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Rana catesbeiana ribonuclease inhibits Japanese encephalitis virus (JEV) replication and enhances apoptosis of JEV-infected BHK-21 cells

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ABSTRACT

Rana catesbeiana ribonuclease (RC-RNase) is a cytotoxic and antitumor RNase isolated from the oocyte yolk granules of the bullfrog *R. catesbeiana*. Our previous studies have shown that RC-RNase possesses antitumor activity by activating proapoptotic caspases. Here, we demonstrate that RC-RNase also possesses antiviral activity. By using cell viability and caspase activation assays, we show that RC-RNase largely enhances apoptosis of Japanese encephalitis virus (JEV)-infected BHK-21 cells by activating caspase-3, caspase-8, and caspase-9. In addition, immunoblotting experiments revealed that JEV infection enhances the internalization of RC-RNase by cells. In sum, these results indicate that RC-RNase provides a beneficial effect on JEV-infected cells by enhancing apoptosis.

1. Introduction

Japanese encephalitis virus (JEV) is a positive-sense, single-stranded RNA virus which belongs to the family of Flaviviridae. JEV infection causes Japanese encephalitis (JE), a leading cause of childhood mortality and morbidity in Southeast Asia and Western Pacific region. Around 3 billion people are at risk of developing JE and approximately 30,000–50,000 cases are reported in Asia annually (Ghosh and Basu, 2009). Among the JE cases reported, about 50% of patients develop permanent neuropsychiatric sequelae and 25–30% of cases results in death (Kaur and Vrati, 2003). Until now, there exists no specific anti-JE therapy other than supportive care (Diagana et al., 2007). Therefore, extensive research has focused on developing an effective therapy to prevent and treat JEV infections.

Rana catesbeiana ribonuclease (RC-RNase), a pyrimidine-guanine sequence-specific enzyme isolated from the oocytes of bullfrogs (Liao, 1992; Liao and Wang, 1994), produces potent antitumor effects on various tumor cells, but is nontoxic to primary and untransformed cell lines (Hu et al., 2001a,b; Huang et al., 1998; Liao et al., 1996; Tang et al., 2005; Wei et al., 2002). This enzyme belongs to the RNase A superfamily and shares high homology to *Rana pipiens* onconase (Rosenberg, 2008), which is currently in Phase IIIb clinical trials to treat malignant mesothelioma (Ardelt et al., 2008). Besides its antitumor activity, onconase also possesses anti-RNA virus activity. As such, onconase specifically inhibits the replication of human immunodeficiency virus type 1 (HIV-1) (Saxena et al., 1996; Suhasini and Sirdeshmukh, 2007; Youle et al., 1994). Other ribonucleases that belong to the RNase A superfamily have been shown to possess anti-RNA virus activity. For instance, the eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) present good antiviral activity against respiratory syncytial virus (RSV) and are critical for antiviral host defense in the respiratory tract (Domachowske et al., 1998a,b; Phipps et al., 2007). Based on these observations, we hypothesized that RC-RNase may also possess anti-RNA virus activity.

In the present study, we found that RC-RNase effectively blocked JEV replication and enhanced the activation of caspase-3, caspase-8, and caspase-9 in JEV-infected BHK-21 cells, thereby inducing apoptosis. Our findings suggest that, in addition to its well-known antitumor activity, the amphibian RC-RNase may represent a potent anti-RNA virus agent.

2. Materials and methods

2.1. Chemicals and cell lines

RC-RNase was purified from *R. catesbeiana* oocytes as described (Liao et al., 1996). The purity of RC-RNase was examined with

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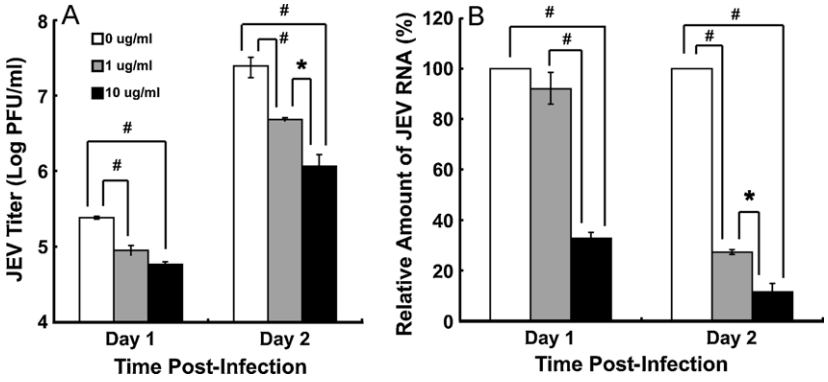

Fig. 1. RC-RNase inhibits JEV replication in JEV-infected BHK-21 cells. JEV-infected BHK-21 cells were treated with various concentrations of RC-RNase for either 1 or 2 days. The titer of nascent JEV virions was examined with a plaque-forming assay (A) and intracellular JEV RNA was quantified by RT-PCR (B). The amount of JEV RNA in JEV-infected BHK-21 cells without RC-RNase treatment (0 μg/ml) was set as 100%. The data represent average from 3 wells for each time indicated and were expressed as mean ± S.D. The symbol * marks values with p-value < 0.05 and the symbol # marks values with p-value < 0.01.

silver stain as described (Hu et al., 2001b). JEV neurovirulent strain RP9 (Chen et al., 1996) was kindly provided by Dr. Y.L. Lin (Academia Sinica, Taiwan). Caspase substrates were purchased from Anaspec (Fremont, CA). The baby hamster kidney cell line BHK-21 was obtained from Bioresource Collection and Research Center (Taiwan). BHK-21 cells were cultivated in RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 100 IU/ml penicillin/streptomycin. A multiplicity of JEV infection of 1:1 was used.

2.2. Cell viability assay

BHK-21 cells were cultured in 96-well cell culture plates overnight and were infected with RP9 using a multiplicity of infection of 1:1. Cells were treated with various amounts of RC-RNase, and XTT assays (Roche, Basel, Switzerland) were performed every 24 h (Hu et al., 2001a).

2.3. Measurements of JEV production

BHK-21 cells were infected with RP9 and treated with RC-RNase. Virus titer in culture media was measured by a plaque-forming assay (Chiou et al., 2003).

2.4. Real-time PCR

Total RNA was reverse-transcribed to cDNA as described (Chen et al., 2009). To examine intracellular JEV RNA copy number, cDNA were mixed with LightCycler 480 SYBR Green I Master (Roche) and the primers JEV-F (5′-^{3126}tttggagaggttaaatcttgcactt^{3150}-3′) and JEV-R (5′-^{3219}ctatggtatgcggaatgatgagttc^{3195}-3′). The amount of hypoxanthine phosphoribosyltransferase 1 (HPRT1) RNA was examined with the primers HPRT1-F (5′-^{578}tgacactggcaaaacaatgca^{598}-3′) and HPRT1-R (5′-^{671}ggtccttttcaccagcaagct^{651}-3′). The mixture was applied to a LightCycler 480 (Roche) for real-time PCR (RT-PCR). Copy number of JEV RNA was normalized against the amount of HPRT1 RNA.

2.5. Propidium iodide staining and flow cytometry

Cells were trypsinized and fixed with 70% ethanol at −20 °C for 30 min. Fixed cells were washed and stained with propidium iodide (PI) (4 μg/ml PI, 1% Triton X-100, 0.1 mg/ml RNase A in PBS, pH 7.4). After 3 h of incubation, cells were washed and analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, Palo Alto, CA).

2.6. Caspase activity assay

Cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) and cell lysates were used for caspase activity assays (Wei et al., 2002).

2.7. Ultrastructural observations

BHK-21 cells were either mock-infected or JEV-infected, with or without RC-RNase (10 μg/ml) for 2 days, prior to preparation for electron microscopy (Chiou et al., 2003).

2.8. Immunoblotting

Proteins were separated by SDS-PAGE and were electroblotted to an Immobilon-P membrane (Millipore, Billerica, MA) (Chang et al., 2004). Proteins were detected using SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL). Monoclonal anti-JEV NS3 antibody was kindly provided by Dr. Y.L. Lin (Academia Sinica). Monoclonal anti-actin antibody was purchased from Millipore.

2.9. Statistical analysis

All data were presented as means ± standard deviation (S.D.) for the number of experiments indicated. Differences between treated and control groups were analyzed using Student's t-test. Statistical analyses were performed using Microsoft Office Excel. A p value < 0.05 was considered statistically significant.

3. Results

3.1. RC-RNase blocks JEV replication in JEV-infected BHK-21 cells

To examine the antiviral activity of RC-RNase, we infected BHK-21 cells with JEV, treated the resultant cells with RC-RNase, and monitored the titer of released viruses and virus replication using plaque-forming assay and RT-PCR, respectively. RC-RNase significantly blocked the production of nascent JEV virions as revealed by the plaque-forming assay (Fig. 1A, p value < 0.01). RC-RNase also inhibited JEV RNA amplification in a dose-dependent manner as shown by RT-PCR (Fig. 1B, p value < 0.01). In JEV-infected BHK-21 cells treated with 10 μg/ml of RC-RNase, the relative amount of JEV RNA dramatically decreased to 30% and below 10% at day 1 and day 2 post-infection, respectively (Fig. 1B). The production of nascent JEV virions was reduced more than 10 fold at day 2 post-infection

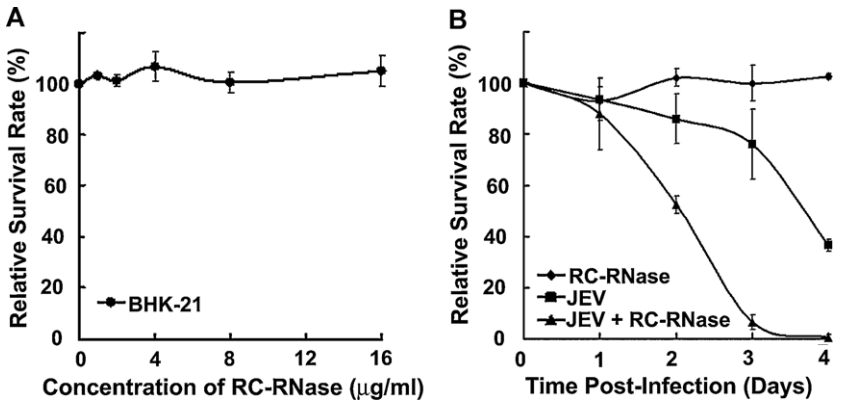

Fig. 2. RC-RNase enhances cell death in JEV-infected BHK-21 cells. The survival rate of BHK-21 cells was examined with the XTT assay after RC-RNase treatment with various concentrations for 4 days (A). The JEV-infected BHK-21 cells were treated with/without 10 μg/ml RC-RNase in a time-dependent manner. The survival rate of RC-RNase-treated BHK-21 was also examined (B). Each data point was calculated from 3 duplicate groups and expressed as mean ± S.D.

(Fig. 1A). These results indicate that RC-RNase significantly blocks JEV replication in JEV-infected BHK-21 cells.

3.2. RC-RNase enhances cell death in JEV-infected BHK-21 cells

Next, experiments were designed to determine if RC-RNase increases cell death in JEV-infected BHK-21 cells. In this case, the survival rate of JEV-infected BHK-21 cells following treatment with RC-RNase was examined with the XTT assay. While the growth of BHK-21 cells was unaffected by RC-RNase (Fig. 2A), even at a concentration of 16 μg/ml for 4 days which is cytotoxic to various tumor cell lines (Hu et al., 2001a,b; Liao et al., 1996; Tang et al., 2005; Wei et al., 2002), the survival rate of JEV-infected BHK-21 cells was shown to decrease following RC-RNase treatment (Fig. 2B). Notably, while JEV induced apoptosis of BHK-21 cells on its own, we observed that RC-RNase further increased the level of apoptosis compared to JEV infection alone (Fig. 2B). For instance, at day 2 post-infection, while JEV infection resulted in 10% of cell death, the RC-RNase treatment produced cell death that reached 50% (Fig. 2B). Similarly, at day 3 post-infection, JEV infection produced 20% cell death, and RC-RNase treatment increased cell death further to 90% (Fig. 2B). At day 4 post-infection, the RC-RNase treatment killed almost all infected cells, while JEV-infected cells still had a 40% survival rate in the absence of RC-RNase treatment (Fig. 2B). These results indicate that RC-RNase enhances cell death in JEV-infected BHK-21 cells. In addition, these observations suggest that RC-RNase may possess antiviral activity.

3.3. RC-RNase enhances apoptosis by activating caspases in JEV-infected BHK-21 cells

In order to elucidate the mechanism of RC-RNase-induced cell death in JEV-infected cells, we monitored the sub-G1 cell population using flow cytometry (Fig. 3A). Mock-infected BHK-21 cells showed a small population of sub-G1 cells for both non-treated (Mock) and RC-RNase-treated groups (Fig. 3A). In JEV-infected BHK-21 cells, the percentage of sub-G1 cells was about 10% at day 2 post-infection without RC-RNase treatment (JEV), indicating that only a few JEV-infected BHK-21 cells underwent apoptosis (Fig. 3A). When JEV-infected BHK-21 cells were treated with 10 μg/ml RC-RNase, however, the percentage of sub-G1 cells dramatically increased to more than 30% (JEV + RC-RNase), indicating that RC-RNase enhances apoptosis of JEV-infected BHK-21 cells (Fig. 3A).

The activation of caspases-3 (Fig. 3B), caspase-8 (Fig. 3C), and caspase-9 (Fig. 3D) was further examined in order to verify activation of apoptosis. In JEV-infected BHK-21 cells, caspase-3, caspase-8, and caspase-9 were still inactive at 24 h post-infection without RC-RNase treatment, but these caspases became active at 48 h (Fig. 3B–D, p value < 0.01). However, with a 10-μg/ml RC-RNase treatment, activation of caspase-8 and caspase-9 was faster (i.e. 24 h), and the activation of all caspases tested was largely increased 48 h post-infection in JEV-infected BHK-21 cells, while the RC-RNase alone (10 μg/ml) did not activate caspases, even when the cells were treated for 48 h (Fig. 3B–D). These results indicated that RC-RNase treatment enhanced the activation of caspase-8, and caspase-9 in JEV-infected BHK-21 cells within 48 h of treatment.

We also used electron microscopy to confirm the effect of RC-RNase on JEV-infected BHK-21 cells. No apoptotic traits such as chromatin condensation or cytoplasmic bubbles were apparent in mock-infected BHK-21 cells, with or without RC-RNase treatment (Fig. 4A and B). However, JEV-infected BHK-21 cells harbored typical cytopathological alterations, such as dilatation of the endoplasmic reticulum (ER), where JEV virions could be observed (Fig. 4C, double arrowheads). JEV-infected cells treated with 10 μg/ml RC-RNase showed swelling and disrupted mitochondria (Fig. 4D, arrows) as well as condensed chromatin (arrowheads), indicative of cells undergoing apoptosis. Taken together, these results indicate that RC-RNase enhances apoptosis of JEV-infected BHK-21 cells by increasing caspase-8, and caspase-9 activity.

3.4. Internalization of RC-RNase by JEV-infected BHK-21 cells

Accumulating evidence indicates that internalization of cytotoxic ribonucleases is critical for the cytotoxicity effects of these enzymes (Saxena et al., 1991). Therefore, we investigated whether JEV infection affects the internalization of RC-RNase. After RC-RNase treatment, the internalization of RC-RNase was examined by immunoblotting with an anti-RC-RNase antibody (Wei et al., 2002). A low amount of intracellular RC-RNase was found in mock-infected BHK-21 cells at 24 and 48 h following RC-RNase treatment (Fig. 5). JEV infection increased the amount of intracellular RC-RNase compared to mock-infected cells, as seen by the 1.6 and 2.3 fold increase at 24 and 48 h in JEV-infected cells, respectively (Fig. 5, p value < 0.05). Altogether, our results indicate that JEV infection enhances the internalization of RC-RNase. In this case, the enhanced internalization of RC-RNase may explain the increased level of apoptosis seen in JEV-infected BHK-21 cells following RC-RNase treatment.

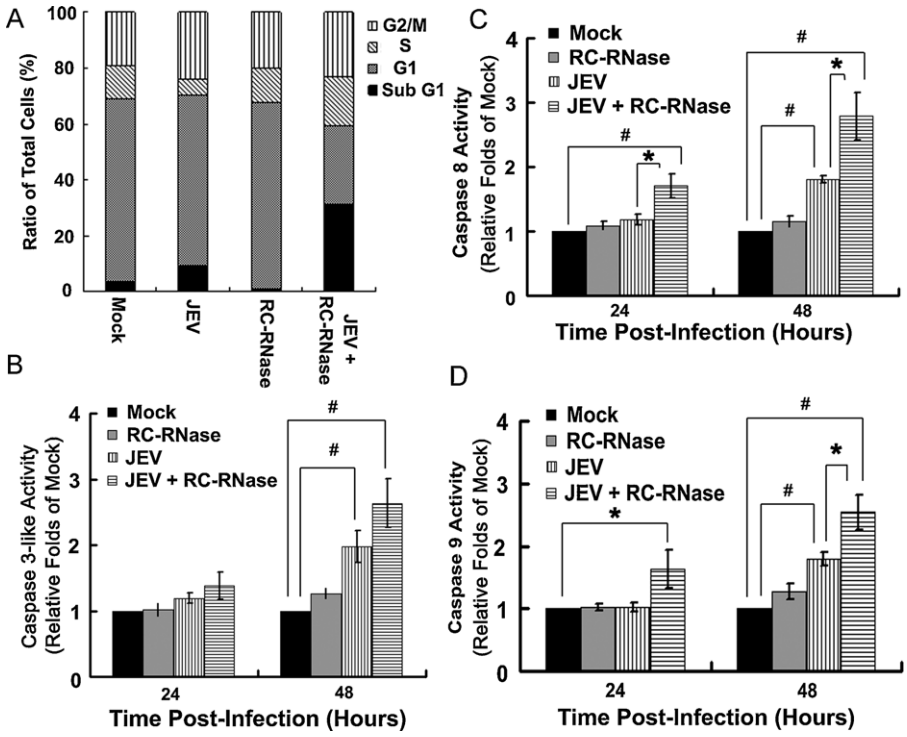

Fig. 3. RC-RNase enhances activation of caspase-3, caspase-8 and caspase-9 and apoptosis in JEV-infected BHK-21 cells. After JEV infection and RC-RNase treatment, the DNA of BHK-21 cells was stained with PI and examined by flow cytometry (A). The activity of caspase-3 (B), caspase-8 (C) and caspase-9 (D) was examined with a caspase activity assay. Data represent averages from 3 wells for the time indicated and were expressed as mean ± S.D. The symbol * marks values with p-value < 0.05 and the symbol # marks values with p-value < 0.01.

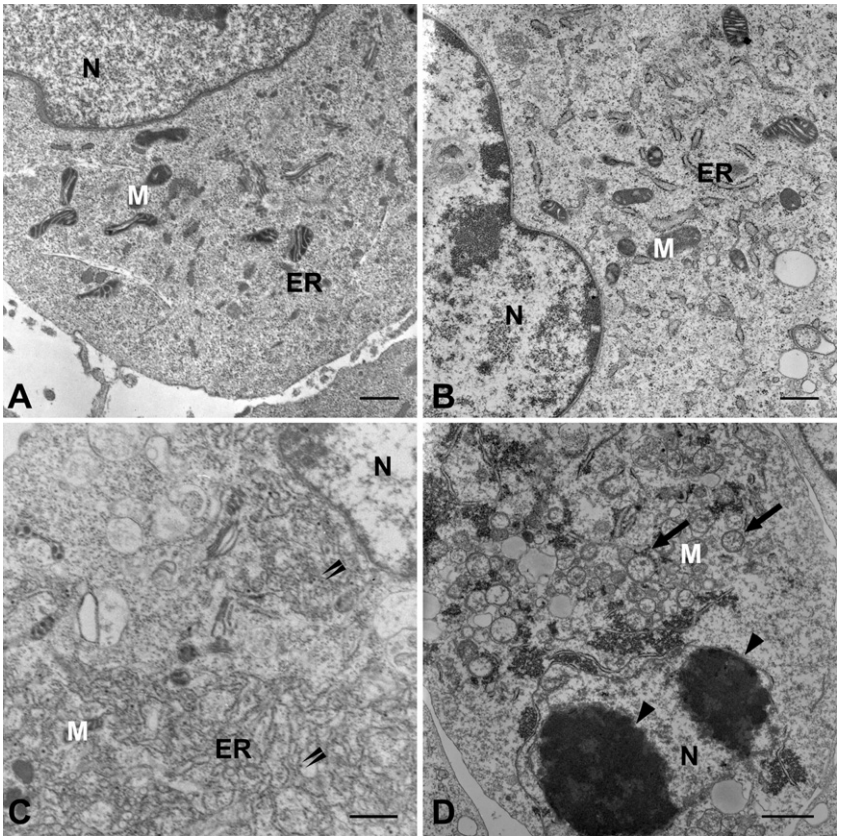

Fig. 4. RC-RNase-induced ultrastructure alterations in JEV-infected BHK-21 cells. JEV-infected BHK-21 cells were treated with (D) or without (C) RC-RNase, prior to processing for ultrathin section and TEM. Mock-infected (A) and RC-RNase-treated (B) BHK-21 cells were used as negative control. Double arrowheads indicate JEV virions in dilated ER. Arrowheads indicate chromatin condensation. Arrows indicates swelling and disrupted mitochondria. N, nucleus; M, mitochondria; ER, endoplasmic reticulum. Bar = 1 μm.

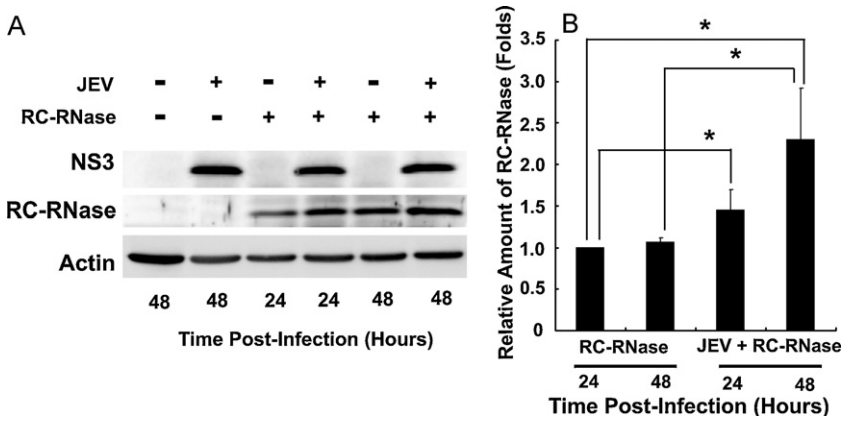

Fig. 5. RC-RNase penetrates into BHK-21 cells. Mock- and JEV-infected BHK-21 cells were treated with 10 μg/ml of RC-RNase for 24 or 48 h. Intracellular RC-RNase, JEV NS3 and actin were examined by immunoblotting (A). JEV NS3 and actin were used as internal controls for JEV infection and protein loading, respectively. Quantization of intracellular RC-RNase was calculated by GeneTools version 4.0 (Synoptics, Cambridge, England) (B). The symbol * marks values with p-value < 0.05.

4. Discussion

In this study, the antiviral activity of RC-RNase was firstly demonstrated. The results of the plaque-forming assay and RT-PCR showed that RC-RNase treatment decreased nascent JEV production and reduced the copy number of intracellular viral genomes (Fig. 1). Moreover, the cell viability assay revealed that RC-RNase produced a dramatically high cytotoxic effect on JEV-infected cells (Fig. 2B). These results indicate that RC-RNase treatment not only reduced the survival of the infected cells but also inhibited viral genome replication, and subsequently reduced nascent JEV particle production. However, the reduction of nascent JEV production and intracellular JEV RNA were only about 10 fold, suggesting that RC-RNase may not directly inhibit JEV replication. Biochemical and morphological investigations indicated that RC-RNase treatment induced apoptosis of JEV-infected cells. In addition, antiviral agents that specifically and directly inhibit virus replication are less cytotoxic (Youle et al., 1994). Therefore, it appears that RC-RNase induces apoptosis of JEV-infected cells, and that this process disturbs JEV replication.

Accumulating evidence suggests that internalization of ribonucleases is the rate-limiting step for their cytotoxicity. As such, ribonucleases that are micro-injected into the cytosol are more toxic than the ones added extracellularly (Saxena et al., 1991). Likewise, conjugating ribonucleases with transferrin (Rybak et al., 1991), growth factors (Futami et al., 1999; Jinno et al., 1996), or antibodies (Newton et al., 2001) increases their cytotoxicity. In this study, immunoblotting experiments showed that JEV infection enhanced the internalization of RC-RNase (Fig. 5). At the same time, the survival rate of JEV-infected BHK-21 cells dramatically decreased when these cells were treated with RC-RNase (Fig. 2B). These data indicate that the entry of RC-RNase into the cytosol is critical for the cytotoxicity of this enzyme. In a previous study, JEV infection was also shown to increase membrane permeability (Chang et al., 1999), an observation which may explain why RC-RNase can readily enter JEV-infected cells.

Additionally, JEV infection modulates the expression of proinflammatory cytokines and chemokines, such as interferons, tumor necrosis factors, and others (King et al., 2003; Diamond, 2009). These JEV-induced cytokines may also contribute to sensitizing JEV-infected cells to RC-RNase. Our previous study showed that the action of RC-RNase can synergize with interferon-γ against tumor cells (Tang et al., 2005). Combination of onconase and tumor necrosis factor-α (Deptala et al., 1998) or interferon-β (Tsai et al., 2002) also enhanced anti-tumor effects in this context. These data suggest that RC-RNase and cytokines induced by JEV infection may trigger synergistic cytotoxic effects on JEV-infected cells.

JEV infection can activate caspase-3, caspase-8, and caspase-9 at 48 h post-infection (Fig. 3B–D) and induce caspase-dependent apoptosis (Fig. 3A) as seen in previous reports (Liao et al., 1997; Tsao et al., 2008). However, the processes of activation of caspases may be cell type specifically. Caspase-3, caspase-8 and caspase-9 are activated 36 h postinfection in JEV-infected N18 cells; whereas caspase-8 and caspase-9 are activated 24 h and 36 h postinfection in JEV-infected MCF-7 cells, respectively (Tsao et al., 2008). When treated with RC-RNase, JEV-infected cells underwent apoptosis rapidly (Fig. 3A). Caspase activity assays also indicated that caspase-8, and caspase-9 were activated at 24 h post-infection in RC-RNase treated JEV-infected cells (Fig. 3B–D), suggesting that RC-RNase enhance caspase-8 and caspase-9 activation in JEV-infected BHK-21 cells. Caspase-3 was activated 48 h post-infection, following the activation of caspase-8 and caspase-9, indicating that activation of caspase-3 may result in the activation of caspase-8 and caspase-9. Furthermore, the activity of caspase-8, and caspase-9 following RC-RNase treatment was much higher than that without RC-RNase treatment (Fig. 3B–D). These data suggest that the enhancement of caspase activation may contribute to RC-RNase enhancing apoptosis in JEV-infected BHK-21 cells. It is worth to note that natural or artificial caspase inhibitors can block JEV-induced apoptosis, but hardly modulate JEV titer in previous reports (Liao et al., 1997; Tsao et al., 2008). However, in the present study, RC-RNase enhances apoptosis in JEV-infected cells and consequently reduces JEV replication, implying a new direction of development of anti-JEV therapy.

In conclusion, our results show that RC-RNase could be use to efficiently inhibit JEV replication and to induce apoptosis in JEV-infected BHK-21 cells. The dual functions of RC-RNase may provide a beneficial effect by eliminating JEV infection. Since there is no specific anti-JEV therapy, many scientists still work on developing new anti-JEV drug, such as N-methylisatin-β-thiosemicarbazone derivatives, which inhibits JEV replication in vitro (Sebastian et al., 2008); N-nonyl-deoxynojirimycin, which blocks the budding and processing of JEV from the endoplasmic reticulum (Wu et al., 2002); designed small interfering RNA (siRNA), which interfere with the expression of essential viral structure proteins (Kumar et al., 2006). Here, while the observed antiviral activity of RC-RNase was not dramatic, we nevertheless provide a new direction for novel anti-JEV drug development. Viruses cannot naturally reproduce outside a host cell. If a drug can specifically affect infected host cells from human patients, this drug will also block virus production. The

results presented in this study show that RC-RNase may be used for this purpose.

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