Aqueous Extracts of *Rhizopus oryzae* Induce Apoptosis in Human Promyelocytic Leukemia Cell Line HL-60

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(Received August 30, 2007; Accepted September 28, 2007; Published online October 1, 2007)

Protection against *in vivo* infection with Salmonella and enhancement of *in vitro* superoxide production by peripheral blood neutrophils are two reported effects of treatment with aqueous extracts of the fungus *Rhizopus oryzae* U-1 (Aq-ROU). Here, we report that Aq-ROU also has antiproliferative activity and can induce apoptosis in a human promyelocytic leukemia cell line, HL-60. During Aq-ROU-induced apoptosis, HL-60 cells undergo genomic DNA fragmentation characteristic of apoptosis after just 6 hr of treatment. Using phosphatidylserine (PS) as an indicator of apoptosis, we also found that the proportion of apoptotic cells increased significantly after 9 hr of treatment. Indeed, induction of apoptosis by Aq-ROU reached 43.3% after 24 hr of treatment, which is comparable to the effects of a known apoptosis-inducer, actinomycin D. Moreover, the activities of caspase-3, -8, and -9 increased in parallel with Aq-ROU treatment, with a peak of activity 9 hr after the initial treatment. Taken together, the results suggest that *R. oryzae* contains one or more water-soluble factor that can reliably and efficiently induce apoptosis in human cells via activation of caspase-3.

Key words —— *Rhizopus oryzae*, apoptosis, HL-60

INTRODUCTION

Fungi of the genus Rhizopus are utilitarian microorganisms that have been used for centuries in Asia to produce alcohol via fermentation and to produce tempeh, a fermented soy food. The Rhizopus species *Rhizopus delemar* [hereafter referred to by the reclassification *Rhizopus oryzae*[1] (*R. oryzae*)] has a broad range of physiological effects on organisms that ingest the fungus. Many of these have the effect of alleviating disorders of the genital systems, including increasing fertilization rates, fecundity, and hatchability in quails and hens;[2] synthetically enhancing the effects of ovarian steroid hormones in rats and rabbits;[3–5] and increasing the rate of pregnancy in cows.[6]

Recently, we reported that an aqueous extract of *R. oryzae* U-1 (Aq-ROU) can alleviate Salmonella infection in rats via activation of peripheral monocytes and production of a more favorable Th1/Th2 balance.[7] In cell culture experiments with human peripheral neutrophils, Zhang *et al.* observed enhanced production of superoxide in response to Aq-ROU treatment.[8] Nevertheless, few reports have described the immunological physiology of *R. oryzae* in terms of its effects on other cells. To address this important question, we examined the effects of Aq-ROU on cell proliferation and induction of differentiation using the HL-60 cell line, which can differentiate into neutrophils or macrophages. We observed strong suppression of cell proliferation consistent with cell death, including the presence of apoptotic bodies.

Apoptosis is an important biological mechanism involved in organogenesis during development; deletion of senescent cells; and cell maintenance and disease prevention via elimination of auto-reactive immune cells, cancer cells, and virus-infected cells.[9, 10] In this study, we observed a new physiological activity of Aq-ROU, namely, the ability to induce apoptosis in a human leukemia cell line, and performed an initial characterization of the activity.

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MATERIALS AND METHODS

Aqueous Extracts of *R. oryzae* U-1 —— The fungal strain used in this study, *R. oryzae* strain U-1 (ROU), was isolated from a fermenting mixture of ground barley and bran. ROU was inoculated into malt extract medium (malt extract, 20 g; polypepton, 1 g; and glucose, 20 g/l; pH 7) and incubated at 24°C for 4 days. After incubation, fungi were suspended in 45°C water (10 g/100 ml) and stirred for 30 min at a constant temperature of 45°C. The solution was then centrifuged at $3000 \times g$ for 20 min and the supernatant was vacuum concentrated to a dried powder. The powder was dissolved in distilled water (1 g/10 ml) and filtered using a 0.20 μm filter. The resuspended aqueous extract was used as the active extract in this study and is referred to as Aq-ROU.

Cell Culture —— The human promyelocytic leukemia cell line HL-60 (RIKEN CELL BANK, Tsukuba, Japan) was maintained in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, U.S.A.) with 10% fetal calf serum (FCS) (Nippon Biotest Lab., Tokyo, Japan) at 37°C in 5% CO_2.

Viability of HL-60 Cells after Treatment with Aq-ROU —— HL-60 cells (2.5×10^5 cells/ml) were incubated for 12 or 24 hr in the presence or absence of Aq-ROU (diluted in media to a final concentration of 0.1, 1, or 5%). The number of viable cells was determined using a MTT Cell Count Kit (Nakalai Tesque, Inc., Kyoto, Japan). The absorbance of each sample at 570 nm was measured using a microtiter plate reader.

Analysis of Apoptotic DNA Fragmentation —— HL-60 cells were incubated at a cell density of 2.5×10^5 cells/ml in the presence or absence of Aq-ROU (1%) for 0, 3, 6, 12, or 24 hr. Actinomycin D was used as a positive control for apoptosis; specifically, HL-60 cells were treated with 5 μg/ml actinomycin D (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) for 9 hr. The cells were collected by centrifugation at $1600 \times g$ for 5 min, and genomic DNA was extracted as described by the manufacturer using a Apoptosis Ladder Detection Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The DNA was separated by electrophoresis on a 1.5% agarose gel and detected with SYBR Green I.

Analysis of Apoptosis by Detection of Phosphatidylserine with Annexin V —— Cell-surface levels of phosphatidylserine (PS) were determined by flow cytometry using EPICS XL and EXPO32 software (Beckman Coulter Inc., Fullerton, CA, U.S.A.). More specifically, PS levels were estimated using an Annexin V FITC Apoptosis Detection kit (Merck KGaA, Darmstadt, Germany). Following treatment with Aq-ROU (1% in the medium; cell density of 2.5×10^5 cells/ml) to HL-60 cells for 0–24 hr, 5×10^5 cells were collected by centrifugation at $500 \times g$ for 5 min, and washed in pre-chilled phosphate buffered saline (PBS). The cells were then stained with FITC-conjugated annexin V and propidium iodide (PI) to visualize PS and nucleic acids, respectively.

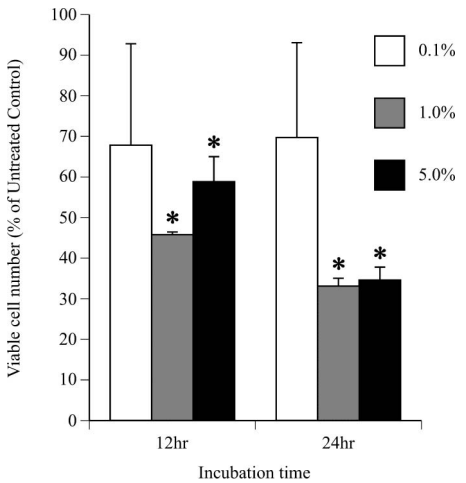

Fig. 1. Reduced Viability of Human Leukemia HL-60 Cells in Response to Treatment with Aq-ROU

The number of viable cells in treated populations is expressed as a percentage of the cell number observed in untreated control cell populations. The bar above the column shows the standard deviation of three independent experiments. A significant difference relative to untreated cells, $^*p < 0.05$.

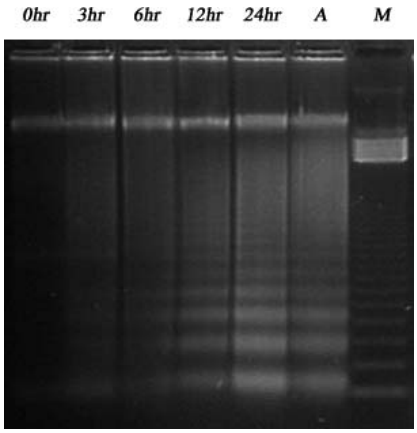

Fig. 2. Detection of Genomic DNA Fragmentation in Aq-ROU Treated HL-60 Cells by Agarose Gel Electrophoresis

Lane A, apoptotic positive control cells treated for 9 hr with Actinomycin D (5 μg/ml). Lane M, 123 bp DNA ladder.

Assay of Caspase Activity —— The activities of caspases-3, -8, and -9 were determined using the Caspase Colorimetric Activity Assay Kit (Chemicon International, Inc., Temecula, CA, USA). Briefly, HL-60 cells were plated at a cell density of 2.5×10^5 cells/ml and incubated in the presence of 1% Aq-ROU for 0–24 hr. The cells were then collected by centrifugation at $1600 \times g$ for 5 min and washed once with PBS. The cells were resuspended in cell lysis buffer at a cell density of 6.25×10^6 cells/ml and incubated on ice for 10 min. Cell debris was removed by centrifugation at $10000 \times g$ for 5 min. The overall protein concentration of the supernatants was determined using the Protein Assay Rapid Kit (Wako Pure Chemical Industries). Equal amounts of protein (60 μg per reaction mixture) were added to the assay buffer and used in colorimetric assays for caspase-3, -8 or -9 activity. Caspase activity was detected by determining the absorbance at 405 nm using a microtiter plate reader.

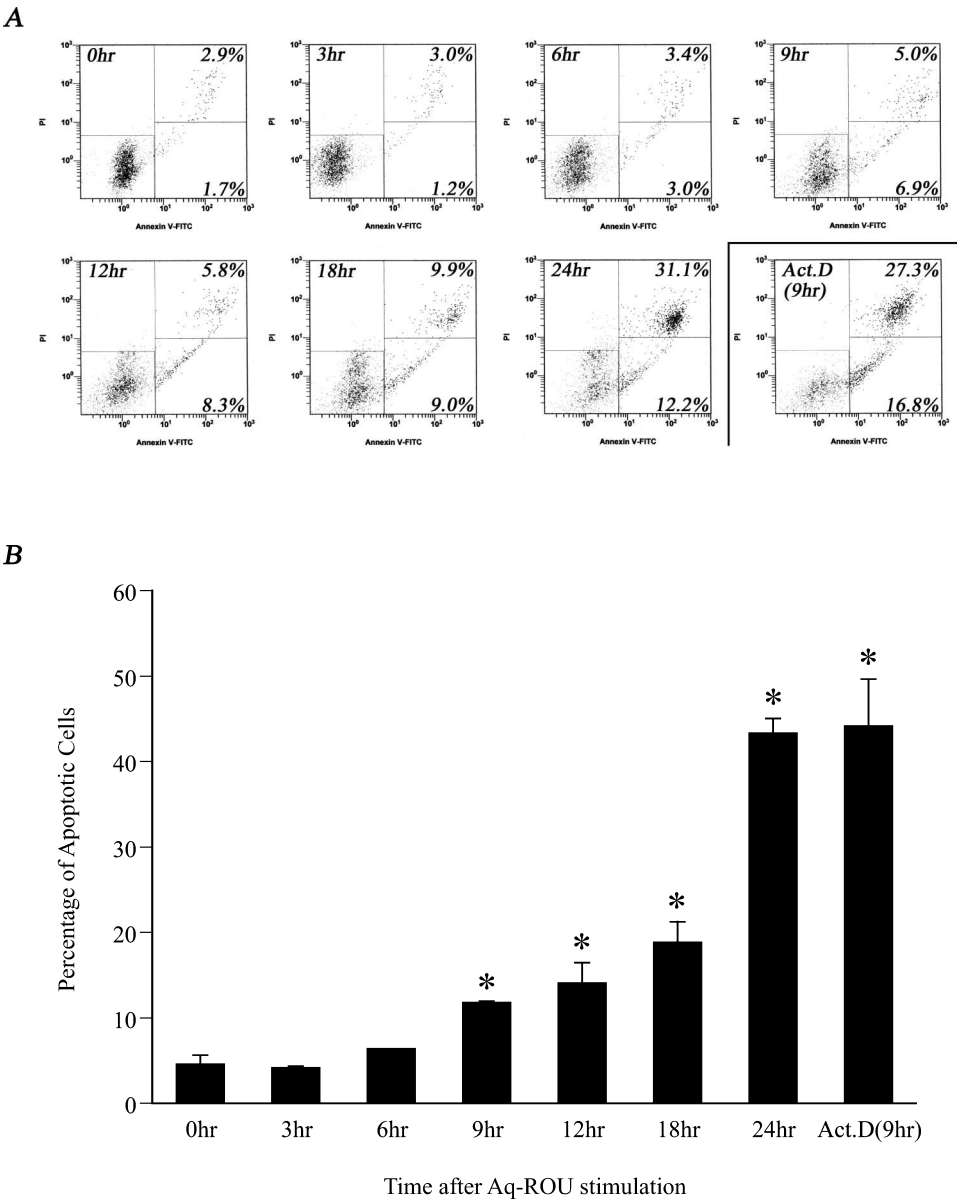

Fig. 3. Kinetics of Aq-ROU-induced Apoptosis in HL-60 Cells

(A) Representative cytograms showing percentages of Annexin V positive cells (lower right quadrants) and Annexin V/PI double positive cells (upper right quadrants). Lower left quadrants, viable cells. (B) Annexin V positive and Annexin V/PI double positive cells were assumed to be apoptotic cells and the ratio of apoptotic cells is expressed a percentage of total cell number. The bar above the column shows the standard deviation of three independent experiments. A significant difference relative to 0 hr incubation, $^* p < 0.05$.

Statistics —— Respective measured values were analyzed using the Student's *t*-test. A value of $p < 0.05$ was inferred to be statistically significant.

RESULTS AND DISCUSSION

Effects of Aq-ROU on Viability of HL-60 Cells

HL-60 cells were incubated in the presence of 0.1–5% of Aq-ROU and the number of viable cells was determined (Fig. 1). We found that proliferation of HL-60 cells was suppressed in a dose- and time-dependent manner. After treatment with a final concentration of 1% or 5% Aq-ROU, the number of viable cells was approximately 50% of that observed for untreated cells after 12 hr, and decreased to 30% of control levels after 24 hr in culture. After exposure to Aq-ROU, treated cells failed to proliferate, and we noted in smear samples that this correlated with the presence of apoptotic bodies and micronuclei in treated but not untreated cells.

Kinetics of Apoptosis in Aq-ROU-treated HL-60 Cells

To further test if Aq-ROU induces apoptosis in HL-60 cells, we looked at markers of apoptotic cells. Towards this goal, we first asked if we detect the ladder pattern of genomic DNA indicative of fragmentation at nucleosomal units on a gel. In HL-60 cells, DNA ladders typical of apoptosis were clearly detectable after 6 hr or more of culture in the presence of 1% Aq-ROU (Fig. 2). Signals that induce phagocytosis, such as an increase in cell-surface levels of PS, are typical of apoptotic cells, as they attract phagocytes such as macrophages which then phagocytose the dying cell; these signals are recognized by phagocytes via death sensors.[11–13] Thus, we next looked at cell-surface levels of PS, which increase on outside of apoptotic cells and can be detected with FITC-conjugated annexin V.

As shown in Fig. 3A and 3B, Aq-ROU-treated HL-60 cells exhibited a significant increase in annexin V staining after 9 hr of culture in the presence of 1% Aq-ROU ($11.8 \pm 0.2\%$). The extent of PS accumulation increased as the cells were incubated for longer time periods (for example, $43.3 \pm 1.7\%$ of cells were positive after 24 hr in culture). When Aq-ROU treated cells were stained with PI to visualize nucleic acids, together with annexin V, we found that the number of double-positive increased with time in treatment (Fig. 3A; upper right quadrants). PI cannot permeate the cell membrane of normal cells; however, in late apoptotic or necrotic cells, in which the normal structure of cell membrane has been compromised or has disintegrated, PI can enter the cell and bind to nucleic acids. As the number of annexin V-positive and PI-positive cells increased in parallel, Aq-ROU does not appear to induce necrosis in HL-60 cells but rather, has characteristics consistent with apoptotic cell death.

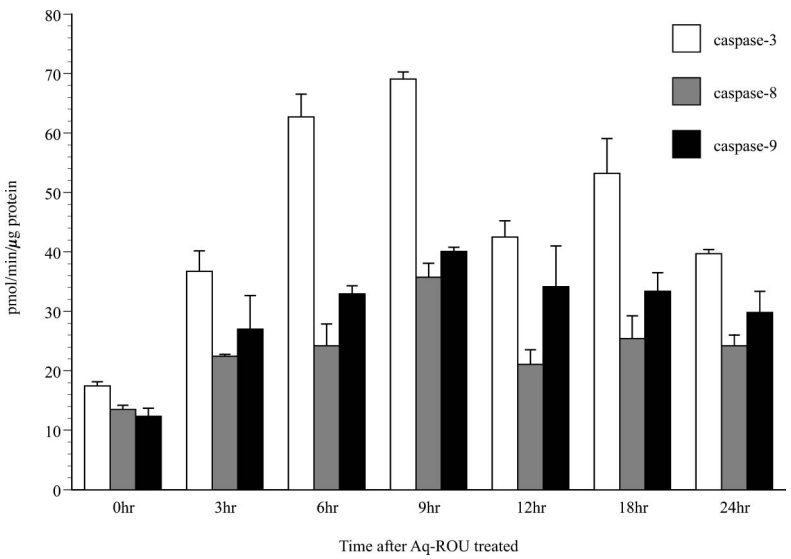

Fig. 4. Caspase Activity in Aq-ROU-treated HL-60 Cells
The bar above the column shows the standard deviation of three independent experiments.

Activation of Caspase-3, -8, -9 in Response to Aq-ROU Treatment

Intracellular activation of caspases can be broadly divided into two inductive systems. In one system, caspase-8 is activated by the binding of a death ligand to a death receptor in the TNF receptor family, such as Fas, TNFR-I, or Death Receptor (DR) 4 and DR5.[14,15] In the other system, caspase-9 is activated by cytochrome c released by mitochondria.[15] Both systems eventually activate caspase-3, triggering limited proteolysis of specific proteins[16] and DNA fragmentation by deoxyribonuclease (DNase) γ.[17] Thus, evaluating caspase-8 activity can provide information about induction via death receptors, whereas evaluating caspase-9 activity provides information about induction via mitochondria.

Activities of caspase-3, -8, and -9 are shown in Fig. 4. The activity of each caspase increased after 3 hr of Aq-ROU treatment and peaked after 9 hr of treatment. After 12 hr, caspase-9 activity remained stable at a level of 30 pmol per min/μg. In contrast, caspase-8 activity declined after 12 hr but increased again after 18 hr. Caspase-3 activity fluctuated, particularly during the period 12–24 hr (with a range of 39.7 ± 0.7 to 53.2 ± 5.9 pmol per min/μg during that period), suggesting that it is sensitive to changes in caspase-8 activity.

Based on our data, we surmise that there is one or more water-soluble factor in *R. oryzae* that can activate apoptosis via induction of caspase-3 and -8 as soon as 6 to 9 hr after treatment. Caspase-8 is known to activate Bid, a *bcl-2* family protein, which leads to activation of caspase-9, enhancing release of cytochrome c by mitochondria.[18] Additionally, previous studies report that caspase-3 is activated via several routes, including though activity of caspase-9, -8 and Bid, which appear to work in concert to amplify apoptotic signals.[19,20] Thus, activation of caspase-9 via Aq-ROU is likely to amplify the apoptotic signal via both the caspase-8 and mitochondrial pathways, which can account for the efficient induction of apoptosis we observed for Aq-ROU-treated HL-60 cells.

In summary, we report a new physiological function attributable to one or more water-soluble factors in the fungus *R. oryzae* that has the ability to induce apoptosis in a human cancer cell line, suggesting that identification and study of the factors may be useful in the development of new treatments for cancer.

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