Induction of FADD Expression and Caspase Cascades Involved in RC-RNase-Induced Apoptosis in Human Cervical Cancer Cells

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AIM: RC-RNase is a novel member of RNase A superfamily from Rana catesbeiana (bullfrog) oocytes. This study is to investigate the cytotoxic effect of RC-RNase on cancer cells. The cytotoxic mechanisms were investigated and discussed.

METHODS: Cytotoxicity of RC-RNase to human cervical cancer HeLa S3 cells was determined by microculture tetrazolium test and trypan blue exclusion assay. Apoptotic characteristics, such as chromatin condensation, blebbing morphology, DNA ladder, phosphatidylserine translocation, caspase activation, and poly (ADP-ribose) polymerase (PARP) cleavage, were analyzed by generally described methods. Cell-cycle phases were analyzed by flow cytometry, and cellular cyclin B1/CDC2 activity was detected by in vitro histone H1 kinase assay. Western blot analyses were performed to examine the levels of Fas, FADD, TNFR1, TRADD, cyclin B1, CDC2, Wee-1, and the cleaved forms of Bid, PARP and caspases-3, 8 and 9.

RESULTS: RC-RNase induced cell death with apoptotic characteristics in HeLa S3 cells. In vitro activity assays suggested that caspases-3, 8, and 9 activities were all increased in RC-RNase-treated cells. The results of Western blot analyses, including induction of FADD expression and increases of the cleaved forms of Bid, PARP and caspases-3, 8 and 9, confirmed that RC-RNase indeed induced the activation of caspases-3, 8, and 9. Although RC-RNase did not significantly affect the cell-cycle distribution, cyclin B1/CDC2 kinase activity was increased in RC-RNase-induced apoptotic cells. Cellular Wee-1 level was reduced by RC-RNase treatment, and CDC2 was kept in Tyr-15-dephosphorylated (active) form.

CONCLUSION: RC-RNase was potent to induce FADD expression, caspase cascades, cyclin B1/CDC2 activation, and ultimately apoptosis in HeLa S3 cells.

Keywords: RC-RNase, FADD, caspase, Wee-1, cyclin B1/CDC2


Introduction

Ribonucleases (RNases) are a group of versatile ribonuclease enzymes with diverse biological functions. Other than performing the fundamental cellular metabolic function of RNA processing and degradation, some RNases have been shown to exhibit various important biological effects including antiviral, antitumor, antiherpetic, antiherbicidal, neurototoxic immunosuppressive, angiogenic, and pollen fertility-activation activities [1-6]. For example, RNase L is a 2',5'-oligoadenylate-dependent RNase and functions in the molecular mechanisms of interferon action; S-RNase from the pistils of some flowering plants can cause the RNA degradation in pollen tubes and is involved in self-incompatibility. Bovine seminal ribonucleases, a homodimer with two identical subunits, exhibit both ribonuclease and antitumor activities [7]. Several RNases have been reported to induce cancer cell death with characteristics of apoptosis, such as RNases in the commercial choric gonadotrophin (hCG) preparations of human urine shown to kill Kaposi's sarcoma Y-1 cell line [8] and Onconase, a representative cytotoxic RNase from embryos of leopard frog that induced apoptosis ex vivo, exhibited anti-tumor activities in animal models [9-11], and is currently in a Phase-I/II clinical trial for malignant mesothelioma and a Phase I / II trial in non-small cell lung cancer (conducted by Alfacell Corporation, Bloomfield, NJ, USA).

We have previously purified RC-RNase from Rana catesbeiana (bullfrog) oocytes and shown it to be a novel member of RNase A superfamily that exhibited pyrimidine-guanine
sequence-cleavage specificity and was cytotoxic to several cancer cell lines [12,13]. RC-RNase is synthesized in the liver and subsequently transported to the oocytes. Expression of RC-RNase in the liver increases with the maturity of female frog during oogenesis and is inducible by estradiol even in the male frog liver [14]. Although there are a large amount of RC-RNase and related RNases stored in the oocytes of frog [15], the physiological role or possible cell growth effect of RC-RNase in the frog oocytes is not known. Similar to Onconase, RC-RNase in combination with IFNγ could produce synergistic cytotoxicity to hepatoma cells [16] and also induced cytotoxic effect with the activation of caspase-7 in human breast carcinoma MCF-7 cells [17]. However, the mechanisms for RC-RNase-induced cell death remain to be elucidated.

Apoptosis is an important pattern of cell death involving the activation of a series of caspase cascades [18]. Caspasas are a group of proteases that contain a Cys residue in the catalytic domain and exhibit a cleaving specificity toward the Asp-Xxx-Axxp family. There are two well-characterized apoptotic pathways leading to caspase activation. The extrinsic pathway is initiated by the engagement of cell membrane death receptors with ligands, recruiting FADD to form a DISC (death-inducing signaling complex) and ultimately resulting in the activation of caspase-8 or caspase-10 [18]. The intrinsic pathway is triggered by cellular response to stress stimuli such as chemicals and viral infection, culminating in the activation of caspase-9. Caspase-8 and caspase-9 are the initiator caspases responsible for cleavage and thus activation of the effector caspases, such as caspase-3 and caspase-7, which cause cell death by cleaving a variety of protein substrates.

Apoptotic cells exhibit characteristic morphological changes somewhat similar to the features of mitosis. The cyclin B1/CDC2 complexes are known to catalyze the chromatin condensation as well as nuclear envelope breakdown during mitosis, thus performing a key and rate-limiting function in the transition from G2 into M phase [19]. The catalytic subunit of the complex, CDC2, is constitutively present throughout the cell cycle. However, the periodic accumulation and destruction of cyclin B1, the regulatory subunit, is mainly responsible for determining the oscillation of CDC2 activity. In addition, activation of CDC2 can be controlled by other mechanisms, such as hyperphosphorylation of CDC2 by Wee-1 or Myt-1 kinase [20,21] and dephosphorylation of CDC2 by CDC25 phosphatase [22]. Inappropriate activation of cyclin B1/CDC2 kinase has contributed to the apoptotic cell death of human cancer cells caused by anti-cancer agents, such as paclitaxel, camptothecin, etoposide, and GL331 [23-26].

In the present study, we investigated the effects of RC-RNase and bovine pancreatic RNase A on the cell proliferation of human cervical cancer HeLa S3 cells. Our data showed that RC-RNase but not RNase A induced apoptosis in HeLa S3 cells, which substrate-dependent with the inducible FADD expression and caspases-3, 8, and 9 activities. In addition, RC-RNase reduced the levels of Wee-1 kinase and Tyr-15-phosphorylated CDC2, and induced cyclin B1/CDC2 activity. Our data suggested a model for RC-RNase-induced apoptosis.

Materials and Methods

Cell culture and RNases

HeLa S3 cells (American Type Culture Collection, Rockville, MD, USA) were grown as a monolayer in Dulbecco’s Modified Eagle Medium (D-MEM) supplemented with 10% fetal bovine serum, 2 mM of L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, and were cultured under 5% CO2 in a humidified 37°C incubator. RC-RNase was purified from mature native bullfrog (Rana catesbeiana) as previously described [15]. Bovine pancreatic RNase A was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cytotoxicity Assay

Cytotoxicity of RC-RNase was evaluated by microculture tetrazolium test (MTT). Three thousands of HeLa S3 cells were seeded on each well of 96-well dishes and incubated overnight at 37°C in a 5% CO2 humidified incubator. Adherent cells were then treated with RC-RNase ranging from 10 nM to 100 μM in 200 μl of fresh culture medium. After a 72-h exposure, 50 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [0.25% (w/v)] in PBS, Sigma] was added to the medium of each well for another 4-h incubation. The medium was then removed and formazan formed was extracted with 50 μl of DMSO and quantitated by measuring absorbance at 550 nm.

Transmission electron microscopy

HeLa S3 cells were treated with 100 μM RC-RNase for 48 h, and were collected and fixed in 2.5% glutaraldehyde and 0.1 M cacodylate buffer. After dehydration with a graded series of ethanol solution, the cells were embedded in eponate-12 and finally examined under a transmission electron microscope (JEM 1230, JEOL, Peabody, MA, USA).

Internucleosomal cleavage assay

Apoptotic DNA fragments were extracted by the method described previously [26]. Briefly, logarithmically growing HeLa S3 cells were treated with RC-RNase or RNase A for 48 h. Both the adherent and floating cells were collected and washed twice with PBS. Cell pellets were suspended in 4°C pre-chilled lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 1% NP-40. The cell lysates were centrifuged at 3,000 xg for 1 min, and the supernatants were brought to 1% SDS and 0.2 μg/ml of RNase A and incubated at 45°C for 1 h, followed by digestion with 2.5 μg/ml of proteinase K at 55°C overnight. After the addition of half volume of 10 M ammonium acetate and 20 μg of glycogen, RNase/protease-digested supernatants were extracted with phenol-chloroform twice, chloroform once and added with -20°C pre-chilled ethanol to precipitate the DNA fragments. The pattern of DNA ladders was examined after 1.2% agarose gel electrophoresis and ethidium bromide staining by Alpha-Innotech IS500 digital Imaging System (Avery Dennison, CA, USA).

Annexin V/propidium iodide staining

The apoptotic cell ratio was measured quantitatively, according to the manual of Apoptosis Detection Kit (R & D Systems Co., Minneapolis, MN, USA). After RC-RNase or RNase A treatments, approximately 105 cells were trypsinized, washed twice with PBS, and suspended as single cells in 200 μl of calcium-containing binding buffer. The cell suspensions were then added with 20 μl of fluorescein isothiocyanate (FITC)-labeled annexin V and 10 μl of propidium iodide (PI), and incubated at 4°C in the dark for 15 min. After diluted with 800 μl of binding buffer, the apoptotic cells with FITC-annexin V staining were measured by flow cytometer FACStar (BD Bioscience, San Jose, CA, USA).

Western blot analysis

Cell lysates for immunoblot analyses were prepared as described previously [27]. Aliquots (40 μg) of protein samples were resolved by 10% or 12% SDS-PAGE, and then electrotransferred onto polyvinylidene difluoride membranes. After blocking with PBST (PBS plus 0.1% Tween-20) plus 5% non-fat milk, the membrane blots were incubated with
indicated primary antibody (in PBST plus 5% non-fat milk) at 4°C for 12 h, then washed three times with PBST buffer, and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing three times with PBST buffer, the protein bands were detected by the enhanced chemiluminescence procedure (Amer sham Biosciences, Piscataway, NJ, USA). The antibodies against caspases-3, 8 and 9 were from Cell Signaling Technology (Danvers, MA, USA). The antibodies against Bid, Fas, FADD, TNFR-α receptor 1 (TNFR1), TRADD, Wee-1, cyclin B1, CDC2, and poly (ADP-ribose) polymerase (PARP) N-terminal part were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cappel horseradish peroxidase-conjugated goat anti-mouse and rabbit immunoglobulins were obtained from Organon Teknika (Dublin, Ireland). The anti-Tyr-15-phosphorylated CDC2 antibody was purchased from New England Biolabs (Beverly, MA, USA).

**Immunoprecipitation and in vitro histone H1 kinase assay**

Cell lysates were prepared for immunoprecipitation and kinase assay according to previously described procedure [28]. Briefly, RC-RNase or RNase A-treated HeLa S3 cells were suspended in lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin-A, and 10 μg/ml leupeptin, and subjected to sonication at 3-4 output and 30-40% of duty cycle for 5 pulses. After centrifugation at 13,000 x g for 20 min at 4°C, supernatants were collected and determined for protein concentrations (Bradford method). For immunoprecipitation, 100 μg of protein samples were incubated with anti-cyclin B1 antibody at 4°C with constant mixing for 15 h. The immune complexes were precipitated by binding to protein A-Sepharose (Sigma), and the immunoprecipitates were washed four times with lysis buffer and twice with kinase buffer (20 mM Tris-HCl, pH 7.4, 7.5 mM MgCl₂, and 1 mM dithiothreitol) plus 0.1 μg/ml of bovine serum albumin. For in vitro histone H1 kinase assay, the anti-cyclin B1 immunoprecipitates were resuspended in 40 μl of kinase buffer plus 30 μg ATP, 50 μCi [γ-32P]-ATP (7000 Ci/mmol, Amersham), and 5 μg of histone H1 (Roche, Indianapolis, IN, USA). The kinase reactions were performed by shaking the mixture at room temperature for 30 min. After separation by 10% SDS-PAGE, proteins were electrotransferred onto polyvinylidene difluoride membranes. The radioactive histone H1 bands were autoradiographed and quantitated by a Phosphomager densitometer (Molecular Dynamics Inc., Sunnyvale, CA, USA).

**Flow cytometric analysis of DNA content**

Cellular DNA content was determined for cell-cycle distribution analysis by flow cytometry as described [29]. After various time periods of RC-RNase treatment, cells were trypsinized and fixed in 75% ethanol at -20°C for at least 30 min. After centrifugation at 140 x g for 5 min at 4°C, cell pellets were suspended in 1 ml of 0.5% Triton X-100 and incubated at room temperature for 5 min to permeabilize the cells, and then mixed with 1 ml of 60 μg/ml PI plus 0.5% (w/v) of RNase A. Ten min later, DNA contents of cell samples were analyzed by the FACStar flow cytometer (BD Bioscience) with an argon laser tuned to the 488-nm line for excitation.

**Assay of caspase activity**

RC-RNase-treated HeLa S3 cells were harvested by plastic scraper, washed twice with PBS and suspended in lysis buffer consisting of 25 mM HEPES, pH 7.5, 5 mM MgCl₂, 5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin-A, and 10 μg/ml leupeptin. After three freeze-thaw cycles, the cell lysates were centrifuged at 15,000 x g for 20 min at 4°C and the supernatants were collected for determination of caspase activities. In brief, each reaction was conducted in a 100-μl mixture including 80 μg of cell lysate, 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol, and 300 μM chromogenic substrate Ac-DEVD-pNA, Ac-IETD-pNA or Ac-LEHD-pNA (Upstate Biotechnology Inc., Lake Placid, NY, USA). The reaction mixtures were incubated at 37°C, and the levels of cleaved p-nitroaniline were measured at 405 nm by a microtiter plate reader (mQuant, Bio-Tek Instrument Inc., VT, USA).

**Results**

**Inhibitory effect of RC-RNase on HeLa S3 cell proliferation**

To study the effect of RC-RNase on the proliferation of HeLaS3 cells, MTT assay was first performed to determine the effects of RC-RNase and bovine pancreatic RNase A on cell viability. HeLa S3 cells were treated with different concentration of RC-RNase or RNase A, ranging from 10 nM to 100 μM, for 72 h. As shown in Figure 1A, only RC-RNase, but not RNase A (even 100 μM), inhibited the viability of HeLa S3 cells. The IC₅₀ of RC-RNase was determined to be around 10 μM. On the other hand, the effects of RC-RNase and RNase A on HeLa S3 cell growth curve were determined...
by trypan blue exclusion assay. Our data showed no difference during first 24 h with 10 μM RC-RNase or RNase A treatment, but thenafter, the growth curve of RC-RNase-treated HeLa S3 cells became declined (Figure 1B).

**Apoptosis induced by RC-RNase in HeLa S3 cells**

Apoptosis is an important pattern of cell death induced by cancer chemotherapeutic agents. To determine whether or not the cell proliferation-inhibitory effect of RC-RNase was attributed by apoptotic induction, morphological observation was conducted. The results were that the apoptotic morphology appeared in HeLa S3 cells after exposure to RC-RNase but not RNase A (Figure 2A-2D). More cells became blebbled and died with increasing concentration of RC-RNase, but no such observation made in the control and RNase A-treated cells. In most cell types, fragmentation of nuclear DNA into internucleosomal size fragments is the biochemical hallmark of apoptosis. Cellular DNA was then extracted from 48-h samples treated with different concentrations of RC-RNase or RNase A and analyzed by agarose gel electrophoresis. Our data indicated that RC-RNase was also more efficient than bovine RNase A to induce apoptotic DNA

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**Figure 2: Apoptosis induced by RC-RNase in HeLa S3 cells.** Nuclear morphology of control cells (A) and the cells treated 48 h with 10 μM RC-RNase (B) was observed after staining with 4,6-diamidino-2-phenylindole (DAPI). Transmission electron microscopy was performed to demonstrate the blebbling morphology and chromatin condensation appeared in 10 μM RC-RNase-treated cells (D) but not control cells (C). Internucleosomal DNA fragmentation was visualized after 1.2% agarose gel electrophoresis and ethidium bromide staining (E). Lane M, 100-bp DNA marker. Lane C, control cell. Lanes A1-A3, cells treated 48 h with 0.5, 2 and 10 μM of RNase A, respectively. Lanes R1-R3, cells treated 48 h with 0.5, 2 and 10 μM of RC-RNase, respectively. For quantitative measurement of the apoptotic cell ratio, logarithmically growing HeLa S3 cells were treated with 0.5, 2 or 10 μM of RC-RNase or RNase A for 48 h and then subjected to annexin V-FITC/PI double-staining flow cytometric analysis (F). The lower right population at each data represents the cells undergoing apoptosis.
Figure 3: Activation of caspase-3 induced by RC-RNase. (A) Caspase-3 activities assayed in 100 μg of cell lysates from HeLa S3 cells treated 48 h with 10 μM RC-RNase or RNase A. It was noted that only RC-RNase was able to induce caspase-3 activation. The data represent mean ± SD of three independent experiments. (B) Caspase-3 activities assayed in 100 μg of cell lysates from HeLa S3 cells treated with 10 μM RC-RNase for 12, 24, 48, or 72 h. It was noted that caspase-3 was activated from 2.5 to 8.3 folds and the activity could be inhibited by the caspase-3 inhibitor DEVD-CHO (R & D Systems). The data represent mean ± SD of three independent experiments.

Figure 4: Activation of cyclin B1/CDC2 induced by RC-RNase. (A) Assay of cyclin B1-associated CDC2 kinase activity in HeLa S3 cells treated with 10 μM RC-RNase for 24, 36, or 48 h. **[3H]**-labeled histone H1 bands were detected and quantitated by the Phospholmager densitometer (Molecular Dynamics). The mean of band intensities from two independent experiments is shown just below the representative band image. C, control cells. RC, RC-RNase-treated cells. A, adherent cells. F, floating (non-adherent) cells. (B-E) Western blot analyses of the cyclin B1 (B), total CDC2 (C), Tyr-15-phosphorylated CDC2 (D), and Wee-1 (E) levels in HeLa S3 cells treated with 10 μM RC-RNase for 24, 36, or 48 h. C, control cells. RC, RC-RNase-treated cells. A, adherent cells. F, floating (non-adherent) cells. The representative results from three independent experiments were shown.

fragmentation. The pattern of DNA ladders could be detected from the cells treated 48 h with 2 μM or 10 μM of RC-RNase but not with bovine RNase A (Figure 2E). A similar result was also observed from the cells treated with 2 μM of RC-RNase for 24 to 96 h with a highest fragmentation at 72 h (data not shown). The annexin V-FITC/PI double-staining flow cytometry was then performed to quantitate the extent of apoptosis. As shown in Figure 2F, the apoptotic cell fraction of HeLa S3 was significantly increased by 7.3% and 13% after incubation with 2 μM and 10 μM of RC-RNase for 48 h, respectively. For comparison, no significant change was seen in 10 μM RNase A-treated HeLa S3 cells. A drastic increase of apoptotic cells from 5.3% to 54% was observed in HeLa S3 cells when treated with 2 μM RC-RNase from 24 h to 96 h (data not shown). Take together, RC-RNase was indeed able to induce apoptosis in HeLa S3 cells.

Caspase-3 activity induced by RC-RNase

Next, we assayed the caspase-3 activity to investigate if it was increased during RC-RNase-induced apoptosis in HeLa S3 cells. HeLa S3 cells were treated with 10 μM RC-RNase or RNase A for 48 h, and cell lysates were prepared for determination of caspase-3 activity. The relative activities were shown in Figure 3A. The caspase-3 activity was increased 5 folds in RC-RNase-treated HeLa S3 cells compared with control or RNase A-treated cells. A time-course induction of caspase-3 activity by RC-RNase was shown in Figure 3B. Caspase-3 activity could be induced from 2.5 (at 12 h) to 8.3 (at 72 h) folds. The increase of caspase-3 activity was validated because its inhibition by the specific caspase-3 inhibitor, DEVD-CHO.

Cyclin B1/CDC2 activity induced by RC-RNase

Some morphological characteristics, such as chromatin condensation and nuclear envelope disassembly, are commonly shared by apoptotic cells and mitotic (M-phase) cells. Cyclin B1/CDC2 is an important M-phase regulatory protein complex. Abnormal activation of cyclin B/CDC2 is associated with the induction of apoptosis by several stimuli including chemotherapeutic agents and γ-radiation. We therefore investigated the cyclin B1/CDC2 kinase activity in RC-RNase-treated cells. HeLa S3 cells were treated with 10 μM RC-RNase for 24, 36, or 48 h. Cellular cyclin B1/CDC2 activity was found to be increased in the floating cell frac-
Figure 5: Activation of caspases-8 and 9 induced by RC-RNase. (A-C) Assay of the activities of caspase-3 (A), caspase-8 (B) and caspase-9 (C) from the lysates extracted from HeLa S3 cells treated with 10 μM RC-RNase for 24, 48 or 72 h. The data represent mean ± SD of three independent experiments. It was noted that cellular activities of caspases-3, 8, and 9 were all significantly increased after RC-RNase treatments (P < 0.05, Student’s t test). (D) Western blot analyses of the levels of cleaved caspase-8 (p18, active form), tBid and β-tubulin in HeLa S3 cells treated with 10 μM RC-RNase for 24 or 48 h. C, control cells. RC, RC-RNase-treated cells. (E) Western blot analyses of the active forms of caspase-9 and caspase-3 in HeLa S3 cells treated with 10 μM RC-RNase for 24 or 48 h. The level of β-tubulin was used as the internal control.

Table 1: DNA contents of the HeLa S3 cells treated with 10 μM RC-RNase for 24 or 48 h

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<td>RC-RNase</td>
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<td>48 h</td>
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The result indicated that the distribution of the cell-cycle stages of HeLa S3 cells was not significantly affected by RC-RNase within a 48-h treatment period (Table 1).

Caspase-8 and caspase-9 activities induced by RC-RNase

Besides caspase-3, we investigated if RC-RNase induced the activities of caspases-8 and 9, the upstream caspases of caspase-3. The equal amount of cell lysates, extracted from HeLa S3 cells treated with 10 μM RC-RNase for 24, 48 or 60 h, were incubated with different substrates (Ac-DEVD-pNA for caspase-3, Ac-IETD-pNA for caspase-8, and Ac-LEHD-pNA for caspase-9). As shown in Figure 5A-5C, the activities of...
apoptotic pathway. Caspase-9 was thus activated and subsequently caspase-3 was also activated. However, the induction of caspase-3 and caspase-9 activities was not completely prevented by the presence of caspase-8 inhibitor (data not shown). We did not rule out the possibility that RC-RNase also exerted a straightforward effect on mitochondria and caspase-3 activation.

Although there was no predominant arresting at any phase of cell cycle induced by RC-RNase, the in vitro kinase activity assay and Western blot analyses showed that the mitotic kinase cyclin B1/CDC2 activity was significantly elevated in RC-RNase-induced apoptotic cell fractions (Figure 4). Induction of apoptosis by several stimuli including anticancer compounds [23-26], Fas-L [30], and γ-radiation [31] has been shown to require activation of cyclin B/CDC2. The mechanism for RC-RNase-induced cyclin B1/CDC2 activation could be due to the cleavage and depletion of Wee-1 by RC-RNase-induced caspase-3. Once the inhibitor kinase Wee-1 was lacking, cyclin B1/CDC2 was abnormally activated and furthermore induced apoptotic characteristics such as chromatin condensation and nuclear envelope disassembly. It has been reported that Wee-1 is associated with premature mitosis and apoptosis [32]. Expression of CDC2AF mutant, a variant that cannot be phosphorylated by Wee-1 and Myt-1, results in premature mitosis and apoptosis. Similarly, dysfunction of Wee-1 results in inappropriate activation of CDC2 kinase and lead to mitotic catastrophe, an aberrant mitotic process that resembles apoptosis [32]. In addition, depletion of Wee-1 is necessary for both human immunodeficiency virus type 1 Vpr- and γ irradiation-induced apoptosis [33]. Wee-1 has been reported to be a target for caspase-3 cleavage when Fas-L induces apoptosis in human Jurkat cells [31]. Our results provide evidence that in human HeLa S3 cells the level of Wee-1 is associated with RC-RNase-induced cyclin B1/CDC2 activity and apoptosis.

Like Onconase, RC-RNase preferentially kills actively growing cancer cells [12,34]. The cytotoxic effect of Onconase has been reported through degradation of specific mRNA and activation of caspases, most likely along the mitochondrial pathway involving caspase-9 as the initiator caspase [35,36]. Although RC-RNase has been reported to induce cell death through activating caspase-7 activity in human breast cancer MCF-7 cells and caspase-9 in human leukemia HL-60 cells, there were no significant apoptotic features in these dead cells [17,37]. It has been proposed that the exogenous RNases entered cells through a receptor-mediated pathway that might activate the activity of caspase-8. Signal transduction from ligand-ligated Fas and TNFR1 to activation of caspase-8 requires the important mediators FADD and TRADD, respectively. We detected the levels of Fas, FADD, TNFR1, and TRADD in RC-RNase-treated HeLa S3 cells by Western blot analyses. The data indicated that RC-RNase was efficient to induce FADD expression, but in contrast, TRADD was decreased by RC-RNase treatment (Figure 6). The cellular levels of Fas and TNFR1 were not obviously altered by RC-RNase treatment within 24 h and were only slightly reduced at 48 h (Figure 6).

Discussion

In this study, we demonstrated that RC-RNase, a novel member of RNase A superfamily, was able to induce the death of human cervical cancer cells through the apoptotic pathways. The RC-RNase-treated cells presented the typical features of apoptosis such as cell blebbing, DNA fragmentation, membrane conversion, and PARP cleavage (Figures 2 and 3). RC-RNase was potent to induce a series of caspase cascades that rendered cancer cells destined to apoptosis (Figures 2 and 3). Our study suggests that through unknown mechanism, RC-RNase was able to induce FADD expression and in turn the caspase-8 activation (Figure 7). Active caspase-8 cleaved Bid, and the produced dBid translocated onto mitochondria to elicit a further mitochondrion-mediated
RC-RNase treatment was observed to efficiently induce FADD expression (Figure 6). It has been known that increased FADD expression is associated with apoptosis mainly via the caspase-8 pathway [40-42]. The mechanism for RC-RNase-induced FADD is still unknown. Recently, microRNAs (miRNAs), endogenous ~21 nucleotide small non-coding RNAs, have shown efficacies to regulate protein-coding gene expression [43]. Because RC-RNase is able to degrade some tRNAs, it remains the possibility that RC-RNase can perturb cellular proteome by targeting and degrading some miRNAs. In several human cancers, specific sets of miRNAs have been identified as new players of cancer development and progression [44-47]. More mechanistic studies on RC-RNase, Onconase and other related RNases will provide implications and aids for future development of cancer target therapy.

In conclusion, RC-RNase is potent to induce FADD expression and a series of caspase cascades and ultimately apoptosis in human cervical cancer HeLa S3 cells.

References


