Caspase activation in response to cytotoxic *Rana catesbeiana* ribonuclease in MCF-7 cells

Chih-Chi Andrew Hu, Chih-Hang Anthony Tang, Jaang-Jiun Wang*

Institute of Biology and Anatomy, National Defense Medical Center, 161, Sec. 6, MinChuan E. Rd., Taipei 114, Taiwan

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Abstract ***Rana catesbeiana*** **ribonuclease (RC-RNase) and onconase were proven to own anti-tumor activity. While molecular determinants of onconase-induced cell death have become more explicit, the RC-RNase-induced death pathway remains presently unknown. Here we demonstrated that RC-RNase-induced molecular cascades in caspase-3-deficient MCF-7 cells did not include activation of initiation caspase-8 and -9. Cleavage timing suggested that procaspase-2 and -6 might be processed by active caspase-7 in MCF-7 cells. Caspase-7 was also responsible for cleavage of the poly(ADP-ribose) polymerase. Furthermore, we reported that overexpression of Bcl-X_L could raise the survival rates of MCF-7 cells treated with RC-RNase and onconase. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Onconase; Caspase; Bcl-X_L; MCF-7; *Rana catesbeiana* ribonuclease

1. Introduction

Several frog oocyte-derived tumoricidal ribonucleases are noted these days, such as RC-RNase from *Rana catesbeiana* and onconase from *Rana pipiens* [1,2]. It is noted that onconase has been at the phase III clinical trials in the USA as an anti-tumor drug [2,3]. The pyrimidine-guanine sequence specific RC-RNase shares 48.2% amino acid sequence homology to onconase. Both contain an identical lectin domain that is thought to be related with cytotoxicity [4]. We previously demonstrated that cytotoxicity of RC-RNase is much more likely to be differentiation-related, but not proliferation-related [5]. Despite their potential in cancer therapy, the internalization of these cytotoxic ribonucleases into cells and the mechanisms triggering death remain currently unclear.

Iordanov et al. Lipofectin-mediatedly delivered onconase into HeLa cells and reported that procaspase-9, -3, and -7 were processed in these cells and caspase-9 activation is Bax- and cytochrome *c*-independent [6]. Since there were no apparent apoptotic or necrotic characteristics observed in the hepatoma cells treated with RC-RNase [5], we undertook to further define this type of cell death. We utilized caspase-3-deficient MCF-7 breast cancer cells to investigate if caspase activation was involved in RC-RNase-induced cell death and how caspase cascades were led to precipitate the dead-end circumstances.

The distinct caspase machinery would be initiated in the process of receptor-mediated apoptosis or mitochondria-mediated apoptosis [7–9]. Binding of ligands onto the cell surface death receptors activates caspase-8 which can in succession activate the executioner caspase-3. Caspase-9 is the apical caspase participating in mitochondria-mediated apoptosis and can directly process procaspase-3 and its close relative procaspase-7. Activation of caspase-8/-3 or caspase-9/-3 and -7 ensures that apoptosis is unavoidable. Poly(ADP-ribose) polymerase (PARP) is one of the principle substrates cleaved by caspases, primarily by caspase-3 and -7; therefore, the cleavage of PARP can further define that the death was from caspase activation [7].

Mitochondrial disruptions were reported in hepatoma cells treated with RC-RNase in combination with IFN-γ [5] and seen in HeLa cells treated with RC-RNase alone (unpublished data). It is also known that Bcl-X_L can effectively reverse mitochondria-mediated cell death [10]. To examine if Bcl-X_L could act against cytotoxicity derived from RC-RNase, MCF-7 cells were permanently transfected with bcl-xL. Herein, we present a different caspase activation pathway in MCF-7 cells treated with RC-RNase and report for the first time that overexpression of Bcl-X_L could significantly rescue MCF-7 cells from death caused by both RC-RNase and onconase.

2. Materials and methods

2.1. Chemicals, cell lines, and culture conditions

Onconase purified from oocytes of *R. pipiens* was a gift from the Alfacell Corp. (USA). PARP, caspase-2 and caspase-8 monoclonal antibodies were purchased from Pharmingen. Caspase-6 and actin monoclonal antibodies were purchased from Chemicon. Caspase-7 and -9 monoclonal antibodies were purchased respectively from Oncogene and Upstate. Bcl-$X_{S/L}$ polyclonal antiserum was purchased from Santa Cruz. The human breast carcinoma cell line MCF-7 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. Oocyte preparation from R. catesbeiana and purification of RC-RNase

Mature female *R. catesbeiana* were anesthetized on ice and ovaries were removed. Oocytes were released from ovaries by cutting them into small pieces, washing in 0.09 M NaCl, and ultracentrifugation at 150 000 × *g* in a Beckman SW41 rotor. Yolk granule pellet was ho-

*Corresponding author. Fax: (886)-2-87 92 31 59.
E-mail address: jaangjwang@pchome.com.tw (J.-J. Wang).

Abbreviations: XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate; RC-RNase, *Rana catesbeiana* ribonuclease; PARP, poly(ADP-ribose) polymerase; CpG, cytidylyl(3′→5′)guanosine

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mogenized in 0.09 M NaCl and stirred at 4°C for 20 min. The soluble fraction was obtained by centrifugation (15 000 × *g*) followed by flowing through cheesecloth and made to contain HEPES (20 mM, pH 7.9) and EDTA (0.1 mM). Precipitates were removed by centrifugation (16 500 × *g*, 25 min) on each of the 4 days. The supernatant was then dialyzed for 3 days in PC buffer (20 mM HEPES, pH 7.9, 0.1 mM EDTA) containing 0.07 M KCl and the precipitated dross was removed by centrifugation at 16 500 × *g* each day. The dialyzed supernatant was loaded onto a phosphocellulose column (P11, Whatman). Active fractions were collected and loaded onto a carboxymethyl cellulose column (CM52, Whatman) [11]. Fractions with ribonuclease activity were collected, analyzed on a 13.3% SDS–polyacrylamide gel, and visualized by silver staining. Pure RC-RNase was concentrated by another phosphocellulose column eluted with 3 M KCl. RC-RNase for cytotoxicity studies was dialyzed in 200 mM phosphate buffer (pH 7.0).

2.3. Ribonuclease activity assay

Purified RC-RNase was serially diluted, incubated with SDS-loading buffer (0.125 M Tris–HCl, pH 8.0, 2% SDS, and 5% glycerol) for 15 min at 50°C, and then analyzed on a 13.3% RNA-cast SDS–polyacrylamide gel. In situ RNA digestion in the gel was visualized by methods described previously [12].

2.4. Dinucleotide CpG cleavage assay

Two micrograms of dinucleotide CpG was incubated with serial dilutions of RC-RNase in 50 mM Tris–HCl, pH 8.0 for 15 min at 37°C. Reaction mixtures were spotted on a PEI-cellulose F thin layer plate (Merck), developed in 1.6 M LiCl:0.5 M acetic acid, and visualized using a UV illuminator at 254 nm [13].

2.5. Cell viability assay

Cells were grown in 96-well cell culture plates overnight and then treated with culture medium containing RC-RNase (20 μg/ml) or onconase (20 μg/ml). Every 24th hour, XTT assays (Roche) were performed according to the manufacturer's instructions and measured at 492 nm.

2.6. Establishment of transfectants constitutively expressing Bcl-X_L

Human bcl-xL/PCR 3.1 plasmid was constructed previously [14]. MCF-7 cells stably expressing Bcl-X_L were cloned from a single cell by the limiting dilution method [15]. Briefly, cells were transfected using lipofectamine (Gibco) and selected by 200 μg/ml geneticin (Gibco). Transfectants were distributed into 96-well microtiter plates to make each well contain one cell. Once cell colonies derived from each single cell became confluent, they were transferred to tissue culture flasks and maintained in complete medium containing 200 μg/ml geneticin. Expression of Bcl-X_L in these cell clones was verified by Western blotting.

2.7. Western blotting

Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Calbiochem). Proteins in cell lysates were size separated in a 13.3% SDS–polyacrylamide gel and transferred to the nitrocellulose membrane. The membrane was blocked with 5% non-fat milk and 1% NP-40 in TBS-T (0.8% NaCl; 0.02% KCl; 25 mM Tris–HCl; 0.05% Tween 20, pH 7.4) for 2 h and then reacted at 4°C with primary antibodies (1:500) overnight. The membrane was then incubated with biotinylated anti-mouse IgG or anti-rabbit IgG antibodies (1:2000). After further incubation with streptavidin–horseradish peroxidase conjugates (1:4000), labeled proteins were visualized by the Super Signal™ Chemiluminescent-HRP substrate system (Pierce, Rockford, IL, USA).

3. Results

3.1. Successful purification of RC-RNase

Our modified purification method with prolonged precipitation time and increased centrifugation force efficiently reduced the dross compared with the previous method. Active eluates from the phosphocellulose column contained RC-RNase and another protein shown beneath the band of RC-RNase (Fig. 1A, lane 3). Pure RC-RNase was gained after elution from the carboxymethyl cellulose column (Fig. 1A, lane 4). RC-RNase possessing ribonuclease activity was demonstrated by in situ RNA digestion in an RNA-cast SDS–polyacrylamide gel (Fig. 1B). We also characterized RC-RNase by offering the specific dinucleotide CpG for its digestion into 2′,3′-cyclic CMP (Cp) and guanosine (G) (Fig. 1C).

3.2. Overexpression of Bcl-X_L rescued death induced by ribonucleases in MCF-7 cells

After transfection with bcl-xL/PCR 3.1 plasmid and selection in medium containing geneticin, the MCF-7 permanent transfectants were detected to constitutively express Bcl-X_L (Fig. 2). The bcl-xL/PCR 3.1 transfectants were less susceptible to RC-RNase and onconase treatment (Fig. 3A,B). Overexpression of Bcl-X_L could act more efficiently against the

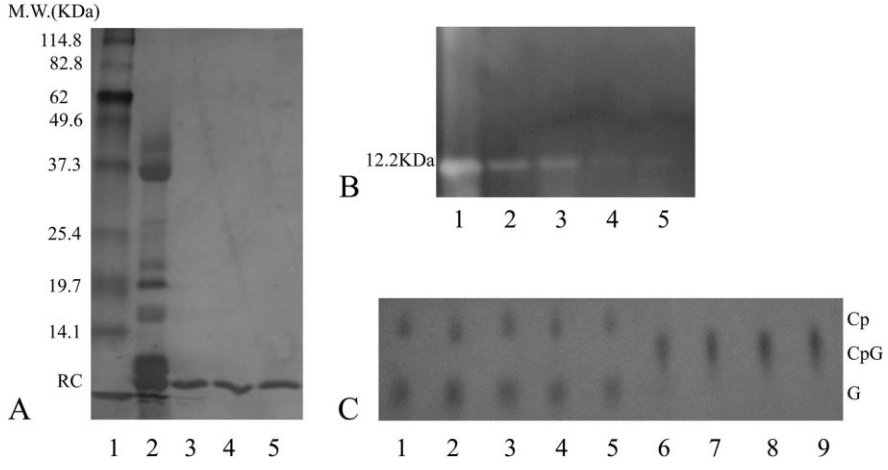

Fig. 1. A: Tracing of RC-RNase during cation exchange column purification by silver staining. Lanes: 1, protein markers; 2, supernatant after precipitation; 3, active eluates of the phosphocellulose column; 4, active eluates of the carboxymethyl cellulose column; 5, concentrated eluates of another phosphocellulose column. B: Identification of RC-RNase by in situ RNA digestion in a 13.3% RNA-containing SDS–polyacrylamide gel. Amount of loaded RC-RNase from lane 1 to lane 5 was as follows: 0.2, 0.1, 0.05, 0.025, and 0.0125 μg. C: Characterization of RC-RNase by its dinucleotide CpG cleavage activity. Amount of utilized RC-RNase from lane 1 to lane 9 was as follows: $10^{-1} \times (1/2)^0$, $10^{-1} \times (1/2)^1$, ..., $10^{-1} \times (1/2)^8$ μg. Dinucleotide cleavage activity in the presence of 2 μg CpG is visualized in lane 5 ($10^{-1} \times (1/2)^4$ μg RC-RNase).

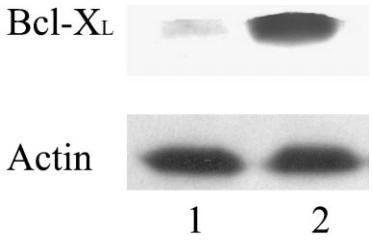

Fig. 2. Western blot analysis of Bcl-X_L expression. Lane 1, endogenous Bcl-X_L expressed in MCF-7 cells; lane 2, Bcl-X_L overexpression in MCF-7 cells transfected with bcl-xL/PCR 3.1 plasmids. Detection of actin served as protein loading control.

cytotoxicity induced by RC-RNase than that by onconase. Observations on MCF-7 cells also showed that 20 μg/ml RC-RNase was more cytotoxic than 20 μg/ml onconase.

3.3. *Caspase activation induced by RC-RNase in caspase-3-deficient MCF-7 cells was different from the two major caspase pathways of apoptosis*

Procaspase-8 and -9 were not cleaved in the caspase-3-deficient MCF-7 cells even after RC-RNase treatment for 96 h (Fig. 4, lane 3). Procaspase-7 was processed to the active caspase-7 within 48 h (Fig. 4, lane 2). Cleavage of procaspase-7 was found to be prior to that of procaspase-2 and -6 (Fig. 4, lanes 2 and 3) because procaspase-2 and -6 were not cleaved after treatment with RC-RNase for 48 h. The processing patterns of procaspases in MCF-7 cells overexpressing Bcl-X_L were almost analogous to those in MCF-7 cells (Fig. 4, lanes 4–6). Judging from the fact that PARP was cleaved

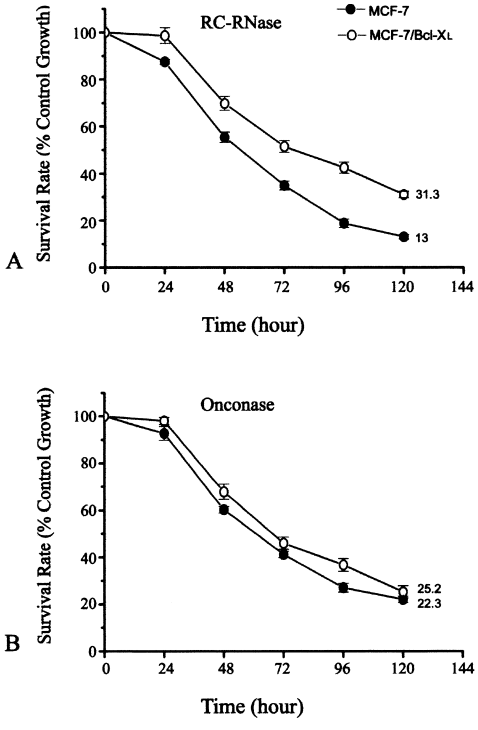

Fig. 3. Survival rates after treatment. The 5-day survival rates of the two indicated cell types were obtained after treatment with RC-RNase (20 μg/ml) (A) and onconase (20 μg/ml) (B). Each data point was calculated from three quadruple groups and represented as mean ± S.D. RC-RNase was more cytotoxic than onconase to MCF-7 cells.

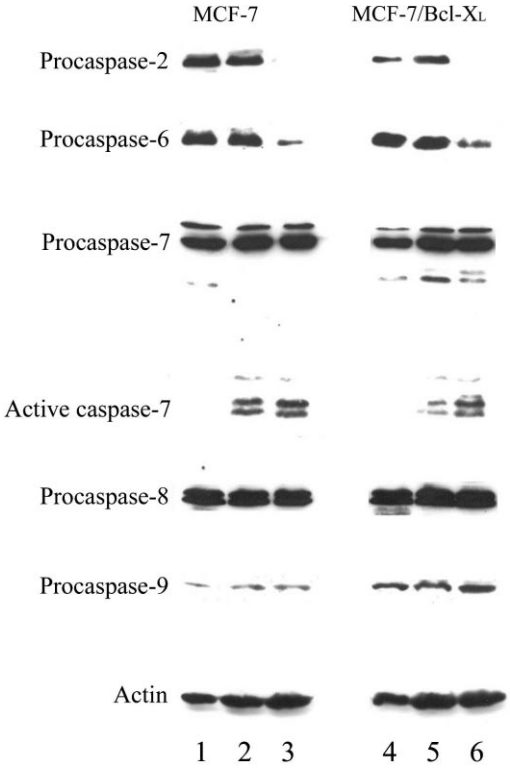

Fig. 4. Caspase activation in response to RC-RNase (20 μg/ml). MCF-7 cells were treated for 0 h (lane 1), 48 h (lane 2), and 96 h (lane 3); MCF-7 transfectants overexpressing Bcl-X_L were treated for 0 h (lane 4), 48 h (lane 5), and 96 h (lane 6). Protein loading controls were done by detecting actin simultaneously.

within 48 h, it is most likely that the activated caspase-7 was responsible for PARP cleavage in the caspase-3-deficient MCF-7 cells treated with RC-RNase. Cleavage of PARP into the major 89-kDa fragment became evident while MCF-7 cells were incubated longer with RC-RNase (Fig. 5, lanes 3 and 4).

4. Discussion

There was accumulating evidence that onconase-treated cells display the characteristics of apoptosis [6]. Although several pivotal hallmarks of apoptosis (e.g. oligonucleosomal degradation of nuclear DNA and exposure of phosphatidylserine) and necrosis (e.g. penetration by dyes) were not examined in RC-RNase-induced cell death, mitochondrial disruptions were reported to be responsible for this mode of cell death [5]. Circumvention of RC-RNase-induced cell death by overexpression of Bcl-X_L in MCF-7 cells offered further evidence that the death is strongly related with mitochondria.

The different capacity of Bcl-X_L on reversing RC-RNase- and onconase-triggered cell death indicated the employment of distinct death pathways by each of the two cytotoxic ribonucleases. Since the targeted cellular substrates for RC-RNase and onconase were rRNA and tRNA, respectively [6,11,16], it is quite reasonable that the two ribonucleases did not initiate the same death pathway. As shown in Fig. 3A, overexpression of Bcl-X_L could partially elevate the survival percentage of RC-RNase-treated cells. Together with the fact that Bcl-X_L did not rescue RC-RNase-induced cell death by inhibiting activation of caspases (Fig. 4, lanes 4–6), we suggested that

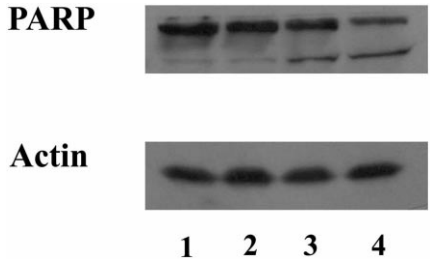

Fig. 5. PARP cleavage after treatment. MCF-7 cells treated with RC-RNase (20 μg/ml) respectively for 0 h (lane 1), 24 h (lane 2), 48 h (lane 3), and 72 h (lane 4) were subjected to Western blotting using an antibody against full-length PARP and its cleaved 89-kDa fragment. Detection of actin served as internal control.

the elevated survival rate was the result of upregulating other survival signals by Bcl-X_L.

Caspase activation ensured the irreversibility of cell death. Cleavage of critical cellular substrates by caspases could finally result in morphological alterations of the cells. Unprocessing of procaspase-8 pointed out that RC-RNase-induced cell death was not initiated from the cell surface death receptors. To our surprise, the apical initiator caspase-9 participating in mitochondria-mediated cell death was also not activated in the cell death triggered by RC-RNase. Caspase-9 activation in onconase-treated cells was considered to be atypical because it was Bax- and cytochrome *c*-independent [6]. Our observation on severe mitochondrial disruptions caused by RC-RNase might explain the lack of cytochrome *c* release; therefore, caspase-9 could not be activated without the participation of this critical coactivator. To account for atypical caspase-9 activation in HeLa cells stimulated by onconase, we should take the Lipofectin-mediated delivery into consideration. As indicated, simply adding onconase without Lipofectin to the medium was not cytotoxic at concentrations of up to 30 μg/ml [6].

Activation of the executioner caspase-7 in caspase-3 null MCF-7 cells treated with RC-RNase suggested that caspase-7 might functionally compensate for the lack of caspase-3. Procaspase-2 and -6 were known to be cleaved chiefly by caspase-3 [7]. That cleavage of procaspase-2 and -6 fell behind activation of the executioner caspase-7 offered evidence that caspase-7 might play a role in processing procaspase-2 and -6 under the circumstances where caspase-3 was defected (Fig. 4, lanes 2, 3, 5 and 6). Because cleavage of PARP was earlier observed (Fig. 5) within 48 h, we thought that PARP was cleaved by caspase-7 in MCF-7 cells. Caspase-2 or -6 could not process PARP due to cleavage specificity [7]. It is, however, worth investigating what factors stand upstream from caspase-7 activation.

In view of the distinct caspase activation in MCF-7 cells stimulated with RC-RNase, we propose that another death pathway exists that could account for the above-mentioned atypical apoptotic characteristics.

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