RC-RNase-induced cell death in estrogen receptor positive breast tumors through down-regulation of Bcl-2 and estrogen receptor

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Abstract. RC-RNase exerts anti-cancer effects on many tumors. However, the mechanisms by which RC-RNase induces cytotoxicity in different tumor cells are unclear. Currently, estrogen receptor (ER)-positive and negative breast tumors are treated with RC-RNase. Our data demonstrate that RC-RNase induces cell death on ER-positive but not on ER-negative breast tumors. This study also shows that down-regulation of ER and Bcl-2 is found on RC-RNase-treated ER-positive breast tumors. Additionally, Bcl-2 overexpression can prevent ER-positive breast tumors from cell death treated with RC-RNase. In summary, this study demonstrates that RC-RNase-induced cell death of ER-positive breast tumors is through regulation of ER and Bcl-2.

Introduction

The anti-cancer effects of RNases have been demonstrated in several studies (1-3). Onconase, derived from *Rana pipiens* and RC-RNase from *Rana catesbeiana* both belong to the RNase family exerting anti-cancer activities (4-7). Presently, onconase has been used in the treatment of tumors in some clinical trails done in the USA and Europe (8-10). RC-RNase, with about 50% of its amino acid sequences homologous to that of onconase, exerts similar anti-cancer activities (5,7,11). Many studies have demonstrated that onconase and RC-RNase can induce cell death on many tumor cells (5,7,11-13). However, the mechanisms of RNase-induced cytotoxicity and therapeutic target have remained unclear.

Our previous study has demonstrated that RC-RNases can induce different cytotoxicity on different tumor cells in humans and showed that RC-RNase induces cell death on breast tumors, hepatic tumors and leukemia through different caspase pathways (11). Additionally, many studies have also demonstrated that the degree of cytotoxicity induced by RC-RNase correlates with the stages of differentiation of tumor cells (6,14,15). These studies indicated that RC-RNase strongly exerts its anti-cancer activity on poorly-differentiated tumors, however, exerts a much lower activity on those tumors that are well-differentiated. However, the site where the RC-RNase will target the tumor cells to exert its cytotoxic effect is still unclear.

In this study, our primary data showed that RC-RNase can induce cytotoxicity on MCF-7 and ZR-75-1 breast tumors in estrogen receptor positive breast tumors through down-regulation of Bcl-2 and estrogen receptor.
tumors but cytotoxicity was not inducted on MDA-MB-231 and ZR-75-30 containing tumors. This result indicated that RC-RNase can induce different cytotoxicity on breast tumors depending on its receptors. Therefore, MCF-7, MDA-MB-231, ZR-75-1 and ZR-75-30 containing breast tumors are widely used to study the target sites of RC-RNase activity on breast tumors.

Many reports have shown that ER-positive breast tumors have MCF-7 and ZR-75-1 (16-19) while ER-negative tumors have MDA-MB-231 and ZR-75-30 (20-22). Primarily, data presented in this report show that RC-RNase induces cell death on MCF-7 and ZR-75-1 tumors, but not on MDA-MB-231 and ZR-75-30 tumors. That is, RC-RNase only induces cell death on ER-positive breast tumors. We further study estrogen receptor level on RC-RNase-treated ER-positive breast tumors. Our data showed that RC-RNase can induce down-regulation of ER. Therefore, ER is an important target of RC-RNase-induced cytotoxicity on ER-positive breast tumors. In addition, our data showed that down-regulation of Bcl-2 was found on RC-RNase-treated ER-positive breast tumors. Previous studies indicated that Bcl-2 and Bcl-XL belong to Bcl-2 family and demonstrated that overexpression of Bcl-2 or Bcl-XL has anti-apoptosis effects (23-26). However, our previous study demonstrated that overexpression of Bcl-XL can not inhibit RC-RNase-induced cytotoxicity on ER-positive breast tumors (5). We investigated whether Bcl-2 can inhibit RC-RNase-induced cytotoxicity. Our study showed that overexpression of Bcl-2 can inhibit RC-RNase-induced cytotoxicity on ER-positive breast tumors. Overall, we firstly demonstrated that RC-RNase induces cytotoxicity on ER-positive breast tumors, but not on ER-negative breast tumors through ER and Bcl-2 down-regulation.

Materials and methods

Reagents and cell culture. RC-RNase was purified with the modified methods described in previous studies (5,6,11). Ac-DEVD-pNA (Acetyl-Asp-Glu-Val-Asp-p-nitroanilide) was purchased from Anaspec (San Jose, CA). XTT assay kit was procured from Roche (Mannheim, Germany). Bcl-2 antibody was purchased from Upstate. ER and actin antibodies were procured from Roche (Mannheim, Germany) and Chemicon Laboratories (Temecula, CA), respectively. Dr Jiang (Tzu Chi General Hospital) provided human breast carcinoma cells with MCF-7, MDA-MB-231, ZR-75-1 and ZR-75-30 containing breast tumors.

Establishment of transfectants overexpressing Bcl-2 and Bcl-XL. Human Bcl-2/PCR 3.1 and Bcl-XL/PCR 3.1 plasmids were constructed employing methods as mentioned previously (5). MCF-7 cells were transfected with Bcl-2/PCR 3.1 or Bcl-XL/PCR 3.1 plasmids using lipofectamine (Gibco BRL) and lysed in RIPA buffer (10 mM Tris-base, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Calbiochem, La Jolla, CA). Equal amounts of the total proteins were loaded into a 13.3% SDS-polyacrylamide gel and underwent electrophoresis. They were then transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech). The membranes were blocked with 5% skim milk and 1% NP-40 in TBS-T (0.8% NaCl, 0.02% KCl, 25 mM Tris-HCl, 0.05% Tween-20, at pH 7.4) for 1 h, incubated with the primary antibody (1:500 dilution in the blocking buffer) at 4°C overnight, and subsequent incubation was done with biotinylated anti-mouse or anti-rabbit IgG (1:1000 or 1:10000 dilution in the blocking buffer) and streptavidin-horseradish peroxidase conjugates (1:2000 dilution in PBS). The membranes were developed using the Super Signal™ chemiluminescent-HRP substrate system (Pierce, Rockford, IL).

Results

RC-RNase induces cytotoxicity and caspase-3-like activity on MCF-7 and ZR-75-1 breast tumors. The following observations were made and recorded in the course of this study. The survival rate of MCF-7 and ZR-75-1 breast tumor cells was below 50%, noted on day 3 after treatment with RC-RNase while >80% of MDA-MB-231 and ZR-75-30 breast tumors.
tumor cells survived after treatment with RC-RNase (Fig. 1A). The data indicated that RC-RNase induces cytotoxicity on MCF-7 and ZR-75-1 breast tumor cells. However, RC-RNase induces lesser cytotoxicity on MDA-MB-231 and ZR-75-30 tumor cells. Caspase-3 like activity was also tested in this study. The result showed that RC-RNase induces caspase-3-like activity on MCF-7 and ZR-75-1 breast tumor cells with no activity observed on MDA-MB-231 and ZR-75-30 tumor cells (Fig. 1B). These observations are highly suggestive of RC-RNase ability to induce cytotoxicity on MCF-7 and ZR-75-1 breast tumor cells through the caspase pathway.

**Down-regulation of estrogen receptor and Bcl-2 on ER-positive breast tumors with RC-RNase treatment.** MCF-7 and ZR-75-1 cells are ER-positive breast tumor cells. They cannot survive without estrogen. However, MDA-MB-231 and ZR-75-30 cells are ER-negative breast tumor cells and can survive without estrogen. As shown in Fig. 1A, RC-RNase induce cytotoxicity and caspase-3 like activity on MCF-7 and ZR-75-1 breast tumor cells but not on MDA-MB-231 and ZR-75-30 breast tumor cells. That is, RC-RNase has a stronger cytotoxicity on ER-positive breast tumor cells than ER-negative breast tumor cells. This result indicates that ER may be one of targets on RC-RNase-treated ER-positive breast tumors. To demonstrate this idea, ER was determined by Western blot analysis. Our result showed that degradation of ER can be found on RC-RNase-treated ER-positive breast tumor cells in a dose-dependent manner (Fig. 2). Additionally, degradation of Bcl-2 was found on RC-RNase-treated ER-positive breast tumor cells (Fig. 2). Based on the observations and results in this study, it is highly suggestive that RC-
RNase induces cytotoxicity on ER-positive breast tumor cells through down-regulation of ER and Bcl-2.

**Overexpression of Bcl-2 inhibits RC-RNase-induced cytotoxicity on ER-positive breast tumors.** Transfectants over-expressing Bcl-2 and Bcl-XL was selected successfully in this study. As shown in Fig. 3A, MCF-7/Bcl-2 transfectants can express Bcl-2 and MCF-7/Bcl-XL transfectants can express Bcl-XL. MCF-7 cells and MCF-7/PCR 3.1 transfectants were used as a negative control. The survival rates of MCF-7, MCF-7/Bcl-2, MCF-7/Bcl-XL and MCF-7/PCR 3.1 with RC-RNase treatment showed that only MCF-7/Bcl-2 transfectants inhibited RC-RNase-induced cytotoxicity (Fig. 3B) while MCF-7/Bcl-XL transfectants did not (Fig. 3B). We can deduce from our study that only Bcl-2 can inhibit RC-RNase-induced cytotoxicity on MCF-7 cells despite Bcl-2 and Bcl-XL both belonging to the anti-apoptosis protein family.

**Overexpression of Bcl-2 inhibits down-regulation of Bcl-2.** We further determined the expression of ER and Bcl-2 on RC-RNase-treated MCF-7/Bcl-2 transfectants. The result is shown in Fig. 4. Comparing with Fig. 2, down-regulation of Bcl-2 is clearer on RC-RNase-treated MCF-7 cells than RC-RNase-treated MCF-7/Bcl-2 transfectants. Furthermore, our data indicate that down-regulation of ER was observed clearly on RC-RNase-treated MCF-7/Bcl-2 transfectants at day 3. This result is similar to that of ER down-regulation on RC-RNase-treated MCF-7 cells (Fig. 2).

**Discussion**

Data from previous literature demonstrated that RC-RNase can induce cytotoxicity on breast tumors (MCF-7 cells) through caspase-7 activation (5,11). However, the target sites where RC-RNase will induce cytotoxicity on breast tumors are still unclear. In this study, our data showed that RC-RNase induces cytotoxicity on ER-positive breast tumors (MCF-7 and ZR-75-1) but fails to induce cytotoxicity on ER-negative breast tumors (MDA-MB-231 and ZR-75-30). In other words, RC-RNase has an anti-cancer effect only on ER-positive breast tumors. Additionally, our study demonstrates that RC-RNase can induce down-regulation of ER on ER-positive breast tumors. ER-positive breast tumor treatments have demonstrated that cell proliferation can be inhibited on ER-positive breast tumors by blocking the ER signal pathway (27-30). Based on these studies and our results, we suggest that ER is an important target of RC-RNase-induced cytotoxicity on ER-positive breast tumors.

Bcl-2 with anti-apoptotic functions and survival effects have also been demonstrated in some studies (31-33). These studies indicated that various cell types cannot survive when Bcl-2 level decreased. Our study shows that down-regulation of Bcl-2 is expressed on ER-positive breast tumors treated with RC-RNase. Many studies have demonstrated that down-regulation of Bcl-2 can induce cell death on ER-positive breast tumors (34-36). These results are similar to our study. We therefore consider that Bcl-2 is also a target site of action for RC-RNase to induce cytotoxicity on ER-positive breast tumors. Our study demonstrates that RC-RNase can down-regulate ER and Bcl-2 levels resulting in cell death on ER-positive breast tumors.

Various reports have indicated that Bcl-2 and Bcl-XL are anti-apoptotic factors (23-26). These reports suggested that overexpression of Bcl-2 and Bcl-XL can inhibit cell death and the down-regulation of Bcl-2 and Bcl-XL can induce cell death. However, previous studies demonstrated that Bcl-2 overexpression cannot prevent hyperoxia-induced cell death on epithelial cells (37) and Bcl-XL overexpression cannot inhibit apoptosis on hepatocytes (38). Additionally, it has been reported that Bcl-2 and Bcl-XL inhibit cell death in a different manner (39). These studies indicated that anti-apoptotic effects between Bcl-2 and Bcl-XL on different cells vary depending on the target site of action and cellular function (37-40). In this study, our results demonstrate that Bcl-XL overexpression cannot inhibit RC-RNase-induced cytotoxicity on MCF-7 cells. This result is similar with our previous study (5). Furthermore, our study shows that Bcl-2 overexpression can inhibit RC-RNase-induced cytotoxicity on ER-positive breast tumors indicating that the anti-apoptotic effect on RC-RNase-treated ER-positive breast tumors is dependent on Bcl-2 functions but not on Bcl-XL functions.

In summary, this study is able to demonstrate that RC-RNase induces cell death on ER-positive breast tumors but not on ER-negative breast tumors through down-regulation of ER and Bcl-2. In addition, the anti-cancer effect on RC-RNase-treated ER-positive breast tumors is related to Bcl-2 overexpression, but not to Bcl-XL overexpression.

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