of IFN-β were analyzed by RT-PCR from control PK-15 (P) and C26 cells (C) at 1 and 6 h after either poly IC treatment or CSFV infection. (B) Band intensities of RT-PCR products were determined by densitometry and compared with band intensities for β-actin-specific RT-PCR products from the same RNA samples. This data are representative of three independent experiments.

4. Discussion

Here we showed that both intracellular expression and exogenous treatment of 3D8 scFv antibody with RNA-hydrolyzing activity provided resistance against CSFV infection to cells. It is obvious that 3D8 scFv exhibits antiviral activity even at a low protein concentration, since we could detect 3D8 scFv with ELISA from the C26 cell lysates (Fig. 1C) but not Western blotting from the C26 cell lysates (data not shown). 3D8 scFv is somewhat cytotoxic. Overexpression of 3D8 scFv in PK-15 cells by transient transfection with the 3D8 scFv gene has caused cell death, possibly resulting from inhibition of protein synthesis by intense damage to host RNAs [7]. Our trials of several times to establish stable clones that constitutively express 3D8 scFv have allowed us to acquire the clones which express 3D8 scFv protein only in subdetectable levels on Western blots, including C26 clone. Once clones were established, however, no noticeable adverse effects by 3D8 scFv expression were observed on the amounts and integrity of 28S and 18S rRNA, and the expression level of β-action mRNA, compared to those of PK-15 cells (data not shown), except slight retardation of the cell growth (Supplementary Fig. S1). Delayed cell cycle was observed in the case of the exogenous treatment of RNases which are able to penetrate cell membrane [15].

Instead of intracellular expression of 3D8 scFv gene, the exogenous treatments of PK-15 cells with 3D8 scFv protein that was prior to CSFV infection (Fig. 2C and D) resulted in the suppression of viral replication. According to our previous study with HeLa cells, 3D8 scFv (10 μM) treated to cells enters the cells via caveolae-lipid raft endocytosis and then released from caveosome, an endocytic vesicle bearing caveolin protein, to the cytosol over 24 h, and then hydrolyzes endogenous RNAs [7]. In 3D8 scFv-treated PK-15 cells, 3D8 scFv released to the cytosol might attack CSFV RNAs, leading to the reduction of the viral RNA levels as shown in Fig. 3B. Furthermore, when we carried out a flow cytometric analysis in which PK-15 cells were first infected with CSFV at m.o.i. of 1 for 1 h at 37 °C and then treated with 3D8 scFv protein (5 μM) for 48 h, E2-positive cells were significantly reduced (67.8%) in 3D8 scFv-treated cells, compared to untreated cells (41.8%) (Supplementary Fig. S2). Cytotoxicity by the treatment of 3D8 scFv in the concentration of 5 μM was not observed in cells for 48 h. This finding indicates that antiviral activity of 3D8 scFv was not by the blockade of the viral infection step itself, but by a function of 3D8 scFv after infection.

A representative endogenous RNase involved in anti-viral defense is IFN-induced RNase L. RNase L is expressed at low levels as enzymatically inactive form and is activated by a conformational change induced by 2′-5′ linked oligoadenylates which is synthesized by 2′-5′-oligoadenylate synthetase (OAS) of which levels are increased by viral stresses and activated by cofactor dsRNA, causing cellular and viral RNA degradation. Finally, activation of RNase L results in the inhibition of protein synthesis and then apoptosis of cells [16]. Recently, studies of a new endogenous IFN-induced RNase, ISG20, have shown that its anti-viral effect against RNA genomic viruses was due to its RNase activity on ssRNA [17,18]. Surprisingly, however, cell toxicity by ISG20 overexpression was not observed, even at the detectable expression level on an immunoblot with cell lysates, in cells that maintained resistance to viral replication [17], unlike in the case of 3D8 scFv.

In the cell-penetrating RNase (onconase) that is a RNase protein from the eggs of a frog (*R. pipiens*), the exogenous treatment of onconase to H9 cells under sub-lethal concentrations resulted in selective degradation of RNAs of HIV without degrading rRNA or mRNAs [1]. Onconase shows preference for cellular tRNA in hydrolysis, but also hydrolyzes dsRNA and *in vitro* transcribed mRNAs without sequence specificity [19]. It means onconase could attack both cellular and viral RNAs. There have been no evidences that onconase is able to discriminate cellular RNAs between viral RNAs and then selectively degrade viral RNAs. At this point we could not give the exact mechanism of anti-viral effect by 3D8 scFv. However it is conceivable that the antiviral activity of 3D8 scFv against CSFV is due to its RNase activity, similarly to onconase which degrades RNAs without sequence specificity. As proposed by Saxena et al. [1], perhaps proteins associated with RNA protect endogenous cellular RNAs from degradations by 3D8 scFv to a greater extent than the viral RNAs.

Since CSFV belongs to the Pestivirus genus, an enveloped RNA virus with a (+) ssRNA genome [8], viral genome (+) ssRNA as well as dsRNA formed as a replicative intermediate could be targeted for degradation by 3D8 scFv in the cytoplasm, where multiplication of most of RNA genomic viruses takes place. It should be noted that 3D8 scFv degrades both ssRNA and dsRNA (Supplementary Fig. S3 and [7]). To assess the effect of 3D8 scFv expression on the replication of other RNA virus, C26 cells were infected with (−) ssRNA-genomed vesicular stomatitis virus (VSV) which is able to infect PK-15 cells at m.o.i. of 1. VSV replication in C26 cells was considerably suppressed compared to PK-15 cells (Supplementary Fig. S4A). C26 cells were also resistant against apoptotic cell death, cytopathic effect by VSV infection [20] (Supplementary Fig. S4B). These results indicate that antiviral activity would not be restricted to the viruses with specific genomic sequence.

It is known that CSFV interfere with IFN-α/β production in the infected cells by virtue of two proteins (N^{pro} and E^{rns}) of CSFV [13,14,21]. In order to investigate the possibility that antiviral activity of 3D8 scFv against CSFV was resulted from breaking the suppression of IFN-α/β induction, we analyzed mRNA levels of IFN-β in control PK-15 cells and C26 cells infected with CSFV. Interestingly the level of IFN-β mRNA induction at 6 h after CSFV infection was lower in C26 cells rather than in PK-15 cells (Fig. 4). The level of IFN-β mRNAs at 9, 12, and 24 h after CSFV infection also revealed the same tendency with observations at 6 h (data now shown). Therefore, it seems that antiviral activity of 3D8 scFv against CSFV is not mediated by the induction of IFN-α/β at least. Slight increase of IFN-β mRNA level upon CSFV (LOM strain) was observed by RT-PCR, in contrast to the reports in which IFN-α/β proteins were not detected in PK-15 cells [13,21].

In conclusion, we have demonstrated the antiviral activity of RNA-hydrolyzing 3D8 scFv against CSFV by using a 3D8 scFv-expressing cell line and by the exogenous treatment of 3D8 scFv protein to cells. 3D8 scFv strongly suppressed CSFV replication at the viral RNA levels without involving IFN-β induction. Further study is needed to determine whether this anti-viral effect will be applicable to various RNA or DNA genomic viruses with different mechanisms for multiplication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.04.032.

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