Induction of differentiation rescues HL-60 cells from *Rana catesbeiana* ribonuclease-induced cell death

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Abstract ***Rana catesbeiana*** **ribonuclease (RC-RNase) exerted strong anti-tumor activity and its cytotoxicity was shown to correlate with differentiation stages of three different hepatoma cell lines. In this study, we demonstrate different RC-RNase cytotoxicity in undifferentiated HL-60 cells and in those that had been induced to differentiate by retinoic acid or dimethylsulfoxide. RC-RNase showed cytotoxicity in undifferentiated HL-60 cells, but not in HL-60 cells undergoing terminal differentiation. Furthermore, the caspase-9/caspase-3 pathway was activated when RC-RNase induced death in undifferentiated HL-60 cells and induction of differentiation led to a reversal of the caspase activation pathway.**

Key words: *Rana catesbeiana* ribonuclease; Differentiation; Caspase; HL-60 cell

1. Introduction

Ribonuclease from *Rana catesbeiana* (RC-RNase) and onconase from *Rana pipiens* are cytotoxic ribonucleases extracted from frog oocytes and have been proposed to be anti-tumor reagents [1–5]. In addition to having 48.2% amino acid sequence homology, both RC-RNase and onconase contain a lectin domain which may account for the tumoricidal activity [6]. Although cell death machineries initiated by the two frog oocyte-derived ribonucleases still remain unclear, onconase is undergoing phase III clinical trials in the USA as an anti-tumor drug [2,7].

Tumor-specific cytotoxicity of RC-RNase has been reported [8,9]. Using hepatocellular carcinoma cell lines with distinct differentiation stages, we established a rather simple correlation between RC-RNase-induced cytotoxicity and cell differentiation stage [9]. That is, RC-RNase-induced cytotoxicity correlates with differentiation given the evidence that RC-RNase has a strong tumoricidal activity on poorly differentiated hepatoma cells, a lower activity on intermediately differentiated ones, and a much lower activity on well-differentiated ones. RC-RNase is not cytotoxic to normal or immortalized fibroblasts which both represent well-differentiated cells. To further strengthen the previous correlation hypothesis, we wanted to conduct experiments in a cell system in which we could actively induce differentiation so that a clearer causal relationship between RC-RNase-induced cytotoxicity and cell differentiation could be demonstrated.

Differentiation of hematopoietic cells has been well studied. For instance, undifferentiated human promyelocytic leukemic HL-60 cells can be induced with several differentiation-inducing agents (e.g. dimethylsulfoxide (DMSO), retinoic acid, and actinomycin D) which stimulate terminal differentiation leading to the appearance of granulocyte-like characteristics as judged by morphological and immunocytochemical means [10–12]. This model has been used to investigate responses of differentiated and undifferentiated cells to cytotoxic agents for years. By adopting this well-established hematopoietic differentiation model, we aimed at establishing a causal relationship between RC-RNase-induced cytotoxicity and cell differentiation.

Initiation of the caspase-8/caspase-3 cascade is mediated by ligands binding to death receptors (e.g. FasL/Fas) while activation of the caspase-9/caspase-3 cascade is induced by homeostatic alterations in mitochondria [13–15]. Being highly homologous to caspase-3 in amino acid sequence, caspase-7 also acts as an executioner protease. Both caspase-3 and -7 can induce cleavage of other procaspases and cellular substrates such as poly(ADP-ribose) polymerase (PARP) [13]. In this study, we induced terminal differentiation in HL-60 cells with DMSO and retinoic acid and showed that differentiated HL-60 cells were much less susceptible to RC-RNase than their undifferentiated counterparts. We also found that RC-RNase can induce death in undifferentiated HL-60 cells by activating the caspase-9/caspase-3 cascade and induction of terminal differentiation can reverse caspase activation and rescue the cells from death.

2. Materials and methods

2.1. Chemicals, cell lines, and culture conditions

All-*trans*-retinoic acid (RA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and nitroblue tetrazolium (NBT) were purchased from Sigma. DMSO was bought from Merck. Caspase-3 monoclonal antibody and caspase-9 monoclonal antibody were procured from Transduction Laboratory and Upstate respectively. Actin monoclonal antibody was purchased from Chemicon. Caspase-7 monoclonal antibody and

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Abbreviations: RA, all-*trans*-retinoic acid; DMSO, dimethylsulfoxide; RC-RNase, *Rana catesbeiana* ribonuclease; PARP, poly(ADP-ribose) polymerase; NBT, nitroblue tetrazolium; TPA, 12-*O*-tetradecanoyl-13-phorbol acetate

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PARP monoclonal antibody were bought from Biomol Research Laboratories. Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) and acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (Ac-LEHD-pNA) were procured from Anaspec. Human promyelocytic leukemia HL-60 cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids.

2.2. Purification of RC-RNase

The methods of RC-RNase purification from yolk granules in oocytes of *R. catesbeiana* have been reported [5]. Briefly, the soluble fraction of crude yolk granule homogenate was obtained by centrifugation and made to contain HEPES and EDTA to allow dross precipitation. Using a phosphocellulose column and then a carboxymethylcellulose column, we purified RC-RNase. Ribonuclease activity was confirmed by in situ RNA digestion in an RNA-cast sodium dodecyl sulfate (SDS)-polyacrylamide gel and the dinucleotide CpG cleavage assay as we have described before [5].

2.3. Induction of differentiation

RA dissolved in 95% ethanol and DMSO were prepared. Cultured HL-60 cells were treated with 1 μM RA or 1.3% DMSO in growth medium for indicated days. All manipulations were performed under subdued light.

2.4. NBT reduction assay

HL-60 cells were incubated with an equal volume of 0.2% NBT containing 200 ng/ml TPA for 40 min at 37°C. We could identify differentiated cells by their intracellular blue formazan deposits. A minimum of 200 cells was counted under a light microscope for each experiment.

2.5. Preparation of differentiated HL-60 cells

HL-60 cells were induced to differentiate into mature granulocytes by RA or DMSO. The differentiation percentage could be determined by the NBT reduction assay. Differentiated cells were then cultured in fresh growth medium without RA or DMSO.

2.6. Cell viability assay

Cells were grown on 24-well cell culture plates overnight and then treated with RC-RNase (2 μg/ml or 20 μg/ml in culture medium). Every 24 h, trypan blue exclusion assays were performed and viable cells were counted.

2.7. Western blotting

Cell lysates were obtained in RIPA buffer supplemented with protease inhibitors (cocktails from Calbiochem). Proteins in cell lysates were run in a 13.3% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Blocked with 5% non-fat milk in TBS-T (0.8% NaCl, 0.02% KCl, 25 mM Tris–HCl, 0.05% Tween 20, pH 7.4), the membrane was reacted at 4°C with primary antibodies (1:500) overnight. Incubated with biotinylated anti-mouse IgG or anti-rabbit IgG antibodies (1:2000), the membrane was subsequently incubated with streptavidin–horseradish peroxidase conjugates (1:4000). Labeled proteins were visualized by the Super Signal™ Chemiluminescent-HRP substrate system (Pierce, Rockford, IL, USA).

2.8. Caspase activity assay

Cells were resuspended in lysis buffer (50 mM Tris–HCl, 120 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, pH 7.5) supplemented with protease inhibitors. Cell lysates were obtained after centrifugation (15 000 × *g*) for 20 min at 4°C. Caspase activity assays were performed as previously described [16]. In brief, the whole reaction contained 40 μl cell lysates (80 μg total protein), 158 μl reaction buffer (20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol, 100 mM HEPES, pH 7.5), and 2 μl fluorogenic Ac-DEVD-pNA or Ac-LEHD-pNA substrates (100 μM final concentration). Samples were incubated for 6 h at 37°C and enzyme-catalyzed release of *p*-nitroanilide was monitored at 405 nm in an ultra-microplate reader (Bio-Tek instruments).

3. Results

3.1. Successful induction of differentiation in HL-60 cells

The NBT reduction assay is a sensitive and easy method to distinguish cell differentiation. In the absence of inducers, undifferentiated HL-60 cells scarcely reduced NBT (Fig. 1A), but the differentiated HL-60 cells obtained from RA or DMSO inductions could reduce NBT and generate blue-black formazan (Fig. 1B,C). Formazan deposits were found in approximately 3–8% of undifferentiated HL-60 cells. After 7 days incubation with 1 μM RA, more than 90% HL-60 cells differentiated into mature granulocytes. There were approximately 80% HL-60 cells differentiating into mature granulocytes after 8 days induction with 1.3% DMSO (Fig. 2A). Interestingly, proliferation of HL-60 cells treated with RA or DMSO was found to be significantly inhibited from day 6 (Fig. 2B). These cells could no longer proliferate even when differentiation inducers were removed.

3.2. Differentiated HL-60 cells were very resistant to RC-RNase-induced cytotoxicity

Compared with undifferentiated cells (Fig. 3A), RA- or DMSO-induced differentiated cells were found to be much less responsive to RC-RNase (Fig. 3B,C). As we have also noted, RC-RNase-triggered cytotoxicity was concentration-dependent in undifferentiated HL-60 cells (Fig. 3A). However, the concentration-dependent relationships became much less significant in differentiated cells (Fig. 3B,C).

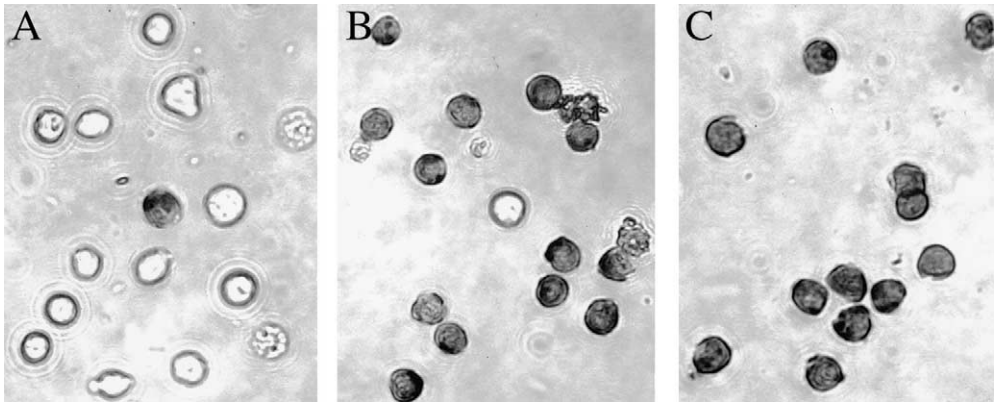

Fig. 1. Terminal differentiation of HL-60 cells. NBT reduction assays were performed in unstimulated cells (A), cells treated with 1.3% DMSO for 9 days (B), and cells treated with 1 μM RA for 7 days (C). Note NBT reduction-positive cells contain intracellular blue-black formazan deposits.

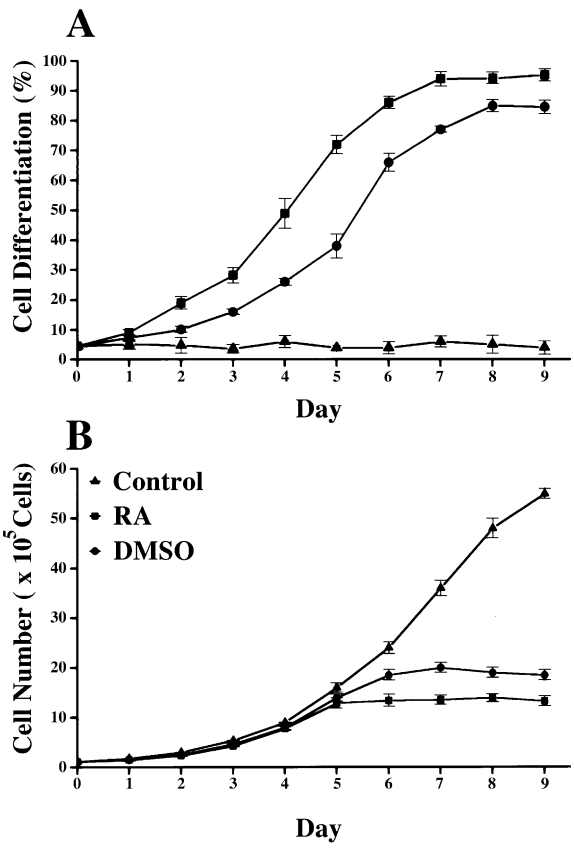

Fig. 2. Differentiation rate and growth curves of HL-60 cells after treatments. Differentiation rate after 9 days treatment with 1 μM RA or 1.3% DMSO (A). Growth curves plotted for cells treated with 1.3% DMSO or 1 μM RA (B). Each data point was obtained from three triplicate groups and expressed as mean ± S.D. Note growth arrest for cells treated with DMSO or RA was significant on day 6.

3.3. *Differentiation rather than proliferation rescued HL-60 cells from RC-RNase-induced cytotoxicity*

As we had observed in Fig. 2B, there was no proliferation difference between HL-60 cells treated with RA for 5 days and those treated with RA for 7 days. However, approximately 70% of RA-treated cells were identified as differentiated cells on day 5 and approximately 90% of RA-treated cells on day 7 (Fig. 2A). Comparing the results from the two groups of cells differentiated with RA for 5 days and 7 days, we found RC-RNase was less cytotoxic to cells differentiated by RA for 7 days (Fig. 4A). This indicated that differentiated cells became less susceptible to RC-RNase treatments. Control experiments in which the same two groups of differentiated cells were treated with H_2O_2 were conducted here to further demonstrate that differentiation-dependent cytotoxicity of RC-RNase was not a common event (Fig. 4B).

3.4. *RC-RNase-triggered caspase activation was modulated by differentiation in HL-60 cells*

After undifferentiated HL-60 cells were treated with RC-RNase (20 μg/ml), procaspase-9, procaspase-3, and PARP were cleaved time-dependently (Fig. 5A, lanes 1–3). The lowest procaspase-9 and procaspase-3 levels were seen after treatments with RC-RNase for 96 h. Cleavage of the intact PARP (116 kDa) into the 89 kDa fragment was also found to be the most evident after 96-h treatments with RC-RNase. No difference in caspase-7 activation was detectable even when proteolysis of PARP had been so severe in cells stimulated with RC-RNase for 96 h. Interestingly, in differentiated HL-60 cells obtained after 7 days induction with RA, cleavage of procaspase-3 and procaspase-9 was not detected after treatment with

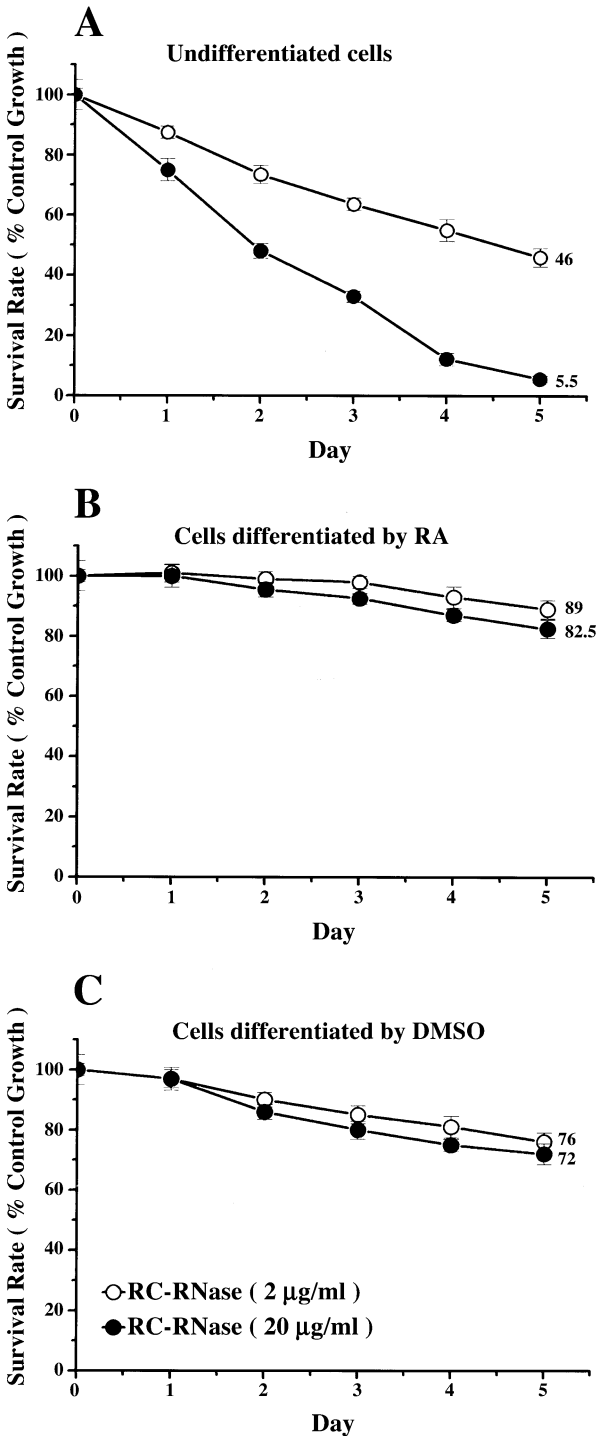

Fig. 3. The 5-day survival rates of undifferentiated and differentiated HL-60 cells. Differentiated cells were obtained after treatment with 1 μM RA for 7 days or with 1.3% DMSO for 9 days. Indicated concentrations of RC-RNase were used to stimulate undifferentiated cells (A) and differentiated cells from RA treatment (B) or DMSO treatment (C). Each data point was calculated from three quadruplicate groups and expressed as mean ± S.D. Note RC-RNase was much less cytotoxic to differentiated cells.

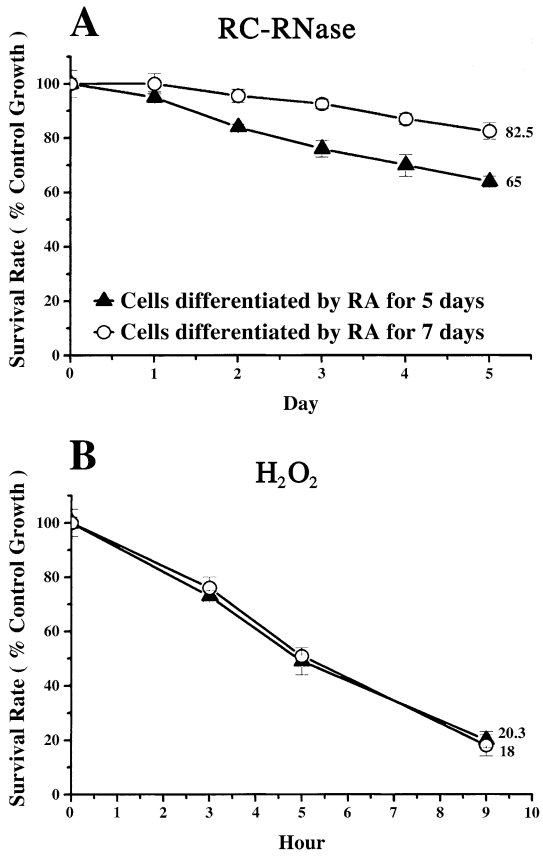

Fig. 4. Differentiation-dependent cytotoxicity of RC-RNase. HL-60 cells differentiated with RA for 5 days or 7 days were treated with RC-RNase (20 μg/ml). The 5-day survival rates for the two groups of cells were plotted from data points calculated from three quadruplicate experiments and shown as mean ± S.D. (A). Treating the same two groups of differentiated HL-60 cells with 50 μM H_2O_2 was used as control and the data points were also calculated from three quadruplicate experiments and expressed as mean ± S.D. (B). Note cytotoxicity triggered by H_2O_2 was not differentiation-dependent.

RC-RNase for 48 h or 96 h (Fig. 5A, lanes 4–6). PARP, known as the best characterized proteolytic substrate for caspases, also was hardly cleaved in these differentiated HL-60 cells after RC-RNase treatments. To further demonstrate that caspase-3 and caspase-9 were less activated in differentiated HL-60 cells after RC-RNase treatments, we adopted substrate cleavage assays in which cell lysates were incubated with fluorogenic Ac-DEVD-pNA or Ac-LEHD-pNA. There were much lower caspase-3 activity and much lower caspase-9 activity in differentiated HL-60 cells (from 7 days induction with RA) than in undifferentiated HL-60 cells no matter which RC-RNase treatment groups (48 h or 96 h) were compared (Fig. 5B,C).

4. Discussion

HL-60 cells provide a convenient system for studying differentiation. HL-60 cells can be induced to differentiate into macrophage-like, monocyte-like, or granulocyte-like characteristics by different inducing chemicals and different culture conditions [10–12,17]. In this study, we induced HL-60 cells to differentiate into mature granulocytes by using DMSO or RA. Compared with DMSO, RA had higher differentiation-induc-

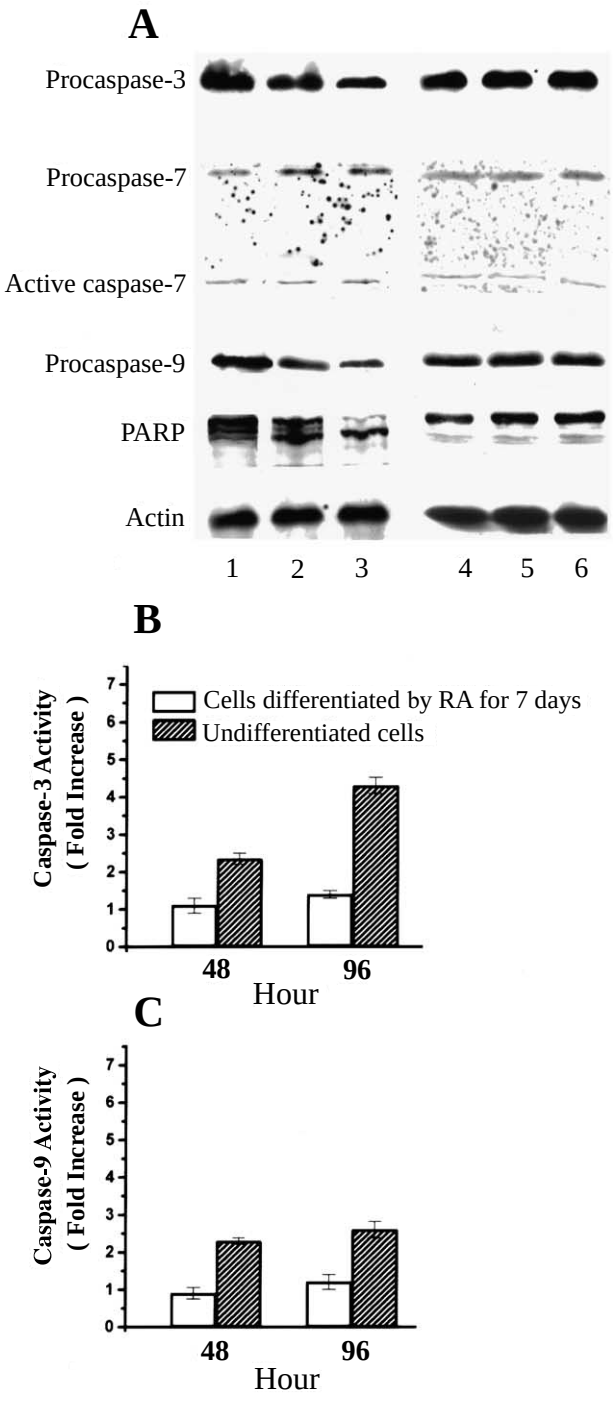

Fig. 5. RC-RNase-induced caspase activation was modulated by differentiation. Cleavage of procaspases and PARP was analyzed by Western blotting (A). Undifferentiated HL-60 cells were treated with RC-RNase (20 μg/ml) for 0 h (lane 1), 48 h (lane 2), or 96 h (lane 3); differentiated HL-60 cells obtained from 7 days induction by RA were treated with RC-RNase for 0 h (lane 4), 48 h (lane 5), or 96 h (lane 6). Detection of actin served as internal control. Note cleavage of procaspase-3, procaspase-9, and PARP was only seen in undifferentiated HL-60 cells treated with RC-RNase. Caspase-3 activity (B) and caspase-9 activity (C) were determined in undifferentiated HL-60 cells and differentiated HL-60 cells (7 days induction by RA) after these cells were treated with RC-RNase (20 μg/ml) for 48 h or 96 h. Bars represented mean ± S.D. from three independent triplicate experiments.

ing activities to HL-60 cells. We also found HL-60 cells gradually died after treatment with RA or DMSO for 2 weeks. Therefore, to avoid cytotoxicity from RA or DMSO, we carefully removed RA or DMSO before conducting experiments on differentiated HL-60 cells.

Most malignant tumor cells are highly proliferative and poorly differentiated. Previous studies have demonstrated that RC-RNase can kill a series of malignant cells, but not normal cells [8,9]. On the basis of a nice correlation, we hypothesized that RC-RNase exerted different cytotoxicity on distinct hepatoma cell lines according to the differentiation stage of the cell lines [9]. Onconase, homologous to RC-RNase, has been reported to induce tumor cell death and its cytotoxicity has been reported to associate with proliferation [18]. To further demonstrate a causal relationship between cell differentiation and RC-RNase-induced cytotoxicity, we compared survival rates of the RC-RNase-treated HL-60 cells that were differentiated by RA for 5 days or 7 days prior to RC-RNase treatments. As we have seen, the two groups of cells no longer proliferated (Fig. 2B) and were still able to resist RC-RNase-induced cell death (Figs. 3B and 4A). Cells differentiated by RA for 7 days were much more resistant to RC-RNase treatments than those differentiated by RA for 5 days. There was approximately 17.5% difference between the survival rates of the two groups of cells after 5 days induction with RC-RNase (Fig. 4A). Therefore, in terms of differentiation and proliferation, differentiation seemed to be a significant factor that decided susceptibility of the cells to RC-RNase-induced cytotoxicity. Since RC-RNase is only found in the oocytic and embryonic stages of bullfrog development [19] and was shown to be much more cytotoxic to undifferentiated or poorly differentiated cells, we thought RC-RNase might play a role in differentiation regulation during bullfrog embryogenesis.

It has been reported that onconase induced activation of the caspase-9/-3 cascade [20]. Here we reported activation of the same cascade in undifferentiated HL-60 cells after RC-RNase treatments. However, morphological characteristics of apoptosis such as chromosome condensation were not apparent in HL-60 cells after RC-RNase treatments (data not shown) and the data were congruent with the findings in RC-RNase-treated hepatoma cells [9]. Dye penetration was seen in some of the cells treated with RC-RNase for 5 days indicating that these cells began to undergo necrosis. Cytotoxicity of RC-RNase has been shown to relate to mitochondrial disruptions [5,9] and here we demonstrated RC-RNase could initiate the mitochondrial death pathway. Comparing our results with the mitochondrial death pathway triggered by H_2O_2 in HL-60 cells [21,22], we found ZVAD (a caspase inhibitor) rescued HL-60 cells from H_2O_2-induced death but not RC-RNase-induced death (data not shown). Along with previous studies showing that apoptotic and necrotic features were not observed in RC-RNase-treated hepatoma cells [9] and that executioner caspase-7 was activated in RC-RNase-treated MCF-7 cells even when there was no activation of initiator caspase-8 or -9 [5], we therefore proposed that RC-RNase might induce cell death through multiple pathways. Due to genetic heterogeneities of different cell lines, RC-RNase might trigger disparate caspase activation cascades in different cell lines.

In the present study, activation of the caspase-9/-3 cascade was seen in RC-RNase-treated undifferentiated HL-60 cells even though some of the cells were observed to be necrotic after longer RC-RNase treatments. The Fas (CD95) receptor is known to be upregulated by RA or DMSO [23]. Our data showing RC-RNase was much less toxic to cells pretreated with RA or DMSO clearly demonstrated that RC-RNase did not induce death by binding to the Fas death receptor or by activating the FasL/Fas death receptor.

Many morphological and functional changes have been reported in differentiated HL-60 cells, including generation of superoxide [24], reassembly of the cytoskeleton [25], decrease of RNA synthesis [26,27], formation of chromatin sheets [28], and alterations of enzyme metabolism [29,30]. These changes might be the reason why differentiated HL-60 cells became resistant to RC-RNase. Since RC-RNase preferred to degrade ribosomal RNA [8] and the ribonuclease activity of RC-RNase was closely related to its cytotoxicity [31], a decrease of RNA synthesis in differentiated HL-60 cells could be a reason why differentiated HL-60 cells could be more resistant to RC-RNase treatments. As we have shown here, differentiated HL-60 cells acted against RC-RNase-induced cytotoxicity by placing a curb on the activation of the caspase-9/-3 cascade (Fig. 5). We thought that there might be some anti-apoptotic proteins upregulated in the differentiated HL-60 cells and these proteins inhibited cleavage of procaspase-9 and procaspase-3 and rescued cells from death.

In conclusion, this is the first report directly demonstrating that differentiation is a significant factor to account for the selective tumoricidal effects of RC-RNase.

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