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RC6 Exerts an Anticancer Effect Through the Caspase-dependent Apoptosis Pathway

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Abstract

Objective: To demonstrate that RC6 exerts an anticancer effect on MCF-7 breast cancer cells.
Materials and Methods: MCF-7 breast cancer cells were treated with RC6. The survival rate was determined by XTT assay. Apoptosis was observed based on nuclear staining and FACS analysis. Caspase activation was determined by substrate cleavage assay.
Results: RC6 is able to inhibit the cell growth of MCF-7 cells (breast cancer) but not BHK-21 cells (fibroblast). The survival rate of MCF-7 cells is less than 50% at day 3 after RC6 treatment. Nuclear staining and FACS analysis demonstrated that the RC6-induced cytotoxicity of MCF-7 cells occurred via the apoptosis pathway. In addition, caspase activation was found to occur because caspase-3-like activity was present in the RC6-treated MCF-7 cells. However, caspase-8 and caspase-9 were not activated in the RC6-treated MCF-7 cells.
Conclusion: RC6 is able to induce caspase-3 like activity and this exert an anticancer effect on MCF-7 cells. (*Tzu Chi Med J* 2010;22(4):189–194)

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1. Introduction

Ribonucleases have been shown to have anticancer effects (1,2), and onconase and RC-RNase belong to the ribonuclease family. Onconase purified from *Rana pipiens* is able to inhibit the cell growth of many tumors (3,4). Similarly, RC-RNase purified from *Rana catesbeiana* also inhibits the cell growth of tumors (5,6). Although the mechanism(s) of onconase-induced/RC-RNase-induced cytotoxicity are still unclear, it should be noted that onconase has been used as an anticancer drug in a number of American clinical trials (7,8). RC6 can be purified from bull frog and its amino acid sequence is similar to that of onconase and RC-RNase (9). Therefore, it seems likely that RC6 will have an anticancer effect that is similar to onconase and RC-RNase.

MCF-7 cells belong to estrogen receptor (ER)-positive breast cancer cells (10,11). In the present study, MCF-7 cells were used to study if cell growth was inhibited

and caspases were activated after RC6 treatment. Previous studies have shown that cell death is divided into apoptosis and necrosis depending on the status of the cell [12,13]. Many studies have also indicated that dead cells created via the apoptosis pathway are removed by phagocytes [14,15] and that this process has fewer side effects than when cell death occurs via the necrosis pathway.

Previous studies have indicated that apoptosis can be induced via caspase-dependent or caspase-independent pathways [16,17]. Substrate cleavage assays can be used to determined caspase activities [5,18].

This study investigated whether RC6 has a cytotoxic effect on MCF-7 cells and whether side effects occur via the apoptosis or necrosis pathway. It was found that RC6 exerts a time-dependent cytotoxicity on MCF-7 cells and that RC6 does not induce a similar effect in fibroblasts. DNA fragmentation, condensation and the presence of sub-G1 phase cells after treatment with RC6 indicated the induction of apoptosis in MCF-7 cells. Under these circumstances, caspase-3-like activity could be detected in RC6-treated MCF-7 cells, but not caspase-8 and caspase-9 activity. In summary, RC6 exerted an anticancer effect on MCF-7 breast cancer cells through a caspase-dependent apoptosis pathway and the results suggest that RC6 may have potential in cancer treatment.

2. Materials and methods

2.1. Reagents and cell culture

RC6 was purified from bull frog using a previously published method [9]. Ac-LEHD-pNA (acetyl-Leu-Glu-His-Asp-*p*-nitroanilide: caspse-9 substrate), Ac-DEVD-pNA (Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide: caspase-3 like substrate), and Ac-IETD-pNA (acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide: caspase-8 substrate) were purchased from AnaSpec Inc. (Fremont, CA, USA). The XTT assay kit was obtained from Roche (Basel, Switzerland). Human breast carcinoma cells (MCF-7), human fibroblasts (HS68 and WI38) and mouse fibroblast (BHK-21) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 2 mM L-glutamine (Gibco), 100 IU/mL penicillin/streptomycin (Gibco), and 0.1 mM non-essential amino acids (Gibco). Human mammary epithelial cells (MCF-10A) were a gift from Dr. Yung-Luen Yu (from the Center for Molecular Medicine, China Medical University Hospital) and cultured in DMEM/F12 (Gibco) supplemented with 5% horse serum, 1% penicillin/streptomycin, 10 μg/mL bovine pancreatic insulin, 20 ng/mL epidermal growth factor and 0.5 μg/mL hydrocortisone.

2.2. Cell survival assay

Cell survival was measured using an XTT {sodium 3'-{1-(phenylamino-carbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate} kit that determines mitochondrial dehydrogenase activity. In brief, 5×10^3 cells were grown in each well of a 96-well plate. On the second day, the cells were treated with RC6 (20 μg/mL). XTT assays were carried out everyday thereafter for 4 days according to the manufacturer's instructions. The absorbance at 492 nm and 620 nm were determined using a multi-well ELISA reader (Molecular Devices Inc., Sunnyvale, CA, USA).

2.3. Nuclear staining

DNA condensation and fragmentation can be observed during apoptosis and these were assessed by nuclear staining and FACS analysis. Nuclear staining was performed using Hoechst 33342. The cells were treated with 10 μg/mL Hoechst 33342 for 10 minutes, and then DNA condensation and fragmentation were observed by fluorescence microscopy (excitation, 352 nm; emission, 450 nm).

2.4. FACS analysis

Sub-G1 phase cells occur during apoptosis and can be detected by FACS analysis. In brief, cells were fixed with 70% alcohol overnight. The next day, the cells were treated with 50 mg/mL propidium iodide, 100 mg/mL RNase A and 0.1% Triton X-100 for 40 minutes. After washing with phosphate buffered saline, the cells in sub-G1 phase were assayed using a FACS machine (CyFlow SL and FloMax; Partec GmbH, Görlitz, Germany).

2.5. Caspase activity assay

In order to analyze the activity of various caspases, cells were lysed with lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 1 mM EDTA, 1% NP-40, pH 7.5) in the presence of protease inhibitors. The cell pellets were discarded after centrifugation at 15,000*g* for 30 minutes at 4°C. Caspase activity was assessed in a working reaction containing 40 μL cell lysate (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 substrate, either Ac-LEHD-pNA or Ac-DEVD-pNA or Ac-IETD-pNA (100 μM

final concentration); the reaction was incubated at 37°C for 6 hours. Cleavage of the fluorogenic substrate released p-nitroanilide, which was measured at 405 nm in an ultra-microplate reader (BioTek, Winooski, VT, USA). The fold increase in caspase activity was calculated using the following formula: (A405sample − A405control)/A405control.

2.6. Statistical analysis

The results are presented as mean ± standard deviation and analyzed using Student's *t* test. A value of $p < 0.01$ was considered statistically significant.

3. Results

3.1. RC6 induced cell cytotoxicity in MCF-7 breast cancer cells

Initially, it was found that RC6 was able to induce cytotoxicity in MCF-7 cells and that this effect was dose-dependent (Fig. 1A). RC6 in the range of 20–200 μg/mL was able to effectively inhibit the cell growth of MCF-7 cells, but there was no significant difference between treatment with 20 μg/mL RC6 and 200 μg/mL RC6. As a result, we used 20 μg/mL RC6 throughout this study for cell treatment. To compare RC6-induced cytotoxicty between breast cancer cells and normal cells, MCF-7, BHK-21, MCF-10A, WI38 and HS68 cells were treated with RC6 (20 μg/mL) for 4 days (Fig. 1B). The results indicated that RC6 was able to induce significant cytotoxicity in MCF-7 cells. However, no obvious cytotoxicity was induced in BHK-21, MCF-10A, WI38 or HS68 cells. Therefore, we suggest that RC6 exerts a specific anticancer effect against MCF-7 cells.

3.2. RC6 induced cell death in MCF-7 cells through apoptosis pathway

As shown in Fig. 1, RC6 induced cytotoxicity in MCF-7 cells. Therefore, whether RC6 induces cytotoxicity through the apoptosis or the necrosis pathway was determined next. Many studies have shown that DNA condensation and fragmentation are observed during apoptosis [19,20]. In this study, nuclear staining was performed using Hoechst 33342 and it was found that, compared with the control cells (Fig. 2A), DNA condensation and fragmentation were present in the RC6-treated cells (Fig. 2B). About 35% of RC6-treated cells showed nuclear fragmentation and condensation. Previously, PARP cleavage was shown to occur during apoptosis [11]. Western blot analysis at day 3 showed that PARP was cleaved and reduced in size from 116 kD to 89 kD when MCF-7 cells were treated with RC6 (Fig. 2C). These findings support the hypothesis that RC6-induced cytotoxicity in MCF-7 cells occurs via the apoptosis pathway.

In addition, the presence of a sub-G1 phase cell population is a good indicator of cell death via the apoptosis pathway [21,22]. FACS analysis showed that sub-G1 phase cells could be found among RC6-treated cells on day 3 and that the percentage of RC6-treated cells in G1 phase was about 27% by FACS analysis. These results, when taken together, suggest that RC6-induced cytotoxicity in MCF-7 cells occur via the apoptosis pathway.

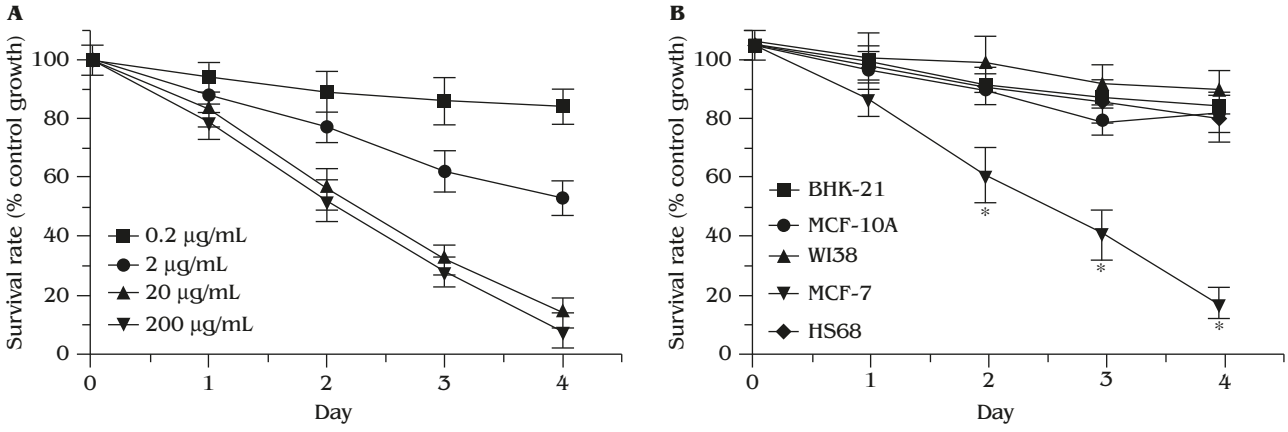

Fig. 1 — The 4-day survival rates. (A) MCF-7 cells treated with RC6 (0.2–200 μg/mL). (B) MCF-7, BHK-21, MCF-10A, HS68 and WI38 cells treated with 20 μg/mL RC6. The results were obtained from four independent triplicate experiments and are presented as mean ± standard deviation. On comparing RC6-treated MCF-7 with other RC6-treated cells, there were significant differences: *$p < 0.01$.

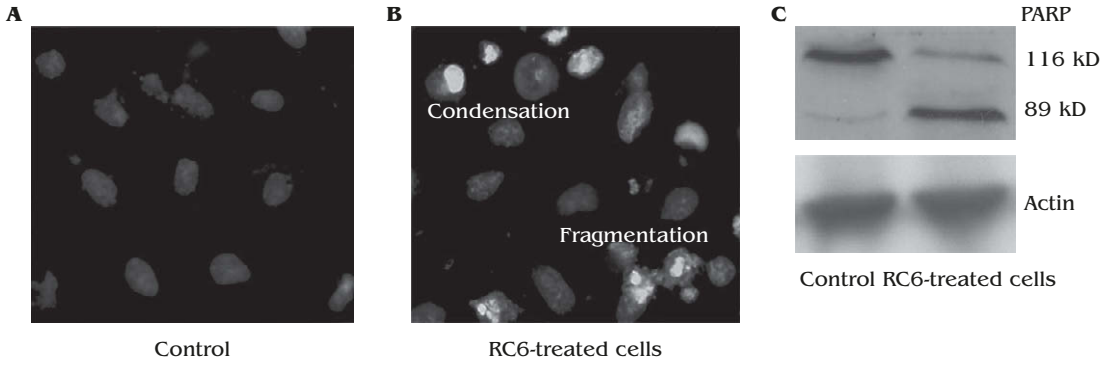

Fig. 2 — Nuclear staining and PARP cleavage. (A) MCF-7 cells without RC6 treatment. DNA condensation and fragmentation cannot be found. (B) MCF-7 cells after RC6 treatment for 3 days. DNA condensation and fragmentation are found. (C) PARP was cleaved from 116kD to 89kD in the RC6-treated MCF-7 cells. Actin served as the internal control.

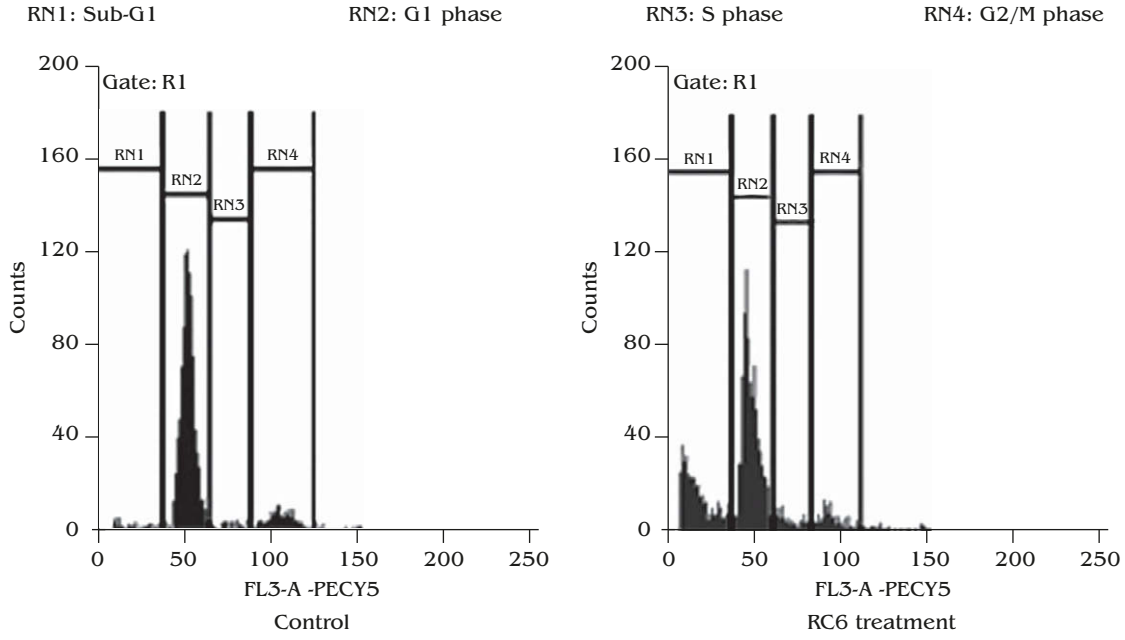

Fig. 3 — The cell cycle was determined by FACS analysis. Sub-G1 phase cells were found in MCF-7 cells at day 3 after RC6 treatment.

3.3. Caspase-3-like activity was activated in RC6-treated MCF-7 cells

Caspase-3-like, caspase-8 and caspase-9 activities were measured by incubating cell lysate with an appropriate fluorogenic substrate, Ac-LEHD-pNA or Ac-DEVD-pNA or Ac-IETD-pNA. Caspase-3-like activity increased in a time-dependent fashion when MCF-7 cells were treated with RC-6 (Fig. 4A). In contrast, no caspase-8 and caspase-9 activity could be detected in the RC-6-treated MCF-7 cells (Figs. 4B and 4C). RC-6-treated BHK-21 cells were used as the negative control. Neither caspase-3-like, caspase-8 or caspase-9 activity was found in RC6-treated BHK-21 cells. Thus, the present study is the first to demonstrate that RC6 is able to induce cytotoxicity in MCF-7 cells via the apoptosis pathway by activating caspase-3-like activity.

4. Discussion

Many studies have demonstrated that RC and onconase are able to exert an anticancer effect on breast tumors (5,11,23,24). In this study, we are the first to show that RC6 also exerts a similar anticancer effect on MCF-7 breast cancer cells. Hu et al showed that the 5-day survival rate of onconase-treated MCF-7 cells was 22% (11), and Tang et al showed that the 5-day survival rate of RC-RNase-treated MCF-7 cells was about 20% (5). In this study, the 5-day survival rate of

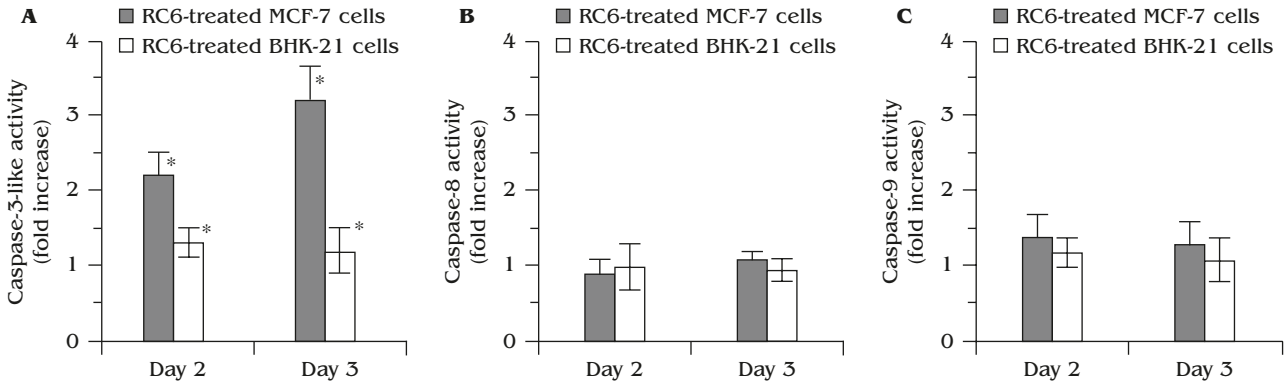

Fig. 4 — (A) Caspase-3-like, (B) caspase-8 and (C) caspase-9 activity were determined in MCF-7 and BHK-21 cells after RC6 (20μg/mL) treatment. Note that caspase-3-like activity was significantly increased in RC6-treated MCF-7 cells (*$p<0.01$). The results are presented as mean±standard deviation from four independent triplicate experiments.

RC6-treated MCF-7 cells was about 14% (Fig. 1). Therefore, we considered that RC6 has a stronger cytotoxic effect on MCF-7 cells than either RC-RNase or onconase. However, previous studies have indicated that onconase is also able to induce obvious cytotoxicity in normal fibroblasts [5]. In addition and importantly, onconase has been used as an anticancer drug in clinical trials in the U.S. [7,8]. The present study showed that RC6 does not induce obvious cytotoxicity in normal fibroblasts (Fig. 1). Taking the above into account and in comparison with previous studies [5,11], the present results (Fig. 1) suggest that RC6 has potential as an anticancer treatment.

Cell death has been divided into the apoptosis and necrosis pathways [12,13]. DNA condensation, fragmentation and the presence of sub-G1 phase cells are the major characteristics of apoptosis [19–22]. These apoptotic characteristics were found when MCF-7 cells were treated with RC-6 (Figs. 2 and 3), and the present results support the hypothesis that RC6 induces apoptosis in MCF-7 cells.

Previous studies have shown that cytotoxic RNases are able to induce apoptosis in cancer cells [25–27]. Though the mechanism(s) by which cytotoxic RNase-induced cytotoxicity occurs have remained unclear, RC6 is similar to the previously described cytotoxic RNases in that it induces apoptosis in cancer cells. Previous studies have demonstrated that apoptosis may occur through either a caspase-dependent or a caspase-independent pathway [16,17]. As shown in Fig. 4, caspase-3-like activity was found in RC6-treated MCF-7 cells. Our results support the hypothesis that RC6 induces apoptosis via a caspase-dependent pathway. In addition, many studies have shown that caspase-3/-8-like and caspase-3/-9-like activities are the major caspase cascade pathways [28–30]. However, neither caspase-8 nor caspase-9 activity was found in RC6-treated MCF-7 cells (Figs. 4B and 4C). We suggest that the RC6-induced caspase cascade may occur through an atypical pathway, which is similar to a number of previous studies [11,31,32]. Earlier studies have indicated that downregulation of inhibitors of apoptosis proteins (IAPs) is able to induce apoptosis via XIAP, CIAPs and survivin [33,34]. IAP-induced apoptosis via caspase-dependent and caspase-independent pathways have been demonstrated [35,36]. Recently, a number of studies have shown that IAPs are able to activate effector caspases (caspase-3, caspase-7) without the activation of initiator caspases (caspase-8, caspase-9) [37–39]. Our study shows that RC6 only induces caspase-3-like activity and not caspase-8 and caspase-9 activity. Thus, the levels of various IAPs (survivin, XIAP and CIAPs) were determined by Western blot. However, downregulation of these IAPs was not found in RC6-treated MCF-7 cells. We therefore suggest that unknown mechanisms that induce caspase-3-like activity but bypass caspase-8 and caspase-9 activation may be responsible, and these unknown mechanisms will be investigated in future.

Taken together, this study is the first to demonstrate that RC6 induces apoptosis in MCF-7 cells via the caspase-3-like pathway via a novel route. Our studies also suggest that RC6 may be useful for anticancer treatment in the future.

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