Synergism of *Rana catesbeiana* ribonuclease and IFN-γ triggers distinct death machineries in different human cancer cells

Chih-Hang Anthony Tang[a,b,1], Chih-Chi Andrew Hu[c,1], Chyou-Wei Wei[a,d,1], Jaang-Jiun Wang[b,e,*]

[a] *Neuromedical Scientific Center, Buddhist Tzu-Chi General Hospital, Hualien 970, Taiwan*
[b] *Institute of Biology and Anatomy, National Defense Medical Center, Taipei 114, Taiwan*
[c] *Department of Pharmacology, New York University School of Medicine, New York, NY 10016, USA*
[d] *Tzu-Chi College of Technology, Hualien 970, Taiwan*
[e] *The College of Medicine, Fu Jen Catholic University, 510 Chung-Cheng Rd., Hsin-Chuang, Taipei Hsien 24205, Taiwan*

Abstract *Rana catesbeiana* ribonuclease (RC-RNase) possesses tumor-specific cytotoxicity, which can be synergized by IFN-γ. However, it is unclear how RC-RNase and RC-RNase/IFN-γ induce cell death. In this study, we use substrate cleavage assays to systematically investigate RC-RNase- and RC-RNase/IFN-γ-induced caspase activation in HL-60, MCF-7, and SK-Hep-1 cells. We find that RC-RNase and RC-RNase/IFN-γ induce mitochondria-mediated caspase activation in HL-60 and MCF-7 cells but not in SK-Hep-1 cells, although death of SK-Hep-1 cells is closely related to mitochondrial disruptions. Our findings provide evidence that RC-RNase and RC-RNase/IFN-γ can kill different cancer cells by distinct mechanisms. Compared with onconase, RC-RNase seems to harbor a more specific anti-cancer activity.
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1. Introduction

Rana catesbeiana ribonuclease (RC-RNase) derived from oocytes of *R. catesbeiana* and onconase from those of *Rana pipiens* share ~50% amino acid sequence identity and contain an identical lectin domain that may be responsible for the tumoricidal effect of the two ribonucleases [1,2]. Cell death machineries initiated by these frog oocyte-derived ribonucleases still remain unclear.

Cellular proteases named caspases are known to mediate apoptotic cell death and two autonomous but cross-talking caspase activation pathways have been identified [3–5]. For receptor-mediated apoptosis, the initiator caspase-8/executioner caspase-3 pathway is activated via binding of ligands onto the cell surface death receptors (e.g., FasL/Fas). For mitochondria-mediated apoptosis, the initiator caspase-9/executioner caspase-3 pathway is activated in response to homeostatic alterations of mitochondria. As a close relative to caspase-3, caspase-7 can also be activated by caspase-8 or caspase-9 to be an executioner protease. Both caspase-3 and -7 can lead to the proteolytic cleavage of other caspases (e.g., caspase-2 and -6) and numerous cellular substrates like poly(ADP-ribose) polymerase (PARP) [3].

We have previously reported that caspase-7 is activated in the RC-RNase-treated caspase-3-deficient MCF-7 cells, while there is no activation of its upstream caspase-9 or caspase-8; that caspase-7 can in succession process procaspase-2, procaspase-6 and PARP; and that Bcl-X_L, an anti-apoptotic mitochondrial protein, can efficiently rescue MCF-7 cells from RC-RNase-induced cell death [6]. We also found that the caspase-9/caspase-3 pathway is activated in RC-RNase-treated undifferentiated HL-60 cells, suggesting that RC-RNase-induced caspase activation is initiated from mitochondria [7].

RC-RNase and IFN-γ were found to have synergistic tumoricidal effect in three hepatoma cell lines bearing different differentiation stages, and such an effect was shown to be especially prominent in poorly differentiated SK-Hep-1 cells [8]. Although RC-RNase/IFN-γ treatment had caused a massive death in SK-Hep-1 cells, there was neither apoptotic nor necrotic feature observed in these cells [8]. Together with evidence that mitochondrial disruptions were observed in HeLa cells treated with RC-RNase (unpublished data) and in SK-Hep-1 cells treated with RC-RNase/IFN-γ [8], we suggested that RC-RNase-induced cell death might be initiated from mitochondrial disruptions.

In this present study, we used a more sensitive method to systematically investigate RC-RNase- and RC-RNase/IFN-γ-induced caspase activation in HL-60, MCF-7, and SK-Hep-1 cells. We showed that RC-RNase- or RC-RNase/IFN-γ-induced caspase activation could vary in different cell types, suggesting that distinct death machineries were triggered. We also showed that the RC-RNase/IFN-γ regimen could be much more specific in killing tumor cells than the onconase/IFN-γ regimen.

2. Materials and methods

2.1. Reagents and cell culture

RC-RNase was purified using the modified methods published previously [6,9]. Caspase-3, caspase-7, PARP, and actin monoclonal antibodies were purchased, respectively, from Imgenex, Oncogene,

*Corresponding author. Fax: +886 2 8792 1375.
E-mail address: jaangjwang@hotmail.com (J.-J. Wang).

[1] These authors contributed equally to this work.

Pharmingen, and Chemicon. DMSO was purchased from Merck. Ac-LEHD-pNA (acetyl–Leu–Glu–His–Asp–*p*-nitroanilide), Ac-DE-VD-pNA (acetyl-Asp–Glu–Val–Asp–*p*-nitroanilide), and Ac-IETD-pNA (acetyl-Ile–Glu–Thr–Asp–*p*-nitroanilide) were purchased from Anaspec. Sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) assay kits were purchased from Roche. Human HL-60 promyelocytic leukemia cells, human MCF-7 breast carcinoma cells, and human SK-Hep-1 hepatoma cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 2 mM L-glutamine (Gibco), 100 IU/ml penicillin G sodium (Gibco), 100 μg/ml streptomycin sulfate (Gibco), 1 mM sodium pyruvate (Sigma), and 0.1 mM non-essential amino acids (Gibco).

2.2. Cell viability assay

Cell viability was evaluated by using XTT assays that detect activity of mitochondrial dehydrogenases. Briefly, 2×10^3 cells were grown in each well of 96-well cell culture plates overnight. On the next day, cells were treated with RC-RNase (20 μg/ml), onconase (20 μg/ml), IFN-γ (10 ng/ml), or the combined treatments. XTT assays were performed every 24th hour according to the manufacturer's instructions. Absorbance at 492 nm was determined by a multi-well ELISA reader (Molecular Devices).

2.3. Caspase activity assay

Cells were resuspended in the lysis buffer (50 mM Tris–HCl, 120 mM NaCl, 1 mM EDTA, and 1% NP-40, pH 7.5) supplemented with protease inhibitors. Insoluble pellets were removed by centrifugation using $15000 \times g$ at 4 °C for 20 min. The caspase activity assay was performed in a reaction containing 40 μl cell lysates (80 μg total protein), 158 μl reaction buffer (20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol, and 100 mM HEPES, pH 7.5), and 2 μl fluorogenic Ac-LEHD-pNA, Ac-DEVD-pNA, or Ac-IETD-pNA substrates (100 μM final concentration); the reaction was incubated at 37 °C for 6 h (in this condition, all substrates were not used up and the caspase activity could be compared in the linear range). The fluorogenic substrate cleavage readout was the p-nitroanilide release as detected at 405 nm in an ultra-microplate reader (Bio-Tek instruments). Fold increase in caspase activity was calculated by comparing the A405 readout from the induced sample with that from the un-induced control using the following formula: $(A_{405\mathrm{sample}} - A_{405\mathrm{control}})/A_{405\mathrm{control}}$.

2.4. Immunoblot analysis

Cells were harvested by cell scrapers and lysed in RIPA buffer (10 mM Tris–base, pH 7.4; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; and 0.1% SDS) containing protease inhibitors (Calbiochem). Equal amount of the total proteins was loaded onto a SDS–PAGE (13% acrylamide) gel, electrophoresed, and transferred to polyvinylidene difluoride membrane (Amersham). Membranes were blocked with 5% skimmed milk and 1% NP-40 in TBS-T (0.8% NaCl; 0.02% KCl; 25 mM Tris–HCl; and 0.05% Tween 20, pH 7.4) for 2 h, incubated with the primary antibody (1:500 dilution in the blocking buffer) at 4 °C overnight, and subsequently incubated with biotinylated anti-mouse IgG (1:2000 dilution in the blocking buffer) and streptavidin–horseradish peroxidase conjugates (1:4000 dilution in TBS). The membranes were developed using the Super Signal™ Chemiluminescent-HRP substrate system (Pierce) for protein visualization.

3. Results

3.1. IFN-γ enhanced RC-RNase-induced cell death in SK-Hep-1 cells and MCF-7 cells but not in HL-60 cells

We treated HL-60, MCF-7, or SK-Hep-1 cells with RC-RNase (20 μg/ml) and RC-RNase (20 μg/ml)/IFN-γ (10 ng/ml) for 5 days and assessed the cell viability by the XTT assay, which measured the activity of mitochondrial dehydrogenases. While IFN-γ alone showed little adverse effect on the three cancer cell lines, RC-RNase treatments were cytotoxic to these lines. Noticeably, RC-RNase per se could induce much more

devastating death to HL-60 cells (Fig. 1A) and MCF-7 cells (Fig. 1B) than to SK-Hep-1 cells (Fig. 1C), suggesting that it exerted distinct potencies to different cancer cell lines. The combined treatments of RC-RNase (20 μg/ml) and IFN-γ (10 ng/ml) exerted a significant synergistic effect in MCF-7 and SK-Hep-1 cells (Fig. 1B and C), but not in HL-60 cells (Fig. 1A).

3.2. Caspase activation in HL-60, MCF-7, and SK-Hep-1 cells after RC-RNase or RC-RNase/IFN-γ treatments

We first treated the three cell lines with RC-RNase (20 μg/ml), IFN-γ (10 ng/ml), or RC-RNase (20 μg/ml)/IFN-γ (10 ng/ml) for 48 or 96 h and measured caspase activity using fluorogenic substrate cleavage assay. We measured the caspase-9, caspase-3-like, and caspase-8 activities by incubating cell lysates, respectively, with fluorogenic substrates, Ac-LEHD-pNA, Ac-DEVD-pNA, or Ac-IETD-pNA.

In HL-60 cells, caspase-9, caspase-3-like, and caspase-8 activities were all detected after RC-RNase or RC-RNase/IFN-γ treatments (Fig. 2). Caspase-9 was fully activated in the first 48 h after treatments (Fig. 2A); however, caspase-3-
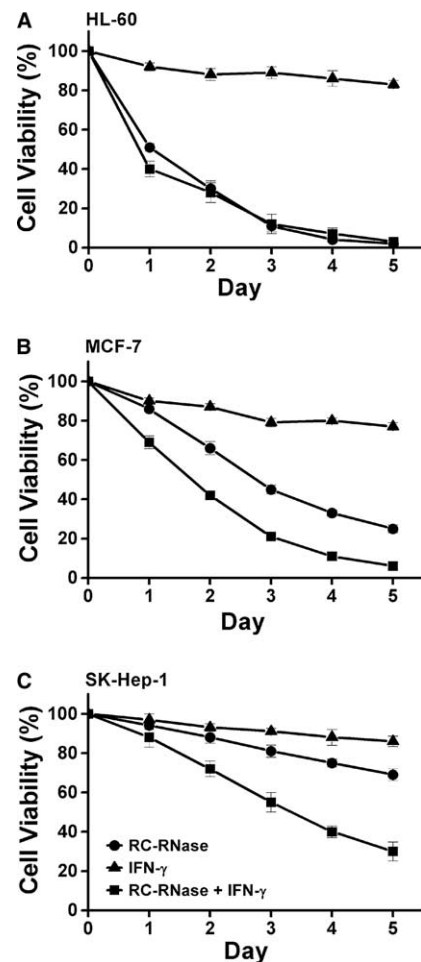

Fig. 1. The five-day survival rates of HL-60 (A), MCF-7 (B), and SK-Hep-1 (C) cells after RC-RNase (20 μg/ml), IFN-γ (10 ng/ml), and RC-RNase (20 μg/ml)/IFN-γ (10 ng/ml) treatments. Note that RC-RNase-induced cytotoxicity was enhanced by IFN-γ in MCF-7 cells and SK-Hep-1 cells, but not in HL-60 cells. Data were obtained from three independent triplicate experiments and presented as means ± S.D.
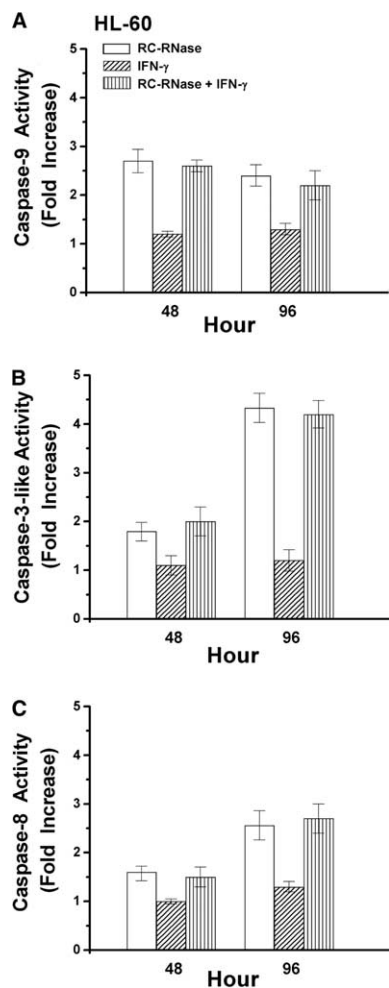
Fig. 2. Caspase activation in HL-60 cells after RC-RNase (20 μg/ml), IFN-γ (10 ng/ml), or RC-RNase (20 μg/ml)/IFN-γ (10 ng/ml) treatments. Note that caspase-9 (A), caspase-3-like (B), and caspase-8 (C) activities significantly increased after RC-RNase or RC-RNase/IFN-γ treatments; that caspase-9 was found to have been fully activated in the first 48 h; and that IFN-γ did not aggravate caspase activation in HL-60 cells. The data are presented as meanss ± S.D. from three independent triplicate experiments.

Fig. 3. Caspase activation in MCF-7 cells after RC-RNase (20 μg/ml), IFN-γ (10 ng/ml), or RC-RNase (20 μg/ml)/IFN-γ (10 ng/ml) treatments. Note that caspase-3-like activity significantly increased after treatments with RC-RNase or RC-RNase/IFN-γ for 96 h, and that IFN-γ could aggravate caspase-3-like activity (B). Caspase-9 (A) or caspase-8 (C) was not activated by any of the treatments. The data are presented as means ± S.D. from three independent triplicate experiments.

like and caspase-8 activities were found to increase in a timely dependent fashion, in which the 96-h treatment induced a more significant activation of the two caspases than the 48-h treatment (Fig. 2B and C). The earlier activation of caspase-9 suggested that RC-RNase-induced death of HL-60 cells should be initiated from mitochondrial alterations. Interestingly, IFN-γ showed no effect in enhancing the RC-RNase-induced caspase activation in HL-60 cells.

While caspase-9 or caspase-8 activity did not increase in MCF-7 cells after RC-RNase or RC-RNase/IFN-γ treatments (Fig. 3A and C), caspase-3-like activity increased significantly after both treatments for 96 h (Fig. 3B). Notably, cells treated with RC-RNase/IFN-γ had much higher caspase-3-like activity than those treated with RC-RNase alone (Fig. 3B), suggesting that IFN-γ could aggravate RC-RNase-induced death via enhancing caspase-3-like activity in these cells. Since MCF-7 cells are caspase-3-deficient, the caspase-3-like activity detected in this cell type should represent caspase-7 activation.
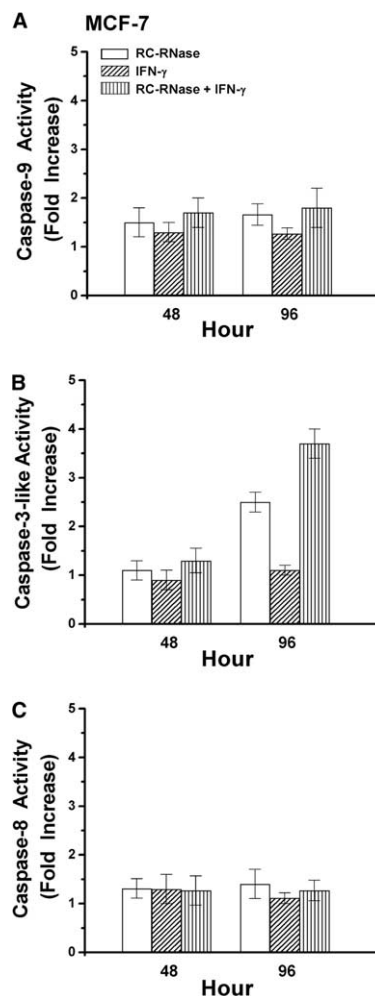
Previously, RC-RNase- or RC-RNase/IFN-γ was shown to induce death of SK-Hep-1 cells, which had atypical apoptotic and necrotic features [8]. Although RC-RNase/IFN-γ had been shown to cause severe mitochondrial disruptions in SK-Hep-1 cells [8], both RC-RNase and RC-RNase/IFN-γ could not induce changes of caspase-9 (Fig. 4A), caspase-3-like (Fig. 4B), and caspase-8 (Fig. 4C) activities in SK-Hep-1 cells after 48-h or 96-h treatments, indicating that such a death mechanism has little to do with caspase activation.

3.3. A distinct executioner caspase was activated in different cell types after RC-RNase and RC-RNase/IFN-γ treatments
In HL-60 cells that express both caspases-3 and caspase-7, only caspase-3 was activated by RC-RNase (data not shown; [7]) and RC-RNase/IFN-γ (Fig. 5, lanes 1–3) treatments, indicating that the increased caspase-3-like activity in RC-RNase- and RC-RNase/IFN-γ-treated HL-60 cells was mainly due to caspase-3 activation (Fig. 2B). In the
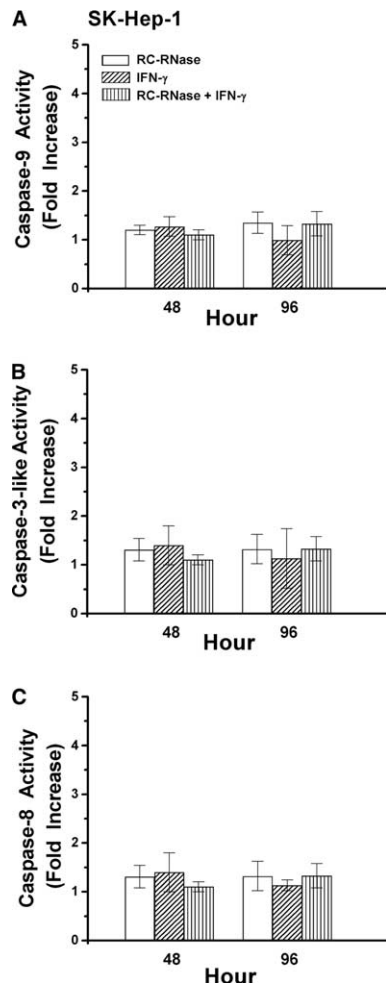
Fig. 4. Lack of caspase activation in SK-Hep-1 cells after RC-RNase (20 μg/ml), IFN-γ (10 ng/ml), or RC-RNase (20 μg/ml)/IFN-γ (10 ng/ml) treatments. Note that none of the three treatments significantly altered caspase-9 (A), caspase-3-like (B), and caspase-8 (C) activities. The data are presented as means ± S.D. from three independent triplicate experiments.
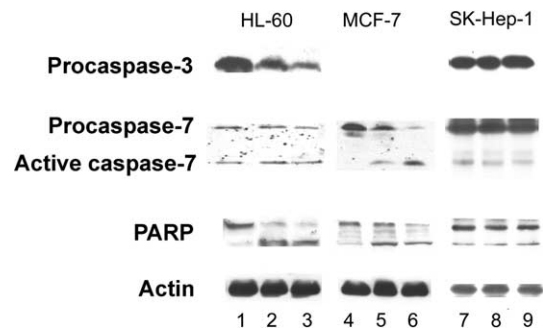

Fig. 5. Cleavage of executioner caspases as a response to the RC-RNase/IFN-γ treatment. HL-60 cells (lanes 1–3), MCF-7 cells (lanes 4–6), and SK-Hep-1 cells (lanes 7–9) were treated with RC-RNase (20 μg/ml)/IFN-γ (10 ng/ml) for 0 h (lanes 1, 4, and 7), 48 h (lanes 2, 5, and 8), and 96 h (lanes 3, 6, and 9); lysed in RIPA buffer; and immunoblotted using antibodies to indicated molecules. Note that the 32-kDa procaspase-3 but not the 35-kDa procaspase-7 was cleaved in HL-60 cells; that the 35-kDa procaspase-7 was cleaved to generate the 20-kDa active caspase-7 in caspase-3-deficient MCF-7 cells; that both procaspase-3 and procaspase-7 were not cleaved in SK-Hep-1 cells; and that the 116-kDa PARP was processed into an 89-kDa fragment in HL-60 and MCF-7 cells, but not in SK-Hep-1 cells. Detection of actin served as the protein loading control.
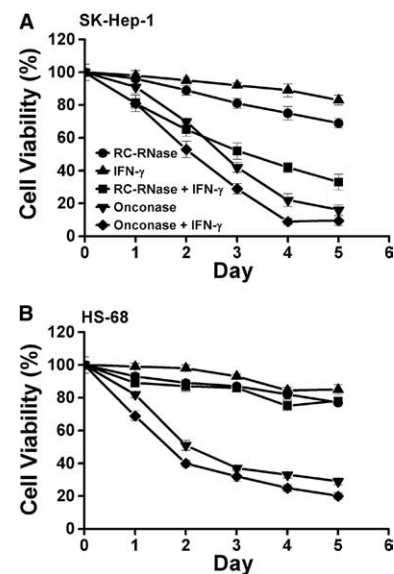

Fig. 6. Specific tumoricidal activity of RC-RNase. The five-day survival rates of SK-Hep-1 cells (A) and HS-68 cells (B) were obtained after treatments with RC-RNase (20 μg/ml), onconase (20 μg/ml), IFN-γ (10 ng/ml), or the indicated combinations. Note that RC-RNase alone had a milder tumoricidal activity to SK-Hep-1 cells when compared with onconase. However, the tumoricidal activity of RC-RNase could be enhanced by IFN-γ synergistically. Also note that RC-RNase and RC-RNase/IFN-γ treatments did not affect the primary HS-68 fibroblasts, while onconase and onconase/IFN-γ treatments did. Data were obtained from three quadruple groups and presented as means ± S.D.

caspase-3-null MCF-7 cells, caspase-7 was activated in response to RC-RNase (data not shown; [6]) and RC-RNase/IFN-γ (Fig. 5, lanes 4–6) treatments; this suggested that the increased caspase-3-like activity in RC-RNase- and RC-RNase/IFN-γ-treated MCF-7 cells was due to caspase-7 activation (Fig. 3B). In SK-Hep-1 cells that express caspase-3 and caspase-7, both caspases were not activated after RC-RNase or RC-RNase/IFN-γ treatments (data not shown; Fig. 5, lanes 7–9). Such results confirmed our data showing no increased caspase-3-like activity in RC-RNase- and RC-RNase/IFN-γ-treated SK-Hep-1 cells (Fig. 4B). In addition, while PARP could be cleaved by caspase-3 in RC-RNase- or RC-RNase/IFN-γ-treated HL-60 cells ([7]; Fig. 5, lanes 1–3) and by caspase-7 in RC-RNase- or RC-RNase/IFN-γ-treated MCF-7 cells ([6]; Fig. 5, lanes 4–6), it was not cleaved in RC-RNase- or RC-RNase/IFN-γ-treated SK-Hep-1 cells (data not shown; Fig. 5, lanes 7–9). This again suggested that the death mechanism activated by RC-RNase or RC-RNase/IFN-γ in SK-Hep-1 cells does not involve caspase activation.

3.4. Comparison between RC-RNase–induced and onconase-induced cytotoxicity in SK-Hep-1 hepatoma cells and primary human HS-68 foreskin fibroblasts

RC-RNase has been shown to be cytotoxic to various human cancer cell lines [6–9], but not to normal or immortalized cells, including human primary HFW fibroblasts [9], human

primary HS-68 foreskin fibroblasts (Fig. 6B), human primary WI-38 lung fibroblasts (data not shown), immortalized hamster kidney BHK-21 cells [8], and immortalized murine NIH-3T3/3 cells [9], suggesting that cytotoxicity of RC-RNase is cancer-specific. The cytotoxicity of RC-RNase can be synergized by IFN-γ in three different hepatoma cells [8] and MCF-7 cells (Fig. 1B). Such synergistic cytotoxicity is also specific to cancer cells, since RC-RNase/IFN-γ treatments do not induce death of primary HS-68 cells (Fig. 6B) and BHK-21 cells [8]. Onconase is known to have strong tumoricidal activity in many cancer cells [10–13], but it is shown here to be very toxic to primary HS-68 cells (Fig. 6B). Although onconase-induced cytotoxicity can be exacerbated by IFN-γ, such exacerbation is minor and can affect not only cancerous SK-Hep-1 cells but also primary HS-68 cells (Fig. 6A and B).

4. Discussion

In RC-RNase- or RC-RNase/IFN-γ-treated HL-60 cells, all three caspases examined are activated (Fig. 2). While caspase-9 activity increases to the maximal extent in the first 48 h and keeps almost consistent in the total 96-h examination period (Fig. 2A), both caspase-3 and caspase-8 activities keep increasing throughout the whole period, resulting in a much more prominent activity after 96-h treatments with RC-RNase or RC-RNase/IFN-γ (Fig. 2B and C). Together with the data showing that caspase-3 has a more prominent effect than caspase-8 after 96-h treatments (Fig. 2B and C) and previous data showing that caspase-3 is critical for caspase-8 activation in mitochondria-mediated apoptosis [14,15], we suggest that in RC-RNase- or RC-RNase/IFN-γ-treated HL-60 cells, caspase-9 is the first to be activated and the activated caspase-9 activates caspase-3, which can in turn activate caspase-8 to amplify death signals and ensure the occurrence of apoptosis. These results clearly show that RC-RNase or RC-RNase/IFN-γ induces caspase-dependent mitochondria-mediated apoptosis in HL-60 cells.

In RC-RNase- or RC-RNase/IFN-γ-treated MCF-7 cells, caspase-7 is activated without detectable activation of the initiator caspase-9 or -8 (Fig. 3) and caspase-7 is also found to process PARP in these treated MCF-7 cells (Fig. 5). It is known that caspase-7 can be activated not only by caspase-9 but also by procaspase-9 because the autocatalytic intra-chain cleavage of procaspase-9 (into capsase-9) has only mild effect on its catalytic activity [16,17] and the catalytic activity of procaspase-9 or caspase-9 is actually dependent on their association with the other two members, Apaf1 and cytochrome c, in the apoptosome [17,18]. Together with our data showing that RC-RNase or RC-RNase/IFN-γ triggers cell death by targeting mitochondria [8] and that Bcl-X$_L$ can reverse RC-RNase-induced death in MCF-7 cells [6], we suggest that RC-RNase- or RC-RNase/IFN-γ-treated MCF-7 cells may also undergo caspase-dependent mitochondria-mediated apoptosis.

Although RC-RNase alone or RC-RNase/IFN-γ can induce death of SK-Hep-1 cells, there is no increased caspase-9, caspase-3-like, or caspase-8 activity detected after treatments (Fig. 4). Such results were confirmed by our immunoblot data showing lack of processing of initiator procaspase-9 or -8 (data not shown), executioner procaspase-3 or -7 (Fig. 5), and PARP (Fig. 5) in RC-RNase/IFN-γ-treated SK-Hep-1 cells. Together with our previous electron microscopy data showing major

mitochondrial disruptions in RC-RNase/IFN-γ-treated SK-Hep-1 cells [8], we suggest that RC-RNase and RC-RNase/IFN-γ may induce death in SK-Hep-1 cells by activating a novel caspase-independent death pathway, which is closely related to homeostatic alterations of mitochondria.

Iordanov et al. [19] showed that the caspase-9/caspase-3 cascade was activated in onconase-treated HeLa tk⁻ cells although activation of this caspase cascade could only be observed when onconase was delivered using a lipofectin-facilitated method. Together with the data showing release of procaspase-9 from mitochondria upon onconase treatment, they suggested that onconase induced apoptosis via mitochondria-mediated caspase activation [19]. By using a pan-caspase inhibitor, Grabarek et al. [20] later showed that caspases were indeed activated in HL-60 cells undergoing onconase-induced apoptosis; however, the activated caspases were not further characterized. In this present study, we systematically investigated caspase activation in three different cell lines treated with RC-RNase, which like onconase is also a cytotoxic ribonuclease derived from frog oocytes. We found that RC-RNase could induce not only caspase-dependent mitochondria-mediated apoptosis (e.g., in HL-60 and MCF-7 cells) but also caspase-independent mitochondria-mediated apoptosis (e.g., in SK-Hep-1 cells). Since RC-RNase and onconase are closely related, it is very likely that both can induce death in different cancer cells using distinct mechanisms.

RC-RNase induces distinct degree of cytotoxicity in HL-60, MCF-7, and SK-Hep-1 cells, possibly because these cell lines have different differentiation stages. We have previously studied three different hepatoma cell lines bearing distinct differentiation stages and reported that RC-RNase-induced cytotoxicity is differentiation-dependent but not proliferation-dependent [8]. Our recent study also showed that induction of differentiation can rescue HL-60 cells from RC-RNase-induced cytotoxicity [7]. We therefore suggest that the different degree of RC-RNase-induced cytotoxicity in HL-60, MCF-7, and SK-Hep-1 cells might also depend on the intrinsic differentiation status of these three cell lines. If we can further show that RC-RNase-induced cytotoxicity is also differentiation-dependent in many other cell types, we think RC-RNase might be able to serve as a useful tool in defining the intrinsic differentiation status of cell lines derived from different tissues.

IFN-γ has been shown to synergize the RC-RNase-induced cytotoxicity; however, this synergistic cytotoxicity is not seen in the HL-60 cells (Fig. 1A). Since RC-RNase-induced cell death is differentiation-dependent [7,8] and IFN-γ is known to induce differentiation of HL-60 cells [21–23], we suggest the reason why there is no synergistic cytotoxicity of RC-RNase and IFN-γ in this cell type might be because the differentiation status of HL-60 cells has been altered by IFN-γ and such a change in the differentiation status protects these cells from the synergistic cytotoxicity.

RC-RNase and RC-RNase/IFN-γ have been shown to be non-toxic to a panel of normal or immortalized cells. Compared with onconase in this study, RC-RNase and RC-RNase/IFN-γ treatments clearly have more tumor-specific cytotoxicity (Fig. 6). While onconase has reached Phase III clinical trials in the US as an anti-cancer drug [24,25], we believe that RC-RNase can also be a promising drug for cancer therapy. Since different cytotoxicity and caspase activation have been observed in HL-60, MCF-7, and SK-Hep-1 cells

after RC-RNase and RC-RNase/IFN-γ treatments, the tumoricidal mechanisms of RC-RNase and RC-RNase/IFN-γ require deliberate investigation before we pursue their clinical applications.

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