Phagocytic clearance of dying cells is found in many phagocytes. It has been shown that dying cells can be phagocytosed by other phagocytic cells through autophagic or apoptotic cellular death. To date, whether cancer cells have such phagocytic activity has not been studied. In this study, our data shows that RC-RNase can trigger cell death in human breast cancer MCF-7 cells through the apoptotic pathway. Interestingly, when treated with cytotoxic protein, the remaining MCF-7 cells can phagocytose the dying MCF-7 cells via autophagocytic activity, demonstrated directly by real-time image observation and electron microscopy analysis. To sum up, this study demonstrates for the first time that RC-RNase can trigger apoptosis and autophagocytosis in MCF-7 cancer cells.

Phagocytic clearance of dying cells is a very important mechanism in the resolution of inflammation (1, 2). Previous studies have demonstrated that dying cells can be phagocytosed by professional or semi-professional phagocytes (3). These phagocytic cells include monocytes, macrophages, dendritic cells and leukocytes (4-6). Many reports showed that most of the dying cells were engulfed by phagocytes through apoptotic or autophagic cellular death pathways (7-9). These results suggested that cellular death triggered by apoptosis or autophagy can be effectively and rapidly removed by phagocytes to prevent further inflammatory response. Therefore, apoptosis and autophagy are important cellular death pathways for animal development, tissue homeostasis and cancer therapy (10-12). Whether cells possess phagocytic activity is less well understood other than monocytes, macrophages, dendritic cells and leukocytes. A recent study showed that human endometrial endothelial cells exhibit phagocytosis of apoptotic trophoblasts (13). This indicates that endothelial cells also have phagocytic activity.

Previous studies found that dying MCF-7 cancer cells, treated with tamoxifen, can be phagocytosed by macrophages through
autophagic death pathway (9, 14). In addition, the study used autophagic marker combined with flow cytometric analysis to demonstrate that the autophagic markers were carried by live MCF-7 cancer cells. In order to investigate whether MCF-7 cancer cells truly have a phagocytic function, we used real-time imaging analysis with electron microscopy in this study.

MCF-7 breast cancer cells, as well as estrogen receptor-positive cells are dependent on the estrogen signal pathway (15, 16). Previous studies have reported that many compounds have anti-tumor activity against MCF-7 cancer cells through estrogen signaling such as benzoxazepine, flaxseed oil, secoisolariciresinol diglucoSIDE, anacardic acid, linoleic acid and tamoxifen (17-21). In addition, previous studies have demonstrated that the cytotoxic protein Rana catesbeiana-RNase (RC-RNase) can activate caspase activity and induce cell death in MCF-7 cancer cells (22, 23). A recent study showed that this cytotoxic protein has anticancer activity in MCF-7 cancer cells related to estrogen signaling (24). However, none of these studies have demonstrated whether this cytotoxic protein can induce cell death in MCF-7 cancer cells without caspase activation. In this study, we aim at investigate the cell death triggered by RC-RNase in human breast cancer MCF-7 cells, and whether the remaining MCF-7 cells may phagocytose the dying MCF-7 cells, directly by real-time image observation and electron microscopy analysis.

Materials and Methods

Chemicals and cell culture. RC-RNase purified as described in previous studies (22-25) was kindly provided by Dr. Jaang-Jiun Wang (Emory University School of Medicine). acetyl-Leu-Glu-His-Asp-p-nitroanilide: caspase-9 substrate (Ac-LEHD-pNA), Acetyl-Asp-Glu-Val-Asp-p-nitroanilide: caspase-3-like substrate (Ac-DEVd-pNA), caspase-8 substrate (acetyl-Ile-Glu-Thr-Asp-p-nitroanilide, Ac-IETD-pNA), general caspases inhibitor (Z-VAD-FMK) and caspase-3-like inhibitor (Z-DEVD-FMK) were bought from Anaspec (San Jose, CA, USA), 2 mM L-glutamine, 100 IU/ml penicillin G sodium, 100 μM sodium pyruvate (Sigma Chemical Co., St. Louis, MO, USA), 2 mM L-glutamine, 100 IU/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.1 mM non-essential amino acids (all Gibco BRL).

Cell survival rate assay. Cell survival rates of MCF-7 cancer cells and ZR-75-1 cells were calculated using the XTT assay as described in previous studies (27-30). Briefly, 5×10⁴ cells were cultured in each well of 96-well plates. On the second day, cells were treated without or with 20 μg/ml RC-RNase. XTT assays were determined every day for four days according to the manufacturer’s instructions. Absorbance at 492 nm and 620 nm were determined using a multi-well ELISA reader (Molecular Device).

Caspase activity assay. Treated and control cells were lysed with lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 1 mM EDTA, 1% NP-40, pH 7.5) and protease inhibitors. Cell pellets were collected by centrifugation under 15,000 xg at 4°C for 30 min. The caspase activity was assessed as previously described (22, 25). Briefly, the caspase activity was studied with a working reaction containing 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-LEHD-pNA, Ac-DEVd-pNA, or Ac-IETD-pNA substrates (100 μM final concentration); the reaction was then incubated at 37°C for 6 h. The fluorogenic substrate cleavage resulting in p-nitroanilide release was detected at 405 nm in an ultra-microplate reader (Bio-Tek instrument). Fold increase in caspase activity was calculated by using the following formula: (A405 sample–A405 control)/A405 control.

Inhibition of caspase activity. Z-VAD-FMK is a general caspase inhibitor which inhibits all caspase activity. Z-DEVD-FMK is a specific caspase inhibitor that inhibits caspase-3 like activity. In this study, MCF-7 cells were pretreated with Z-VAD-FMK or Z-DEVD-FMK prior to RNase treatment. The survival rates of 10⁶ cells in each treatment were determined by XTT assay as described above. Nuclear staining. DNA condensation and fragmentation can be observed in apoptotic cells with nuclear staining method. Nuclear staining was performed using Hoechst 33342. For each treatment, a dish with 106 cells were harvested and treated with 10 μg/ml Hoechst 33342 solution for 10 min. DNA condensation and fragmentation were then observed under a fluorescence microscope (excitation: 352 nm; emission: 450 nm).

Real-time image observation. One day before the RC-RNase treatment, 10⁶ cells were cultured in each plate. Within the 3 days following RC-RNase treatment, the cells in a specific site were continuously observed and counted, and the remaining cells in this specific site could be observed and recorded in pictures every 30 min under an optical microscope.

Electron microscopy observation. MCF-7 cells were treated without or with RC-RNase for 3 days. Cells were then collected and fixed with 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.4) for 1 h. The cells were post-fixed with osmium tetroxide for 1 h then dehydrated in graded alcohol, and were embedded in eponate-12 resin (26). Using an ultra-microtome, thin sections were obtained. After the thin sections were double stained with lead citrate and uranyl acetate, they were observed on a Zeiss 900 electron microscope for real-imaging analysis.

Statistical analysis. The non-picture results were expressed as mean±SEM of three independent experiments. The data were analyzed using Instat software (GraphPad Prism4, San Diego, CA, USA). The Student’s t-test or one-way analysis of variance (ANOVA) with post hoc analysis using Tukey’s multiple comparison method was used for parametric data. P<0.05 was considered to be statistically significant. For each treatment, experiments were performed for at least three times and only one picture was represented. No statistical analysis was needed in this study.
Results

Cell cytotoxicity and caspase-3-like enzyme activation are found in MCF-7 and ZR-75-1 breast cancer cells under RC-RNase treatment. MCF-7 and ZR-75-1 cells are classified as estrogen-positive breast cancer cells (24). Both cell lines were treated with RC-RNase in this study. As shown in Figure 1, our study indicates that RC-RNase induces cytotoxicity in MCF-7 and ZR-75-1 breast cancer cells. The results showed that the survival rates of MCF-7 and ZR-75-1 cells were below 40% at day 3 under RC-RNase treatment (Figure 1A). Moreover, caspase activities were determined in this study and showed that caspase-3-like activity is induced in cytotoxic protein-treated MCF-7 and ZR-75-1 cells (Figure 1B). Caspase-8 and -9 activities were not significantly activated (Figure 1B). The observed findings therefore suggest that RC-RNase induces cytotoxicity and caspase-3-like activity in MCF-7 and ZR-75-1 cells.

RC-RNase induces apoptosis through caspase-dependent and -independent pathways in MCF-7 and ZR-75-1 cells. Whether RC-RNase-induced cytotoxicity depends on caspase activities was determined in this study. RC-RNase still induced cytotoxicity in MCF-7 and ZR-75-1 cells in this study despite blocking caspase activity with caspase inhibitors. Our data shows that the survival rates of RC-RNase -treated MCF-7 cells treated with caspase inhibitors are similar to those without caspase inhibitor treatment (Figure 2). In addition, the characteristic of apoptosis, namely DNA condensation or fragmentation were noted in this study. Figure 3 shows that DNA condensation or fragmentation was found in RC-RNase-treated MCF-7 cells both with and without caspase inhibitor treatment. Similar results were also observed in ZR-75-1 cells (data not shown).

Taken together, the findings of this study suggest that RC-RNase induced cytotoxicity via caspase-dependent and -independent apoptotic pathways.

Autophagocytosis in MCF-7 cells under real-time imaging and electron microscopic observation. Our study has demonstrated that RC-RNase can induce cell death in MCF-7 cells (Figures 1 and 2). However, 40% of cells survive cytotoxic protein treatment at day 3 (Figure 1A). What happens to the remaining cells is noteworthy.
MCF-7 cells were cultured in a specific dish, labeled and numbered. After the MCF-7 cells were treated with RC-RNase for 3 days, the surviving MCF-7 cells were observed in real-time under a microscope half-hourly. It was observed that the shape of the remaining MCF-7 cells was transformed, they extended pseudopodes and engulfed the dying MCF-7 cells (i.e. autophagocytosis) as shown in Figure 4. We further demonstrate the self-phagocytosis of MCF-7 cells under electron microscopy. As shown in Figure 5, MCF-7 cells can extend pseudopodia to contact other MCF-7 cells (Figure 5A) and phagocytose another MCF-7 cell (Figure 5B). In addition, our data shows that cell debris was found in MCF-7 cells (Figure 5C). Thus, these results demonstrate directly that MCF-7 cells have the capacity for autophagocytosis under treatment with RC-RNase.
Two major caspase-mediated death pathways have been reported. The caspase-8 cascade is activated through death receptors while the caspase-9 cascade is activated through alteration of mitochondria (22-25, 31). These studies also showed that the downstream caspase-3-like activity can be activated by caspase-8 or caspase-9 cascade, then inducing cell death. In this study, caspase-3-like activity was found in RC-RNase-treated MCF-7 cells, however activation of caspase-8 and caspase-9 were not found. Our results are the same as previous studies (22, 23). Hence we suggest that caspase-3-like activity does not only depend on the caspase-8 or caspase-9 cascade in MCF-7 cells. Previous studies have indicated that RC-RNase can activate caspase-3-like activity and induce cell death in MCF-7 cells (22, 23). However, whether RC-RNase-induced cell death resulted from caspase-3-like activity was unclear. In this study, we also found out that blocking caspase-3-like activity did not prevent cell death in MCF-7 cells treated with RC-RNase. We conclude that RC-RNase can induce cell death through caspase-dependent and -independent pathways in MCF-7 cells.

Tamoxifen has been used as an anticancer drug in estrogen-positive breast tumors (32, 33). Previous studies have demonstrated that tamoxifen induces cell death in MCF-7 cells through an autophagic death pathway (9, 14). The studies also demonstrated that the dying MCF-7 cells can be phagocytosed by macrophages. Moreover, the autophagic MCF-7 cells and surviving MCF-7 cells were co-cultured and analyzed under flow cytometric method in those studies and results demonstrated that the autophagic markers were found in the surviving MCF-7 cells. Therefore, the studies indicated indirectly that surviving MCF-7 cells may be able to phagocytose autophagic MCF-7 cells (9, 14). Here, we further demonstrate by direct real-time imaging and electron microscopic observation (Figures 3 and 4) that the remaining MCF-7 cells can engulf the dying cells under RC-RNase treatment. In line with previous studies, we consider that MCF-7 cells certainly have a self-phagocytic activity. In addition, while previous studies have shown that autophagic MCF-7 cells can be phagocytosed by surviving MCF-7 cells, our study shows that MCF-7 cells dying through apoptotic pathway can be phagocytosed by the remaining MCF-7 cells when treated with RC-RNase. Hence we suggest that not only autophagic MCF-7 cells but also apoptotic MCF-7 cells can be phagocytosed by the remaining live MCF-7 cells.

In literature, a few studies have previously showed that RC-RNase induced cell death in many types of cancer cell, including cervical cancer cells, leukemia and hepatoma cells (22-26). However, these studies did not demonstrate self-phagocytic activity among these cancer cells. Similar to MCF-7 cells, ZR-75-1 cells are also estrogen receptor-positive breast cancer cell lines (24). As shown in Figure 1, our study indicated that RC-RNase also induces cytotoxicity in ZR-75-1 cells. However, our study has not shown self-phagocytic activity of ZR-75-1 cells and only the surviving MCF-7 cells exhibited self-phagocytic activity. Together with other studies, we could suggest that self-phagocytic activity may be cell specific. Another explanation is that RC-RNase may only induce phagocytic activity in MCF-7 cells or may in fact inhibit phagocytic activity in other cell lines. In
summary, this study is the first to demonstrate that RC-
RNase can induce cell death in MCF-7 cells through both
caspase-dependent and -independent pathways and induce self-phagocytic activity in MCF-7 cells.

Acknowledgements
This work was supported by National Science Council of Taiwan grants NSC99-2320-B-039-030-MY3, NSC99-2632-B-039-001-MY3, and NSC100-2321-B-039-004 (to Y.-L.Y.); and University of Texas MD Anderson-China Medical University and Hospital Sister Institution Fund DMR-101-115 (to Y.-L.Y.).

References


